Stimulation of a Neutral Triacylglycerol Hydrolase from Rat Heart by Phosphatidylethanolamine and Lysophosphatidylethanolamine

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Triacylglycerol hydrolase activity measured at pH 7.5 in a pH 5.2 precipitate fraction from rat heart was increased two- to three-fold by the presence of phosphatidylethanolamine (PE) or lysophosphatidylethanolamine (LPE). This stimulatory effect also could be obtained in assays with particulate and soluble subcellular fractions and was observed with two different methods of preparing triolein substrate emulsions. Ethanolamine and glycerophosphorylethanolamine had no effect on hydrolase activity, whereas phosphatidylcholine (PC) and acidic phospholipids such as cardiolipin were inhibitory. Palmitic acid, palmityl CoA and palmityl carnitine inhibited PE-stimulated hydrolase activity, but ethyl esters of palmitate had no effect. The preparation of acetone-ether powders resulted in a marked reduction of triacylglycerol hydrolase activity, but PE and LPE now stimulated hydrolase activity by ten-fold or greater, suggesting that these phospholipids may have an obligatory role in modulating triacylglycerol hydrolase activity. Triton X-100 also stimulated hydrolase activity in acetone-ether powders.
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The regulation of enzymes by phospholipids has been studied extensively in recent years. An obligatory lipid requirement has been observed for some membrane-bound enzymes when phospholipids are removed first by treatment of membranes by detergents or organic solvents (1,2). For example, the solubilization and purification of an acid lysosomal triacylglycerol hydrolase from rat liver (3) and canine myocardial cells (4) has revealed an obligatory requirement for the acidic phospholipid cardiolipin. The addition of phospholipids to assays with native membranes also can modulate enzyme activities (5,6). Phospholipids also have been demonstrated to influence the activity of soluble enzymes such as bovine milk galactosyltransferase (7) and the multifunctional calcium-dependent protein kinase (C) from rat brain (8).

Phosphatidylethanolamine recently has been found to produce a selective and specific stimulation of the hormone-sensitive triacylglycerol hydrolase from adipose tissue (9). Furthermore, nonactivated and protein kinase-activated forms of the adipose tissue enzyme had the same activity when assayed in the presence of PE (9). Previous studies from this laboratory have shown that the incorporation of PC into triolein substrate preparations resulted in a reduction in triacylglycerol hydrolase activity measured at pH 7.5 in low-speed supernatant fractions (10) and in pH 5.2 precipitate fractions from rat heart (11). Consequently, the objective of this investigation was to examine in more detail the possible effects of other phospholipids on neutral triacylglycerol hydrolase activity from rat heart. PE and LPE have been found to produce a selective stimulation of hydrolase activity.

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MATERIALS AND METHODS

Labeled triolein (glycerol tri[1-^{14}C]oleate) was purchased from Amersham (Oakville, Ontario, Canada) and was purified by Florosil chromatography (12). Triolein, essentially fatty acid-free bovine albumin, palmitic acid, palmityl CoA, DL-palmityl carnitine chloride and palmityl-ethyl ester were obtained from Sigma Chemical Co. (St. Louis, Missouri). Palmitic acid (sodium salt) was dissolved in H_2O; the fatty acyl esters were dissolved in 10 mM acetate, pH 6. Phospholipids were purchased either from Sigma or from Serdary Research Laboratories (London, Ontario). Specific sources of the phospholipids are indicated in the text. Solutions of PE were chromatographically pure as assessed by thin layer chromatography (TLC) on glass silica gel plates (E. Merck, Darmstadt, West Germany) using a solvent system consisting of chloroform/methanol/water/acetic acid (70:30:4:2, v/v/v/v).

A pH 5.2 precipitate fraction from rat hearts was isolated from the high speed (100,000 × g) supernatant as described by Severson and Hurley (11), and finally resuspended in a solution consisting of 0.25 M sucrose, 1 mM EDTA, 10 mM HEPES (N-2-hydroxyethyl-piperazine-N′-2-ethane sulfonic acid) at pH 7.5 (buffer A). The protein content, determined by a fluorometric method using bovine albumin as standard (13), ranged from 8.8 to 14.7 mg/ml. Subcellular fractions were isolated by differential centrifugation of heart homogenates (10).

An acetone-ether powder was prepared by adding 3 ml of acetone (at −20 C) to 1 ml of the pH 5.2 precipitate fraction (ca. 10 mg protein), vortexing and centrifuging for 20 min at 1000 × g. The supernatant was discarded and the pellet was re-extracted with another 3 ml of acetone. After centrifugation, 3 ml of ether (at −20 C) was added to the pellet, and the mixture was vortexed and centrifuged for 15 min at 1000 × g. Again the supernatant was discarded and the pellet was re-extracted with ether. After centrifugation, the pellet was dried carefully under a stream of N_2 gas, placed in a −80 C freezer for at least 10 min and lyophilized to remove the final traces of the organic solvents. The dry acetone-ether powder then was stored at −80 C. Prior to assay, the powder was resuspended in buffer A with the use of a Potter-Elvehjem glass-teflon homogenizer; the protein concentration was 5-8 mg/ml. No appreciable loss of triacylglycerol hydrolase activity was observed in acetone-ether powders stored at −80 C for 1-2 mo.

The phospholipid content of the pH 5.2 precipitate and acetone-ether powder fractions was measured by the method of Chalvardjian and Rudnicki (14) following extraction (15) and isolation of PC and PE by TLC (solvent system consisting of chloroform/methanol/water/acetic acid [70:30:4:2, v/v/v/v]).

Glycerol-dispersed triolein substrates were prepared as described previously (9,16). Each assay (0.2 ml final vol) contained ^{14}C-triolein (125 μM glycerol tri[1-^{14}C]oleate; ca. 200,000 DPM), PIPES (piperazine-N,N′-bis[2-ethane sulfonic acid]) buffer (25 mM, pH 7.5), bovine albumin (0.05%

w/v), glycerol (8.33% v/v) and the enzyme protein (25 to 100 μg). Assays were terminated after 30 min at 30 C by the addition of 3 ml of a fatty acid extraction solution (17); the content of sodium [1-^{14}C]oleate then was determined as outlined previously (16). All assays were performed in duplicate; assay blanks contained buffer A in place of the enzyme protein. For comparative purposes, hydrolase activity also was determined with a sonicated or liposomal triolein substrate prepared as described by Severson and Hurley (9). The final concentration of the radiolabeled triolein, PIPES buffer and albumin (in a final vol of 0.4 ml) was the same as for the assay with the glycerol-dispersed substrate preparation. A unit of hydrolase activity was defined arbitrarily as that amount of enzyme which catalyzed the formation of 1 nmol of free fatty acid (oleate) in one hr at 30 C.

Phospholipids usually were introduced into hydrolase assays by adding an aliquot of the phospholipid solution in chloroform to the ^{14}C-triolein stock solutions prior to the dispersion of the lipids into glycerol or prior to sonication into the buffer solution. Thus, in these experiments, the phospholipids were incorporated into the substrate preparations. In other experiments noted, appropriate quantities of phospholipids *in chloroform* were dried under N_2 and dispersed by sonication into H_2O; aliquots from this dispersion then were added directly to assay tubes.

RESULTS

The incorporation of PC into glycerol-dispersed triolein substrate preparations produced a reduction in triacylglycerol lipase activity, measured at pH 7.5, in a pH 5.2 precipitate fraction from rat heart to 77% of control activity (Fig. 1A), consistent with previous findings (11). The presence of cardiolipin resulted in a more pronounced inhibition to 22% of control; other acidic phospholipids such as phosphatidylserine, phosphatidic acid and phosphatidylinositol also reduced hydrolase activity (results not shown). This same pattern of inhibition was observed when the phospholipids were introduced into the assay directly from sonicated dispersions. In contrast, the presence of PE produced a stimulation of hydrolase activity to 337% of control (Fig. 1A). The specificity of this stimulation then was examined (Fig. 1B). Ethanolamine and glycerophosphorylethanolamine had little or no effect on hydrolase activity, but LPE resulted in the same degree of stimulation as did PE. In contrast, the presence of LPC reduced hydrolase activity to zero.

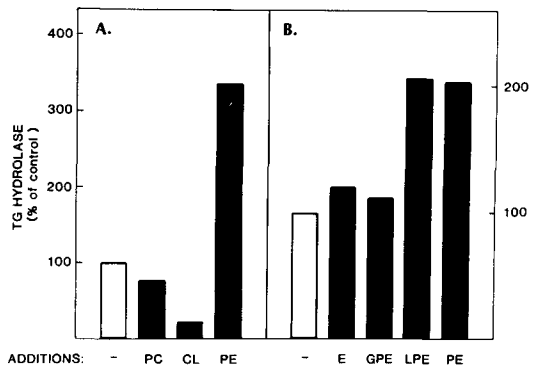

FIG. 1. Effect of phosphoglycerides on triacylglycerol hydrolase activity in a pH 5.2 precipitate fraction from rat heart. Hydrolase activity was measured with a glycerol-dispersed triolein substrate and is expressed as the percentage of control (no additions). Results are the mean of two experiments. A, phosphoglycerides were incorporated into the substrate preparation to give a final concentration of 350 μM; PC (phosphatidylcholine, egg yolk), CL (cardiolipin, bovine heart), PE (phosphatidylethanolamine, egg yolk). B, phosphoglycerides were sonicated into H_2O and aliquots were added to assay tubes to give a final concentration of 350 μM; E (ethanolamine), GPE (glycerophosphorylethanolamine, *E. coli*), LPE (lysophosphatidylethanolamine, pig liver), PE (phosphatidylethanolamine, egg yolk).

The preceding experiments were performed with the pH 5.2 precipitate fraction prepared from the 100,000 × g supernatant fraction after differential centrifugation of heart homogenates; this high speed supernatant fraction contains the majority of the total activity units for the neutral triacylglycerol hydrolase (10). The effect of PE on hydrolase activity was determined for particulate and soluble subcellular fractions (Table 1). The presence of PE produced a comparable increase in hydrolase activity in all the subcellular fractions, so that the percentage distribution of activity did not change. The recovery of hydrolase activity in the pH 5.2 precipitate fraction (70–79%) also was not changed when assays were performed in the presence of PE. The pH optimum for the PE-stimulated hydrolase activity in the pH 5.2 precipitate fraction was 7.5, the same as reported previously for hydrolase activity measured with glycerol-dispersed substrates prepared in the absence of phospholipids (11).

To determine if the stimulation of hydrolase activity by PE was a unique characteristic of assays with the glycerol-dispersed triolein substrate preparation, comparative experiments were performed with a sonicated (liposomal) substrate preparation. The incorporation of 350 μM PE into this substrate preparation also increased hydrolase activity from 58.3 to 169 Units/mg protein in a typical experiment.

The addition of palmitic acid and palmityl CoA to assays with a glycerol-dispersed substrate resulted in an inhibition of hydrolase activity in a pH 5.2 precipitate fraction to 50% and 5% of control, respectively, whereas palmityl carnitine produced a stimulation of hydrolase

TABLE 1

Effect of Phosphatidylethanolamine on Triacylglycerol Hydrolase Activity in Subcellular Fractions from Rat Heart Homogenates

Subcellular fraction	TG hydrolase activity (Units)	
	TO[a]	TO + PE[b]
5,000 × g Pellet	615 (13%)[c]	2,350 (15%)[c]
17,000 × g Pellet	139 (3%)	472 (3%)
100,000 × g Pellet	598 (12%)	1,260 (8%)
100,000 × g Supernatant	3,500 (72%)	11,100 (73%)

[a]Glycerol-dispersed triolein (TO) substrate (n = 2).

[b]Glycerol-dispersed triolein substrate containing 350 μM phosphatidylethanolamine (PE, egg yolk; n = 2).

[c]Percentage of the sum of total activity units.

activity (Table 2); identical results have been reported previously (11). When substrate preparations contained PE, the inhibition of hydrolase activity by palmitic acid and palmityl CoA was unchanged, but palmityl carnitine now produced the same degree of inhibition as did the free fatty acid. In contrast, the ethyl ester of palmitate had no effect on hydrolase activity measured in the absence or presence of PE (Table 2).

In some enzyme preparations, reaction rates determined with various amounts of enzyme protein were curvilinear upwards when assayed in the absence of PE (Fig. 2A). As a consequence, the stimulation of hydrolase activity by PE varied in this experiment from 2.3-fold at the lowest protein content to only 1.2-fold at the highest. This suggested that endogenous phospholipids that coprecipitated at pH 5.2 with the enzyme may modulate hydrolase activity. Therefore, acetone-ether powders were prepared with the result that the content of PC (6.7 nmol/mg protein) and PE (3.3 nmol/mg) in the pH 5.2 precipitate fraction was reduced to 0.46 and 0.08 nmol/mg protein, respectively (mean of two experiments). Hydrolase activity in the acetone-ether powder, measured with a glycerol-dispersed triolein substrate, was reduced substantially (Fig. 2B) relative to the activity in the pH 5.2 precipitate (Fig. 2A) and became very nonlinear with respect to the protein content of the assay. However, the addition of PE produced an even greater magnitude of hydrolase stimulation in the acetone-ether powders, so that the activity approached that measured in the original pH 5.2 precipitate fraction (Fig. 2). When assays were performed with the liposomal triolein substrate, PE (175 μM) increased hydrolase activity in the pH 5.2 precipitate fraction from 71.8 to 230 Units/mg protein (3.2-fold stimulation); by comparison, PE increased hydrolase activity in acetone-ether powders from 19.7 to 189 Units/mg protein for a 9.5-fold stimulation (mean from three preparations).

In experiments with acetone-ether powders, both PE and LPE produced a concentration-dependent stimulation of hydrolase activity measured with glycerol-dispersed (Fig. 3A) and liposomal (Fig. 3B) substrates. With both substrates, the stimulation of hydrolase activity by low concentrations of PE was substantially greater than that by the same concentration of the lysolipid, although the maximal stimulation was very similar. Under these conditions, a substantial stimulation of hydrolase activity was observed when the ratio of PE to triolein was as low as 0.2. Consistent with the earlier results with the pH 5.2 precipitate fraction (Fig. 1), ethanolamine and glycerophosphorylethanolamine had no significant stimulatory effect on hydrolase activity in acetone-ether powders, but cardiolipin, phosphatidylserine and phosphatidylinositol produced an inhibition of hydrolase activity measured in the presence of PE (results not shown).

Since PE and LPE produced such a marked stimulation of hydrolase activity in acetone-ether powders (Fig. 3), the effect of detergents was investigated. Sodium

TABLE 2

Effect of Fatty Acids and Fatty Acid Metabolites on Triacylglycerol Hydrolase Activity in a pH 5.2 Precipitate Fraction from Rat Heart

Additions to assay	TG hydrolase activity (% of control)	
	TO[a]	TO + PE[b]
Palmitic acid (100 μM)	50	52
Palmityl CoA (100 μM)	5	5
Palmityl carnitine (100 μM)	224	56
Palmityl-ethyl ester (100 μM)	103	107

[a]Glycerol-dispersed triolein (TO) substrate (n = 2; 100% = 123 Units/mg).

[b]Glycerol-dispersed triolein substrate containing 350 μM phosphatidylethanolamine (PE, egg yolk; n = 4, 100% = 311 Units/mg).

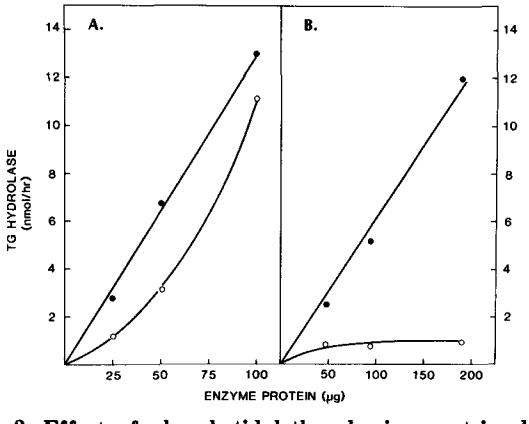

FIG. 2. Effect of phosphatidylethanolamine on triacylglycerol hydrolase activity. Hydrolase activity was measured with the indicated amounts of enzyme protein in a pH 5.2 precipitate (A) or in an acetone-ether powder (B) with a glycerol-dispersed triolein substrate prepared in the absence (○) and in the presence (●) of phosphatidylethanolamine (egg yolk, 350 μM).

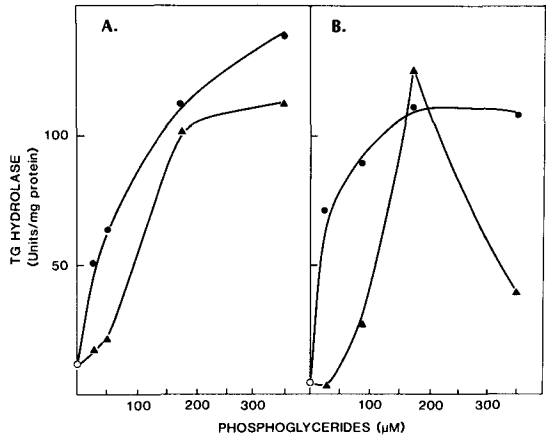

FIG. 3. Effect of phosphatidylethanolamine and lysophosphatidylethanolamine on triacylglycerol hydrolase activity in an acetone-ether precipitate fraction from rat heart. Hydrolase activity at the indicated concentrations of phosphatidylethanolamine (●, egg yolk) and lysophosphatidylethanolamine (▲, pig liver) was determined with either a glycerol-dispersed (A) or liposomal (B) triolein substrate preparation.

dodecyl sulfate resulted in an inhibition of hydrolase activity. In contrast, Triton X-100 produced a stimulation of hydrolase activity in acetone-ether powders, followed by an inhibition with concentrations greater than 60 μM (Fig. 4); this pattern was very similar to that observed with LPE (Fig. 3B). When assays were conducted with substrate preparations containing PE, Triton X-100 did not produce a further increase in hydrolase activity and concentrations greater than 40 μM became inhibitory (Fig. 4). A similar response to Triton X-100 was observed in assays with the pH 5.2 precipitate fraction.

PE from a number of natural sources (egg yolk, bovine liver, bovine brain, *E. coli*) produced the same stimulation of hydrolase activity in acetone-ether powders; in particular, the presence of up to 90% plasmalogens in brain PE preparations resulted in the same activity (167 Units/mg protein) as PE from egg yolk (154 Units/mg protein). Hydrolase activity stimulated by PE in acetone-ether powders had a pH optimum of 7.5. The kinetic mechanism associated with the stimulation of hydrolase activity in acetone-ether powders by PE was investigated. The presence of the phospholipid produced a 5.5-fold increase in maximal velocity with no change in the apparent K_m of 0.6 mM in assays where the concentration of the glycerol-dispersed substrate was varied from 0.05 to 1.5 mM (results not shown).

DISCUSSION

The stimulation of hydrolase activity in a pH 5.2 precipitate fraction from rat heart by PE was observed with two different methods of preparing substrate emulsions. Since the apparent properties of lipid-metabolizing enzymes are influenced markedly by the physical properties of the substrate emulsion (16), it is important to use more than one method of substrate preparation when characterizing a particular enzyme. The stimulation of activity by PE was observed in all subcellular fractions isolated by differential centrifugation of rat heart homogenates, so that the percentage distribution of hydrolase activity was not altered; the majority (73%) of hydrolase activity determined in the presence of PE was still in the high speed supernatant fraction. PE also stimulated (two- to five-fold) triacylglycerol hydrolase activity in a pH 5.2 precipitate fraction from pigeon adipose tissue (9). Although the rat heart hydrolase is a soluble enzyme, it may exist in a lipid-rich environment that could be similar to the environment of membrane-bound enzymes. The rat liver CTP:phosphocholine cytidyltransferase is an ambiquitous enzyme in that it is recovered in both the cytosolic and microsomal fractions of rat liver (18), but only the soluble form is stimulated by phospholipids (19).

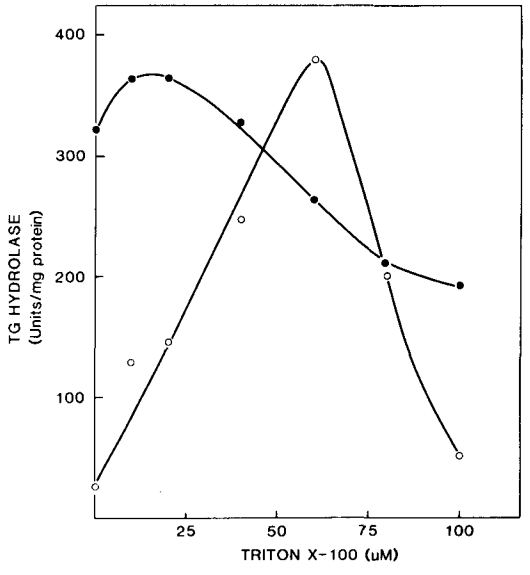

FIG. 4. Effect of Triton X-100 on triacylglycerol hydrolase activity in an acetone-ether precipitate fraction from rat heart. Hydrolase activity at the indicated concentrations of Triton X-100 was determined with liposomal triolein substrates prepared in the absence (○) and presence (●) of phosphatidylethanolamine (egg yolk; 175 μM).

The regulation of PE-stimulated hydrolase activity in the pH 5.2 precipitate fraction by free fatty acids and fatty acid metabolites also was examined. Inhibition of hydrolase activity by palmitic acid and palmityl CoA was not changed when hydrolase activity was increased by the presence of PE, but the stimulatory effect of palmityl carnitine observed previously in the absence of phospholipids (11) was changed to an inhibitory effect when assays were performed in the presence of PE. This alteration in the response to palmityl carnitine likely reflects differences in the properties of the substrate preparations. Hulsmann et al. (20) also have reported that palmityl carnitine inhibited neutral hydrolase activity in heart homogenates. Hearts perfused with ethanol result in the accumulation of fatty acid ethyl esters (21) due to the nonoxidative metabolism of ethanol by a CoA-independent esterification with free fatty acids (22). The induction of mitochondrial dysfunction by the fatty acid ethyl esters (23) could account for alcohol resulting in decreased β-oxidation of fatty acids and accumulation of triacylglycerols (24). However, the triacylglycerol accumulation also could have been due, in part, to an inhibition of cardiac lipase activity by fatty acid ethyl esters, but this seems unlikely since the ethyl ester of palmitate had no effect on hydrolase activity measured in the absence and presence of PE.

The myocardial hydrolase may have an obligatory requirement for phospholipids since the reduction in endogenous phospholipids content by the preparation of acetone-ether powders resulted in a marked decrease in hydrolase activity which was restored to near-control levels by the addition of PE or LPE. The kinetic mechanism associated with this stimulation in acetone-ether powders was due to an increase in maximal velocity with little or no change in the apparent K_m; the same kinetic mechanism also was established for the stimulation of the adipose tissue lipase by PE (9). Hulsmann et al. (25) reported that a short preincubation of a myocardial lipase preparation with phospholipase A_2 and C resulted in a marked reduction of activity, but effects of exogenous phospholipids were not examined.

One of the striking features of this investigation was the specificity of the phospholipid activation for the myocardial hydrolase; only PE and LPE had stimulatory effects. Most other instances of enzyme regulation by phospholipids have involved acidic phospholipids

(4,26-31). In the case of the phosphodiesterase in adipocyte membranes, from five to seven phospholipids and lysophospholipids were stimulatory (5,6).

It is not known whether stimulation of the myocardial hydrolase by PE or LPE is due to an action on the properties of the triolein substrate and/or a direct effect on the enzyme protein. However, the mechanism may be due to a detergent action, since Triton X-100, a neutral detergent, also stimulated hydrolase activity; the combination of PE and Triton X-100 did not produce an additive stimulation of hydrolase activity. Detergents have been shown to stimulate a number of the enzymes that also are regulated by phospholipids (4,6,27-29). The hormone-sensitive lipase in a pH 5.2 precipitate fraction from adipose tissue was stimulated by PE, but 350 μM LPE and 80 μM Triton X-100 had no effect on hydrolase activity (9). This may indicate that phospholipids regulate the neutral triacylglycerol hydrolases from heart and adipose tissue by different mechanisms, although the effects of phospholipids and detergents have not been investigated with acetone-ether preparations from adipose tissue. Future investigations will be directed towards determining a precise molecular mechanism for the activation of the rat heart hydrolase by PE and LPE.

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The Chemistry of Lipid Peroxidation Metabolites: Crosslinking Reactions of Malondialdehyde

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Malondialdehyde reacts readily with amino acids to form adducts containing vinylogous amidine linkages. Crosslinking reactions between nucleic acid bases and amino acids induced by malondialdehyde also have been investigated. The physical data obtained for the adducts provide structural information on the possible mode of crosslinking of proteins and nucleic acids induced by this lipid metabolite.
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The ubiquitous natural compound malondialdehyde (MDA) is produced in substantial quantity in mammalian tissues both as an end product of unsaturated lipid peroxidation and as a side product of prostaglandin and thromboxane biosynthesis (1-4). It also is produced in the γ-irradiation of carbohydrates (5). The measurement of MDA by the thiobarbituric acid test has been used commonly as a method for the detection of peroxidation of unsaturated fatty acids and in the estimation of oxidative rancidity in foods (2,3,6). We have reported recently on a detailed and unambiguous assignment of the structure of this adduct (7). MDA has been reported to be toxic (8,9), carcinogenic (8,10) and mutagenic (10-12). This reported degenerative chemistry of MDA may be the result of its ability to covalently bond and to crosslink a variety of biological macromolecules. For example, MDA is reactive towards nucleic acids, resulting in the loss of their template activity (13,14). Also, it has been suggested that MDA-induced modification of lipoproteins may play a role in atherosclerosis (15-17). Valuable information on both the reactive sites and the structural nature of modification can be obtained through investigation of model systems that represent the vulnerable components. We have shown previously that MDA reacts rapidly at the α-amino group of amino acids to form 1:1 adducts (18), and that reaction occurs with adenine and cytosine bases to form hypermodified products (19). This paper reports some model crosslinking reactions of MDA.

MATERIALS AND METHODS

Melting points are uncorrected and were determined on a Thomas-Hoover melting point apparatus fitted with a microscope. The ^1H and ^{13}C NMR spectra were recorded on a Bruker WM-360 or on a JEOL FX-90Q pulse Fourier transform nuclear magnetic resonance (NMR) spectrometer. Mass spectra were determined on a Hewlett-Packard 5985 gas chromatography/mass spectroscopy (GC/MS) instrument. Ultraviolet data were obtained on a Varian-Cary Model 219 UV-Visible spectrophotometer. Fluorescence spectra were recorded on an SLM-Aminco SPF-500C instrument. Elemental analyses were performed by Galbraith Laboratories. Amino acid derivatives and adenine were purchased from Sigma Chemical Co. (St. Louis, Missouri). MDA *bis*-dimethylacetal was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin) and 2-methyl-3-ethoxyprop-2-enal from Fluka Chemical Co. (Hauppauge, New York). 9-Ethyladenine was prepared as described previously (20).

Sodium MDA was prepared from MDA *bis*-dimethylacetal as described previously (18), except that after hydrolysis the reaction mixture was basified to pH 10 before work up.

Sodium methylmalondialdehyde (sodium MMDA) was prepared from commercial 2-methyl-3-ethoxyprop-2-enal (18).

General procedure for formation of 2:1 adducts from amino acids and MDA and MMDA. In an oven-dried flask with a condenser and a nitrogen bubbler were placed the amino acid methyl ester hydrochloride (2.0 mmol) and sodium MDA or sodium MMDA (1.0 mmol) in dry methanol (10 ml). The reaction mixture was heated under reflux in a nitrogen atmosphere and the progress of reaction was followed by UV spectroscopy. The solvent was removed under reduced pressure and the residue was triturated with 20 ml of 20% CH_3OH/CH_2Cl_2. The precipitated NaCl was removed by filtration and the filtrate was concentrated. The resulting residue was crystallized from CH_3OH/CH_2Cl_2. All adducts gave satisfactory elemental analyses.

Preparation of 9-ethyladenine enaminal **6**. 9-Ethyladenine hydrochloride (1.170 g, 5.85 mmol) in dry methanol (65 ml), was treated with sodium MDA (0.657 g, 5.87 mmol). The mixture was heated (60 C under N_2) and progress of reaction was monitored by UV spectroscopy (19). The reaction was terminated when the 322 nm to 260 nm peak ratio had maximized (about 15 hr). The solvent was removed under reduced pressure and the residue was chromatographed on silica gel plates using 8% CH_3OH/CH_2Cl_2 as the eluent. The band at R_f 0.48 afforded 0.162 g (13%) of **6** as yellow crystals: mp 148-149 C; UV (EtOH) λ max 322 nm (ϵ 42114), 243 nm (ϵ 8810), 223 nm (ϵ 10934); ^1H NMR (Me_2SO-d_6) δ 11.30 (d, 1H, J = 11.5 Hz), 9.41 (d, 1H, J = 8.7 Hz), 8.77 (dd, 1H, J = 11.5, 13.4 Hz), 8.55 (s, 1H), 8.52 (s, 1H), 5.98 (dd, 1H, J = 8.7, 13.4 Hz), 4.28 (q, 2H), 1.45 (t, 3H); ^{13}C NMR (Me_2SO-d_6) δ 191.2, 151.6, 151.3, 149.1, 148.9, 143.6, 120.2, 38.4, 15.0; mass spectrum, m/z (relative intensity) 217 (M^+, 11.3), 189 (M^+ - CHO, 20.6), 188 (100), 160 (29.4), 148 (4.8), 135 (10.3), 120 (purine, 4.2), 119 (10.7). Anal. Calcd. for $C_{10}H_{11}N_5O$: C, 55.29; H, 5.10; N, 32.24. Found: C, 55.64; H, 5.27; N, 31.97.

Reaction of 9-ethyladenine enaminal **6** *with glycine methyl ester hydrochloride.* In an oven-dried flask fitted with a condenser and nitrogen bubbler was placed glycine methyl ester hydrochloride (0.093 g, 0.74 mmol) in dry methanol (22 ml). To this was added 9-ethyladenine enaminal (0.160 g, 0.74 mmol) and 3Å molecular sieves. The reaction mixture was heated under reflux with stirring and under a nitrogen atmosphere. Aliquots were removed

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periodically for kinetic analysis by UV spectral methods. Isolation of **7** in a completely pure form was difficult because of its instability, but strong evidence for its formation came from UV, mass spectral and high-field ^{1}H NMR data.

RESULTS AND DISCUSSION

Crosslinking of proteins by MDA has been reported to occur within 24 hr of reaction (21-23). However, characterization of the products of such modification remains incomplete. The investigation of the structural details of crosslinking of amino acid residues induced by MDA therefore was undertaken as models of protein modification by this natural multifunctional compound. The choice of amino acids for this study was based on several considerations. Initially, the reaction of glycine methyl ester was examined, as its structural simplicity facilitated isolation and identification of the adduct. Lysine, tyrosine, histidine and arginine were chosen because they represent amino acids that were most consistently altered by MDA in studies involving enzymes and other proteins (21-23). These amino acids also have reactive sites at positions other than the α-amino group. Studies with lysine were particularly important as the only primary amino group in protein structures apart from the N-terminal α-amino groups is the ϵ-amino group of lysine. For purposes of comparison, a substituted MDA, MMDA, also was utilized in these studies.

The reactions were carried out by combining solutions of the amino acid methyl ester hydrochloride (two molar equivalents [meq]) and the dialdehyde enolic sodium salt (one meq) in dry methanol (Scheme 1). The reactions were monitored by UV spectroscopy. The disappearance of MDA and the appearance of the vinylogous amidine were monitored at their absorption maxima of ca. 247 and 300 nm, respectively. MMDA reactions were monitored at ca. 252 and 310 nm. The yields, physical properties and UV data of the products are shown in Table 1.

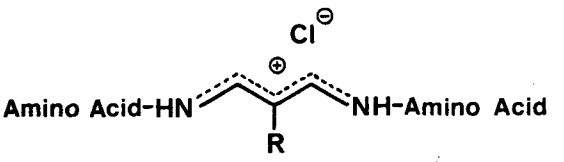
Use of methanol as solvent under acid catalysis provided optimum conditions for conversion to crosslinked adducts in terms of reaction times, product yields and ease of purification. The rates of formation of adducts containing vinylogous amidine linkages were much slower in acetate buffer at pH 4.2, the reported optimum conditions for the reaction of MDA with proteins (18). Considerably faster protein modification by MDA in aqueous buffered systems at pH 4.2 than under the same conditions in these model studies may be due to the presence of a more favorable environment in proteins, not only for crosslinking, but also for survival of the vinylogous amidine linkage. With respect to the latter, our model studies suggest that, once formed, the 1:2 adducts of MDA and amino acids are relatively stable even in aqueous acidic solutions.

Unambiguous evidence for the formation of the adducts **1-5** came from spectral data and elemental analysis. The mass spectral data showed parent ions (minus HCl) for each product. The ^{1}H and ^{13}C NMR spectra of the products provided insight into their stereochemistry and are summarized in Tables 2 and 3. In all cases for the MDA adducts, the coupling constants ($J \geq 11.0$ Hz) for the vinylogous amidine moiety and the single resonance for H_α and C_α in the ^{1}H and ^{13}C NMR spectra suggests an all-*trans* or W form for the stereochemistry of these crosslinked adducts. Although unequivocal assignment with respect to the stereochemistry of the vinylogous amidine moiety cannot be made for the MMDA adducts, it is reasonable to assume that the *trans* form predominates for these compounds as well (24).

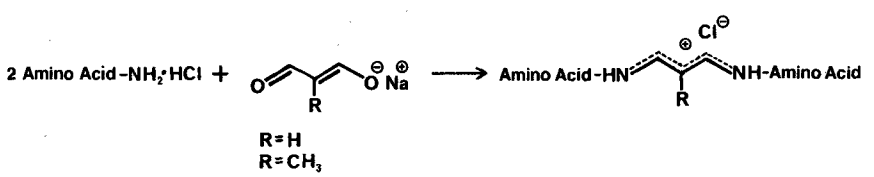
Tappel and coworkers have reported that UV absorp-

TABLE 1

Yields and Physical and Spectral Properties of Vinylogous Amidinium Salts from the Reaction of MDA and MMDA with Amino Acid Derivatives



Compound (amino acid, R)	Yield, %	mp, °C	$UV_{max}(H_2O)(\log \epsilon)$
1a Glycine methyl ester R=H	47	160	298 nm (4.55)
1b Glycine methyl ester R=CH_3	60	98-100	307 nm (4.54)
2a α-N-Acetyllysine, methyl ester R=H	58	Low melting solid	300 nm (4.52)
2b α-N-Acetyllysine, methyl ester R=CH_3	65	Low melting solid	307 nm (4.53)
3a Tyrosine methyl ester R=H	76	92-94	300 nm (4.58)
3b Tyrosine methyl ester R=CH_3	67	93-95	310 nm (4.55)
4a Histidine methyl ester (2HCl) R=H	54	113-115	301 nm (4.55)
4b Histidine methyl ester (2HCl) R=CH_3	60	133-135	310 nm (4.58)
5a Arginine methyl ester (2HCl) R=H	76	85-88	302 nm (4.54)
5b Arginine methyl ester (2HCl) R=CH_3	51	96-98	306 nm (4.57)



SCHEME 1

TABLE 2

^{1}H NMR Data for 2:1 Adducts

Compound	Solvent[a]	H_α	H_β	$-CH_3$	$-\overset{O}{\overset{\|}{C}}OCH_3$	H (others)
1a	Me_2SO-d_6	7.90 d, J = 11.7	5.75 t, J = 11.7	—	3.71	4.29 (s, 4H); 9.55 (bs, 2H)
1b	Me_2SO-d_6	7.70 s	—	1.82	3.71	4.32 (s, 4H); 9.10 (bs, 2H)
2a	CD_3OD	7.74 d, J = 11.5	5.64 t, J = 11.5	—	3.71	1.40–1.75 (m, 12H); 2.00 (s, 6H); 2.84 (t, 4H); 4.38 (t, 2H)
2b	CD_3OD	7.62 s	—	2.00	3.71	1.40–1.75 (m, 12H); 2.00 (s, 9H); 2.83 (t, 4H); 4.38 (t, 2H)
3a	CD_3OD	7.45 d, J = 11.3	5.49 t, J = 11.3	—	3.77	3.29 (m, 4H); 4.12 (t, 2H); 6.75 (m, 4H); 6.95 (m, 4H)
3b	CD_3OD	7.23 s	—	1.61	3.82	3.10 (d, 4H); 4.10 (m, 2H); 6.82 (d, 4H); 6.85 (d, 4H)
4a	D_2O*	7.47 d, J = 11.0	6.10 t, J = 11.0	—	3.43	3.51 (m, 4H); 4.52 (m, 2H); 7.38 (s, 2H); 8.68 (m, 2H)
4b	CD_3OD	7.07 s	—	1.50	3.45	3.40 (m, 4H); 4.30 (t, 2H); 7.37 (s, 2H); 8.30 (s, 2H)
5a	D_2O*	7.93 d, J = 11.0	6.20 t, J = 11.0	—	3.92	1.85–2.06 (m, 12H); 3.35 (m, 2H)
5b	CD_3OD	7.50 s	—	1.57	3.72	1.75–2.90 (m, 12H); 3.10 (m, 2H)
7	Me_2SO-d_6	8.69 bd, J = 11.5	6.70 bt, J = 11.5	—	3.72	1.45 (m); 4.24 (m); 4.36 (m); 8.35 (s); 8.41 (s)

[a]Chemical shifts given are with Me_4Si as internal standard (δ=0), except for *, where external Me_4Si was used.

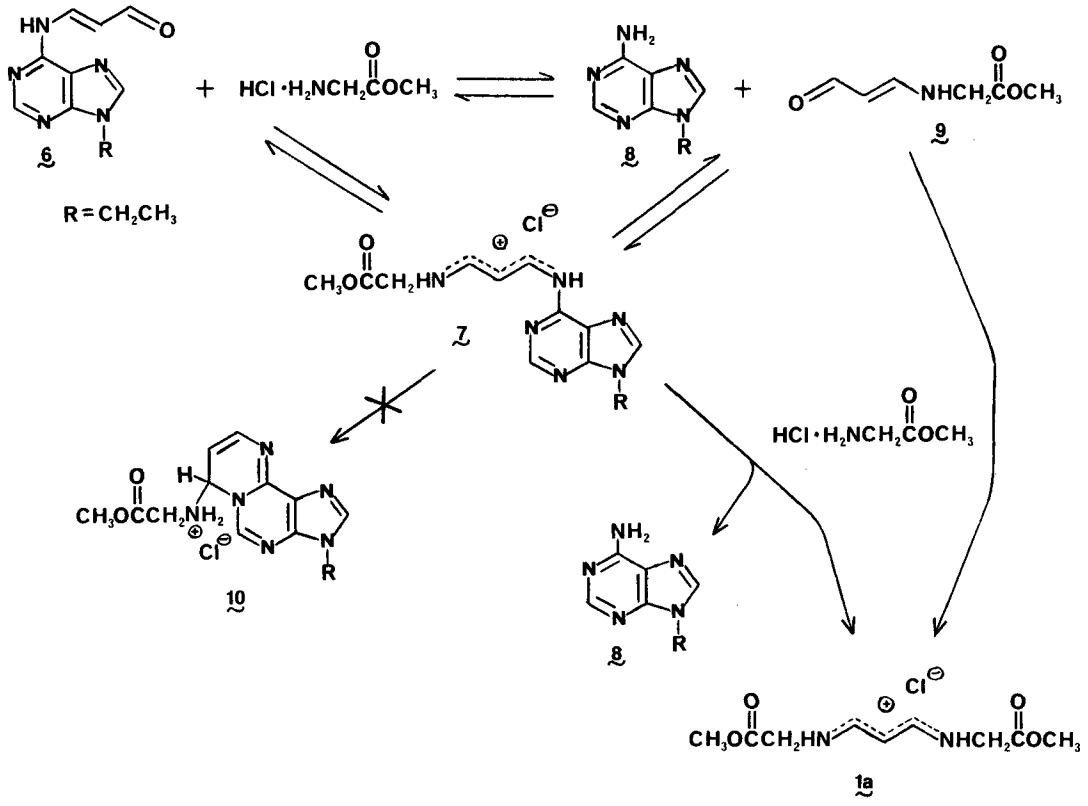
TABLE 3

^{13}C NMR Data for 2:1 Adducts

Compound	Solvent[a]	C_α	C_β	$-CH_3$	$-\overset{O}{\overset{\|}{C}}OCH_3$	C (others)
1a	Me_2SO-d_6	163.3	90.7	—	52.2	44.7; 168.1
1b	Me_2SO-d_6	164.9	101.5	8.3	52.3	48.6; 169.3
2a	CD_3OD	163.4	90.0	—	53.7	22.5; 23.9; 29.9; 32.0; 41.1; 52.6; 173.1; 174.0
2b	CD_3OD	164.3	101.8	7.9	53.7	22.4; 24.0; 29.9; 32.1; 41.2; 52.7; 173.3; 174.1
3a	CD_3OD	158.1	95.5	—	53.1	29.3; 61.7; 115.5; 127.3; 131.2; 157.6; 173.2
3b	CD_3OD	157.6	116.1	7.0	52.6	28.2; 61.0; 116.4; 127.8; 131.2; 157.4; 171.1
4a	D_2O*	166.5	93.4	—	54.5	28.2; 61.8; 118.6; 128.5; 134.8; 171.3
4b	CD_3OD	165.6	104.2	8.3	54.5	27.9; 62.0; 118.6; 129.0; 134.8; 171.4
5a	D_2O*	166.1	93.1	—	54.2	24.9; 28.7; 41.2; 62.3; 157.6; 173.1
5b	CD_3OD	165.1	103.5	8.3	54.2	25.2; 29.1; 41.2; 62.4; 157.5; 173.1

[a]Chemical shifts given are with Me_4Si as internal standard (δ=0), except for *, where internal dioxane (δ=67.4) was used.

tion maxima at approximately 256, 285, 370 and 435 nm result from the products of MDA interaction with proteins (21,25). The model crosslinked compounds isolated in our work gave UV data inconsistent with these observations. The UV data reported previously were very likely for a mixture of products including those containing vinylogous amidine linkages. It also should be explained that MDA generated in situ from the acid-catalyzed hydrolysis of the corresponding *bis*-acetals is contaminated significantly by reactive side products such as β-methoxyacrolein and 3,3-dimethoxypropionaldehyde. We have employed highly purified sodium MDA in this work. Fluorescence spectra of MDA-modified proteins exhibit emission at 440-470 nm with excitation at 370-400 nm (22,25), also indicative of the formation of vinylogous amidines and other linkages. The lysine crosslinked compound **2a** showed a fluorescence emission maximum at 440 nm on excitation at 360 nm, with the other adducts giving similar



SCHEME 2

spectra. These data lend further support for the formation of vinylogous amidine linkages in the modification of proteins by MDA.

MDA is reactive towards nucleic acids, resulting in the loss of their template activity (13,14). The modification of nucleic acids may involve direct modification of the bases, crosslinking between the bases or crosslinking between the bases and proteins (26,27). The direct modification of nucleic acid bases by MDA has been reported by us (19). To provide further basic chemical information on the nature and stability of MDA-induced protein-nucleic acid crosslinks, we studied the reactivity of a 9-substituted adenine with MDA in the presence of glycine methyl ester. No adenine-amino acid crosslinking was observed under these conditions and the only product formed was the glycine adduct **1a**. However, in the absence of the amino acid, 9-ethyladenine was converted slowly to its enaminal **6** by reaction with MDA. No crosslinked base pairs could be detected under a wide variety of conditions. However, if the enaminal were allowed to react with an equimolar amount of glycine methyl ester hydrochloride in scrupulously dry methanol, formation of the base-amino acid crosslinked adduct **7** could be detected at 348 nm in the UV spectrum. Quantitative monitoring of the reaction by UV and high-field ^{1}H NMR spectroscopy showed an initial buildup of **7** to a maximum value of 21% after about one hr. At this point no enaminal **6** remained, and the reaction mixture also contained the glycine adduct **1a** (26.5%) and 9-ethyladenine, **8** (52.5%). After three hr, however, the level of **7** had fallen to 3%, the amount of **1a** had maximized at 35.5% and that of **8** had stabilized at 61.5%. The 2:1 adduct **1a** very likely is formed through the intermediacy of **9**, the initial product of a reaction involving the transfer of the MDA moiety from **6** to glycine. Adduct **1a** also may be produced from the reaction of glycine with **7**, followed by elimination of 9-ethyladenine (Scheme 2).

The crosslinked adduct **7** is much less stable in aqueous solutions than the corresponding amino acid adducts containing vinylogous amidine linkages. However, although adduct **7** could not be isolated in a pure state because of its instability, its structure could be established unambiguously by its UV, high-field NMR and mass spectral data. The mass spectrum showed a parent ion at m/z 288 (M^+-HCl) and appropriate peaks for product fragmentation. The 360 MHz ^{1}H NMR data showed a doublet at δ8.69 (J = 11.5 Hz) and a triplet at δ6.70 (J = 11.5 Hz) corresponding to H_α and H_β of the diazapentadienium moiety. Resonances due to the amino acid components also were observed (see Table 2). The possibility of cyclization of the crosslinked adduct **7** to give **10** (cf ref. 19) may be ruled out by the UV and NMR data.

In summary, we conclude that MDA is capable of crosslinking amino acids through vinylogous amidine linkages. Additionally, MDA may crosslink amino acids with nucleic acid bases or may transfer the MDA moiety from one to the other. The detrimental biological effects of MDA may be mediated by such modifications.

ACKNOWLEDGMENTS

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HPLC Measurement of Testicular Long Chain Acyl-CoA Synthetases with Different Substrate Specificities

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Acyl-CoA synthetase activity with various long chain fatty acid substrates was measured in microsomes from rat testes, isolated spermatids and testes of hypophysectomized adult rats, using reversed-phase high performance liquid chromatography (HPLC). The spectrophotometric HPLC method produced results comparable to those of parallel radiometric assays and was highly specific for acyl-CoA products. At optimal pH and cofactor concentrations, specific activity from whole testis was similar for 18:1, 20:4 and 22:5 but somewhat lower for 16:0 over the substrate range 0.01–3.2 mM. Activity from spermatids or from testes of hypophysectomized rats was much lower with 22:5 than with 18:1 or 20:4, whereas activities with 18:1 and 20:4 were similar at all substrate concentrations. All substrates exhibited Michaelis-Menten type saturation kinetics and linear Lineweaver-Burke plots at lower substrate concentrations but inhibited activity at higher concentrations. Apparent values of K_M for 16:0, 18:1 and 20:4 were more than twice that of 22:5, whereas both observed and calculated maximum velocities were similar for the four fatty acids. Differences in pseudokinetic parameters and differential expression of the testicular acyl-CoA synthetase activities with different fatty acids suggest the presence of multiple enzymes, at least one of which may be hormonally regulated.
Lipids 21, 11–16 (1986).

Most species of animals accumulate substantial amounts of long chain polyenoic acids (LCPA) in testes during germinal cell differentiation. Several lines of evidence suggest that these metabolites of essential fatty acids play a specific and obligatory role during spermatocyte and spermatid differentiation (1-6). Although rats and mice accumulate 22:5n-6 in phosphoglycerides and triacylglycerols of spermatocytes and spermatids, the intimate structural and biochemical associations in the germinal epithelium among these and other cell types, especially the sustentacular Sertoli cells (7), make it difficult to determine the sites of synthesis of LCPA and complex lipids that contain them. Patterns of in vivo and in vitro incorporation of radioactive precursors into various fatty acids and lipid classes have shown that both whole testis and isolated germinal cells are capable of synthesizing all major lipid classes and their constituent fatty acids, including LCPA (8,9). In contrast, the time course of in vivo incorporation of [^{14}C] arachidonate (20:4n-6) into LCPA of rat Sertoli and germinal cells strongly suggests that 22:5n-6 is synthesized primarily in the Sertoli cells (10). However, the distributions of LCPA among testis cell types potentially are dependent not only on the distribution of LCPA synthesis but also on the distributions of other activities involved in mobilization, uptake, metabolism and incorporation into complex lipids. The long chain acyl-CoA synthetase (EC 6.2.1.3), which catalyzes the reaction,

$$RCOOH + ATP + CoASH \rightarrow RCOSCoA + AMP + PP_i$$

is a potential mediator of all of these activities. The rat liver microsomal and mitrochondrial synthetases have been purified and well characterized and are apparently a single enzyme (11). The testicular enzyme has not been characterized, although its activity has been studied indirectly, coupled with phosphoglyceride transacylases in microsomal preparations (12,13). In the present work, we have examined the properties and distribution of the microsomal acyl-CoA synthetases in whole testis and spermatids and the effects of hypophysectomy. Evidence is presented for the existence of at least two distinct enzymes with differential specificities for various long chain fatty acid substrates.

MATERIALS AND METHODS

Acyl-CoA synthetase assay. Long chain acyl-CoA synthetase was measured in microsomes of rat testis from 200-300 g Sprague-Dawley rats by a modification of the procedure of Tanaka et al. (11). Microsomes were isolated by sequential ultracentrifugation as described by Tanaka et al. with the exception that microsomal pellets were obtained at 105,000 × g for 1.5 hr. Unless stated otherwise, assays contained Tris-HCl buffer (0.1 M, pH 8.0), dithiothreitol (1 μmol), KCl (30 μmol), magnesium chloride (3 μmol), ATP (2 μmol), CoA (0.2 μmol), Triton X-100 (0.32 μmol), microsomes (100 μg protein) and fatty acid (.01-1 mM) in a total volume of 0.2 ml. Where indicated, substrates were supplemented with 50,000 dpm [1-^{14}C] labeled oleic or arachidonic acids (New England Nuclear, Boston, Massachusetts) (56 mCi/mmol). The mixture was incubated for 10 min at 32 C and the reaction was terminated by addition of 2.5 ml of isopropanol:n-heptane:1 M H_2SO_4 (40:10:1, v/v/v). Then 1.5 ml n-heptane and 1 ml of water were added, followed by thorough mixing and centrifugation. The n-heptane layer was drawn off and the aqueous phase extracted twice more with 1 ml n-heptane. The aqueous phase then was centrifuged to remove particulates and a 0.1 ml aliquot was taken for analysis by reversed-phase HPLC. Where radioactive substrates were used, an aliquot also was withdrawn for liquid scintillation counting. Reported data points typically present triplicates or quadruplicates with at least one repetition of the experiment.

In preliminary experiments, pH and concentrations of ATP, CoA and protein were varied to confirm that assay conditions were in the optimal ranges for the testicular enzyme. Incubations were carried out for 5-40 min to confirm that activity was linear with respect to time. Results were corrected with reagent blanks and boiled

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controls as required. Protein was determined by the method of Lowry et al. (14). Pseudokinetic parameters were calculated by the statistical method of Wilkinson (15).

Quantitation of acyl-CoA by HPLC. Aliquots (0.1 ml) from postincubation aqueous phases prepared as described above were analyzed for product acyl-CoA by HPLC on a 3.9 mm × 30 cm, MicroBondapak C_{18} (Waters Associates, Milford, Massachusetts) reversed-phase column, eluted at 1 ml/min with a mobile phase prepared by adding tetrabutylammonium hydroxide (40% aqueous solution, Sigma Chemical Co., St. Louis, Missouri) to 80% methanol (HPLC grade) to a concentration of 10 mM. This solution then was titrated to pH 5.5 with a pH meter, by adding 85% H_3PO_4 by drops (certified ACS, Fisher Scientific, Fairlawn, New Jersey). Acyl-CoA products and standards were detected by monitoring effluent for UV absorbance at 254 nm. Products were quantitated by integration of absorbance peaks and calibration of detector response with commercial standards for oleoyl and arachidonoyl CoA (Sigma). Retention time of docosapentaenoyl-CoA was confirmed by collection of the tentatively identified peak, transmethylation with methanolic sodium methoxide and identification of the methyl ester of 22:5 n-6 by capillary gas chromatography (GC) as described by Grogan (16). When radioactive precursor fatty acids were used, column effluent was collected in fractions for liquid scintillation counting. Effluent corresponding to the absorbance peak for each acyl-CoA was collected as a single fraction.

Preparation of fatty acid substrates. Nonradioactive palmitic, oleic and arachidonic acids were purchased from a commercial source (Sigma). Docosapentaenoic acid (22:5 n-6) was isolated from rat testis lipids by reversed-phase HPLC on a preparative column (7.8 mm × 30 cm, MicroBondapak C_{18}, Waters Associates). Rat testes were decapsulated and hydrolyzed in 40% KOH at 50 C for one hr. The alkaline solution then was extracted three times with petroleum ether to remove nonsaponifiables and acidified to pH 2 with concentrated HCl. Fatty acids were extracted into petroleum ether, which then was evaporated to dryness under a stream of N_2. Fatty acids were redissolved in the HPLC mobile phase to a concentration of 4 mg/ml, and 0.5 ml aliquots were injected on the HPLC column. The column was eluted isocratically at a flowrate of 2 ml/min with a mobile phase consisting of acetonitrile/methanol/water (15:1:4, v/v/v), to which 85% H_3PO_4 was added to a final concentration of 4 mM. Unsaturated fatty acids were detected by monitoring effluent at 215 nm with a variable wavelength absorbance detector. Retention time for 22:5 was 35 min under these conditions. Purity (>95%) was confirmed by capillary GC as described by Grogan (16). Both radioactive and unlabeled arachidonic acids (retention time 28 min) were purified by reversed-phase HPLC just prior to use.

Isolation of spermatids. Testes from a Sprague-Dawley rat were decapsulated and dispersed in Eagle's Minimum Essential Media (Flow Laboratories, Rockville, Maryland) with hyaluronidase (Sigma, Type V) and collagenase (Sigma, Type II). Cells were sedimented at 1 × g on an albumin density gradient using a Sta-Put apparatus as described elsewhere (17). Cells were collected in 40 gradient fractions, and aliquots were stained and examined under light microscopy in order to determine cell composition as described previously (17). Fractions enriched in round and condensing spermatids were pooled to obtain a preparation containing >80% spermatids. Microsomes were prepared for these cells by procedures used for processing of whole testis as described above and in Tanaka et al. (11). Acyl-CoA synthetase was determined in triplicate at 0.05, 0.1, 0.2 and 0.4 mM concentrations of 18:1, 20:4 and 22:5 by the HPLC assay. The experiment was repeated in quadruplicate with all fatty acids at 0.2 mM.

Hypophysectomized rats. Four testes from two 250 g hypophysectomized rats (Charles River Breeding Laboratories, Wilmington, Massachusetts) were processed to obtain microsomes as described above for normal rats. Rats were maintained for ca. one yr prior to the experiment. Testis weights were 10% those of nonhypophysectomized controls. Acyl-CoA synthetase activity was determined in triplicate at 0.05, 0.1 and 0.2 mM concentrations of 18:1, 20:4 and 22:5 by the HPLC assay.

RESULTS

HPLC assay. Measurement of product acyl-CoA by HPLC provided a reliable and specific assay for acyl-CoA synthetase activity. The CoA derivatives of 16:0, 18:1, 20:4 and 22:5 eluted from the reversed-phase HPLC column with distinctive retention times of 0.92, 1.00, 0.66 and 0.90, respectively, relative to the retention time of 18:1 (typically 34 min). Analyses of blanks and boiled controls yielded baseline absorbance at these retention times. In the cases of 18:1 and 20:4, results obtained by integration of absorbance (254 nm) of the eluting acyl-CoA were linearly correlated (r=0.984 and 0.956, respectively) with those obtained by measurement of incorporation of radioactivity from ^{14}C-labeled fatty acids into the eluting acyl-CoA (Fig. 1). The methods were in substantial agreement over a broad range of substrate concentrations (.01-3.2 mM) for both fatty acids, although integrated absorbance yielded values consistently somewhat higher than those obtained from the radiometric method.

Recovery of radioactivity in eluent was 100% of that injected on the HPLC column within the limits of counting and aliquotting errors (±5%). A variable amount of radioactivity was measured in fractions other than that corresponding to the acyl-CoA (data not shown). The bulk of this radioactivity was identical in retention time to the unesterified fatty acid, increased as a linear function of the substrate concentration and was present in boiled controls and reagent blanks. In the case of 20:4, other components, intermediate in retention time between 20:4 (1.6 relative to arachidonoyl-CoA) and its CoA derivative, sometimes accounted for as much as 50% of the water soluble radioactivity, were not present in boiled controls and were not associated with peaks of UV absorbance. Samples were stable for at least three days at −20 C as judged by repetitive assays.

Properties of the acyl-CoA synthetase. Activity of the acyl-CoA synthetase from both whole testis and spermatocytes was linear with respect to time (5-40 min, 1 mM substrate) and protein concentration (up to 0.5 mg/ml) for 18:1, 20:4 and 22:5 (data not shown). Optimal activity

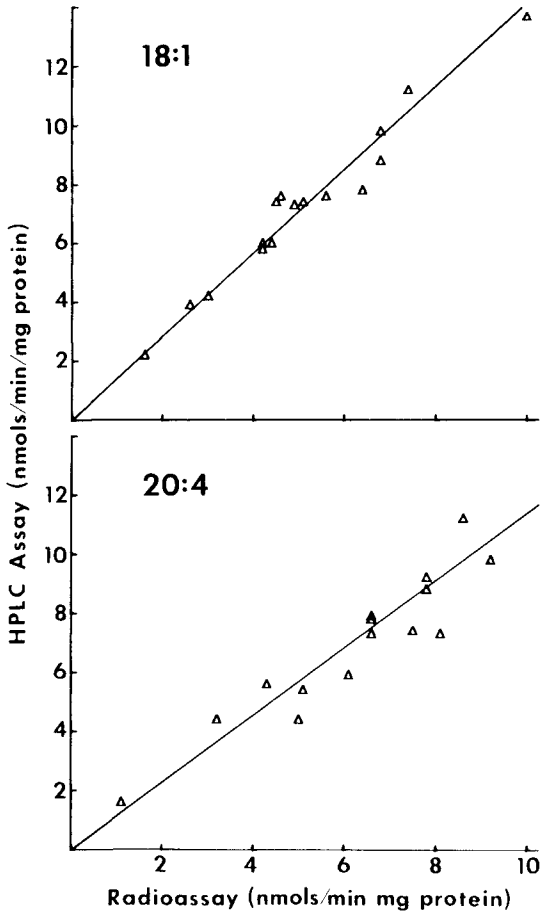

FIG. 1. Comparison of spectrophotometric (HPLC) and radiometric acyl-CoA synthetase assays. Microsomes (0.1 mg) were incubated for 10 min with optimal concentrations of ATP and CoA and 0.01-3.2 mM [1-^{14}C]oleic (18:1) or [^{14}C]arachidonic (20:4) acids at pH 7.8. Product acyl-CoAs were separated by HPLC and measured by monitoring absorbance of effluent at 254 nm and radioactivity of effluent fractions corresponding to acyl-CoAs. Data points are means for duplicate or quadruplicate assays (SEM<10%). Data was fitted to a line by linear regression analysis.

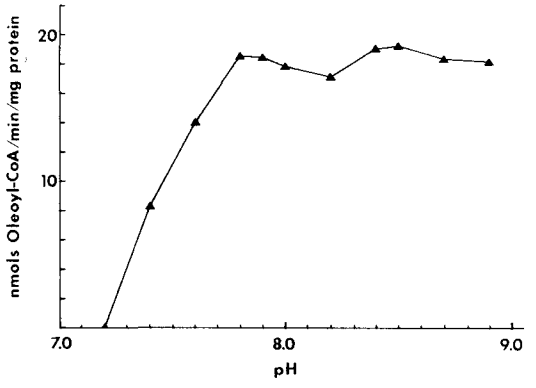

FIG. 2. Testicular microsomal acyl-CoA synthetase activity as a function of pH. Microsomes were incubated for 30 min with 1mM [1-^{14}C]oleic acid and optimal levels of ATP and CoA. Activity was measured by the radiometric method.

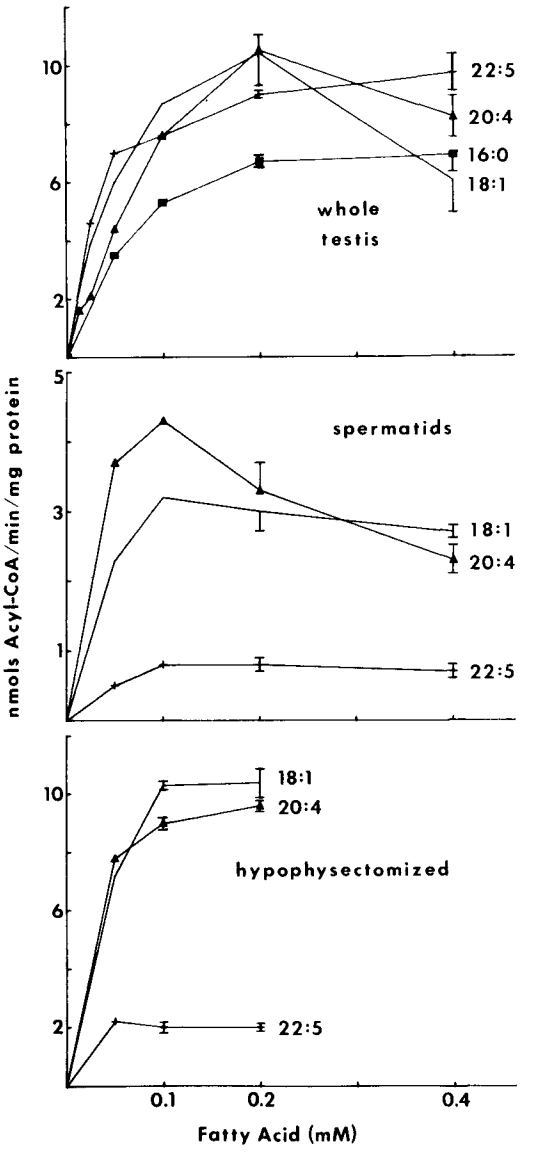

FIG. 3. Microsomal acyl-CoA synthetase activity with various fatty acid substrates as a function of substrate concentration. Microsomes from testes of normal and hyphophysectomized adult rats and from isolated spermatids were incubated for 10 min with optimal concentrations of ATP and CoA and the various fatty acids at the concentrations indicated. Product acyl-CoAs were separated by HPLC and measured by monitoring absorbance of effluent at 254 nm. Error bars at higher concentrations represent standard errors of the means and also are typical of values at lower concentrations. Data points represent triplicate or quadruplicate assays.

was obtained over ranges of 2.5-10 mM ATP and 0.25-1.0 mM CoA. The activity exhibited a broad pH optimum from 7.8 to 9.0, beyond which activity was not determined (Fig. 2). Below pH 7.8, activity dropped off sharply, disappearing at about pH 7.2. Synthetase activity from whole testis exhibited apparent Michaelis-Menten kinetics up to a fatty acid substrate concentration of 0.2-0.8 mM, depending on the fatty acid used (Fig. 3). Activity with 18:1, 20:4 and 22:5 was inhibited by higher substrate concentrations (Fig. 4). Apparent Michaelis constants (K_M), calculated from data which conformed to linearity in double reciprocal (Lineweaver-Burke) plots (Fig. 5), were 74 μM,

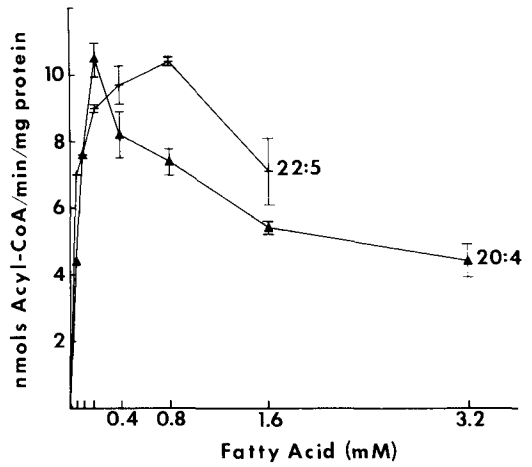

FIG. 4. Testicular microsomal acyl-CoA synthetase activity with polyenoic acids as a function of higher substrate concentrations. Activity was determined as described for Fig. 3. Activity of 18:1 closely paralleled that of 20:4 and thus was omitted for clarity. Error bars represent standard errors of the means.

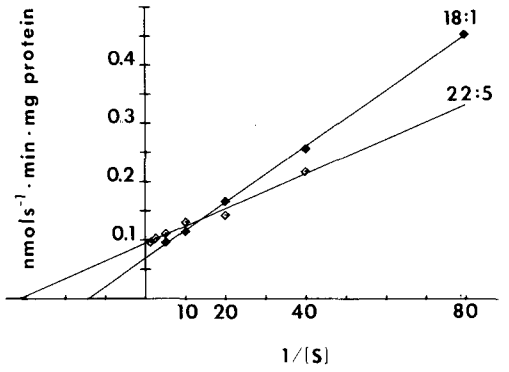

FIG. 5. Typical Lineweaver-Burke (double reciprocal) plots of activity vs substrate concentration for the testicular microsomal acyl-CoA synthetase. Analyses were carried out as described for Fig. 3. Data represent triplicate or quadruplicate analyses. Data points were fitted to a line and pseudokinetic constants were calculated by the statistical method of Wilkinson (15).

69 μM, 86 μM and 32 μM for 16:0, 18:1, 20:4 and 22:5, respectively. The corresponding calculated maximum velocities, 9, 14, 12 and 11 nmols/min/mg protein, are similar to those achieved at optimal substrate concentrations, suggesting that substrate inhibition does not play a significant role until the enzyme approaches saturation with substrate (Figs. 3-4).

The whole testis synthetase showed some relatively small but statistically significant differences ($p<.05$) in activity depending on the substrate fatty acid used. For example, at 0.1 and 0.2 mM, 16:0 yielded lower activity than the other three fatty acids. However, these differences were abolished or even reversed at other concentrations. With the exception of activities with 20:4 and 22:5 at 0.2 mM concentration, which differed by only about 15% ($p<.05$), there were no significant differences among activities with 18:1, 20:4 and 22:5 over the range 0.05-0.2 mM.

Acyl-CoA synthetase of spermatids. Activity of acyl-CoA synthetase in microsomes of isolated spermatids was substantially lower than that in microsomes of whole testis for 18:1, 20:4 and 22:5 substrates within the concentration range 0.05-0.4 mM (Fig. 3). This difference was significant ($p<.05$) at all concentrations tested with the exception of 20:4 at 0.05 mM. At all concentrations, spermatid synthetase activity with 22:5 was much lower ($p<.001$) than spermatid activity with 18:1 or 20:4 or whole testis activity with any of the four substrates used. Spermatid activities with 18:1 and 20:4 did not differ significantly from one another. As with the synthetase from whole testis, optimal activity was achieved at a substrate concentration of 0.1-0.2 mM for 18:1 and 20:4. Optimal concentration for 22:5 was more difficult to estimate due to the low levels of activity, but appeared to fall within the same range.

Acyl-CoA synthetase of hypophysectomized rats. Microsomal acyl-CoA synthetase activities from testes of hypophysectomized rats were quite similar to those of testes from normal rats when 18:1 or 20:4 was used as substrate (Fig. 3). Activities with both substrates approached apparent maximum velocities of about 10 nmol/min/mg protein at 0.1-0.2 mM. In contrast, activity with 22:5 approached an apparent maximum velocity of about 2 nmols/min/mg protein and was significantly lower than activities with 18:1 ($p<.01$) or 20:4 ($p<.005$) within the concentration range 0.05-0.2 mM. Activity with 22:5 also was lower ($p<.001$) than that obtained with microsomes of normal testis for 16:0, 18:1, 20:4 or 22:5 at 0.1 and 0.2 mM. However, activity with 22:5 was higher ($p<.005$) than spermatid synthetase activity with 22:5 at 0.1 and 0.2 mM.

DISCUSSION

HPLC assay. The assay for acyl-CoA synthetase described above is based on integration of the UV absorbance of acyl-CoA products as they elute from a reversed-phase HPLC column. This method has several advantages over radiometric or colorimetric procedures described previously (11) and, as this study shows, produces results comparable to those of the radiometric procedure (Fig. 1). The method obviated the necessity for purchase or synthesis of radioactive substrates, allowing the use of 22:5 n-6, which is not available from commercial sources but can be readily isolated from testis lipids as we describe here. The assay was absolutely specific for the individual substrates since HPLC retention times were characteristic of acyl-CoA products with specific acyl group moieties. The method also was insensitive to other enzymatic activities competing for substrate. This is an especially important consideration for the polyenoic acids, which are potential precursors to oxygenated derivatives (18-20). In the case of 20:4, detection of variable amounts of radioactivity with HPLC retention times other than those of the acyl-CoA or unesterified fatty acid suggested the presence of some of these competing activities in the assay system. In the case of the radiometric assay, isolation of the acyl-CoA by HPLC prior to measurement alleviated contamination of water soluble products by radioactive substrates (data not shown) that, without the HPLC separation, would require substantial corrections from boiled con-

trols at each substrate concentration used. The HPLC spectrophotometric method described here required no blank corrections.

Properties of the acyl-CoA synthetase. The microsomal acyl-CoA synthetase is a key enzyme for incorporation of long chain fatty acids into glycerolipids, by virtue of the requirement for CoA thioesters by the microsomal transacylases (12,13) and the collocation of these activities. The long chain acyl-CoA synthetase of rat testis microsomes is similar in properties to the single enzyme isolated from both microsomes and mitochondria of rat liver (11). Both enzymes exhibit a broad alkaline pH optimum (pH 7.4-9.1 for the purified liver enzyme) and show broad specificity for 16-20 carbon fatty acids (Figs. 2-4). As with the liver enzyme, the testis synthetase apparently utilizes the unsaturated fatty acids (18:1, 20:4 and 22:5) somewhat more efficiently than the saturated 16:0 (Fig. 3). Specific activities under optimal conditions are an order of magnitude lower than those reported in liver microsomes, but several-fold higher than those reported for the combined activity of the synthetase and lysolecithin acyltransferase (EC 2.3.1.23) in a similar testicular system (13). A similar coupling of the synthetase with the testicular diacylglycerol acyltransferase (EC 2.3.1.20) resulted in 20-30% of the specific activity of the synthetase alone (unpublished results from this laboratory). These data suggest that the synthetase is not rate-limiting for acylglycerol or phosphoglyceride synthesis in the whole testis.

Linearity of the synthetase activity with time up to 40 min suggests that affinity of the enzyme for acyl-CoA is relatively low and average velocities are a reliable estimate of initial velocities. Although lack of information concerning the effective concentrations of micellar substrates and apparent substrate inhibition at higher concentrations makes interpretation of kinetic data difficult, the enzyme activity appears to adhere to classical Michaelis-Menten kinetics over a broad range of substrate concentrations, as judged by linear double reciprocal plots (Fig. 5). Apparent values of K_M are similar for 16:0, 18:1 and 20:4 (74, 69 and 86 μM, respectively), and the synthetase attained maximum specific activities at about the same substrate concentration for each of these substrates. These observations are consistent with a single enzyme with broad specificity as reported for the liver (11). However, the apparent K_M for 22:5 (32 μM) is less than half that for each of the other substrates and substrate inhibition was not observed until 1.6 mM, at least four-fold the concentration required for inhibition with 18:1 or 20:4 (Fig. 4). This differential behavior with 22:5 is consistent with the presence of more than one enzyme activity.

Acyl-CoA synthetase of spermatids. Glycerolipids of the postmeiotic haploid germinal cells, the spermatids, contain the highest concentrations of 22:5 among the testis cell types (4-6). The microsomal acyl-CoA synthetase of isolated spermatids consistently was lower in specific activity than that of the whole testis (Fig. 3). Behavior of the enzyme with respect to substrate concentration did not seem different from that of whole testis in the case of 18:1 or 20:4, although insufficient data was available for calculation of reliable pseudokinetic constants. However, unlike the whole testis activity, the spermatid activity with 22:5 was only 15-30% of that with the other two substrates at all concentrations tested. Moreover, spermatid activity with 22:5 was more than an order of magnitude lower than that of whole testis. This differential decrease in activity with 22:5 suggests the involvement of at least two testicular enzymes with differing specificities for fatty acid substrates. It is possible that the overall decreases in synthetase activity in spermatids are due to the cell separation procedure, although previous studies have shown these cells to be metabolically competent in the mouse (8,9). However, even in this event, activity with 22:5 is differentiated by a disproportionate decrease in activity.

Acyl-CoA synthetase of hypophysectomized rats. Hypophysectomy is known to prevent differentiation of germinal cells, which accumulate 22:5 in rat testes (21,22). The acyl-CoA synthetase of whole testis microsomes from hypophysectomized rats had specific activities with 18:1 and 20:4 equivalent to those of whole testis from normal adults. In contrast, activity with 22:5 is much lower than in normal rats. This differential expression of activities with different fatty acids once again suggests multiple synthetases. Hypophysectomy apparently selectively inhibits expression of the activity with 22:5, suggesting the presence of a hormonally regulated synthetase that is specific for 22:5 and expressed under conditions which permit spermatogenesis. It is unclear whether this activity is regulated directly or simply reflects the presence of different cell populations. The observation that the spermatids, the largest class of testicular cells failing to develop in hypophysectomized rats, have very low levels of synthetase activity with 22:5 is evidence that this decrease is not merely a reflection of the differential in cell composition.

Thus, the acyl-CoA synthetase activity specific for 22:5 can be differentiated from that specific for other fatty acids by pseudokinetic parameters, by differential distribution within the testis and by differential response to hormonal deprivation. Differential expression of the 22:5-specific acyl-CoA synthetase suggests a role for the synthetase in specifying the characteristic distribution and metabolism of 22:5 in rat testis. A better understanding of this role may shed light on the specific and obligatory function of long chain polyenoic acids in spermatogenesis.

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Altered Arachidonic Acid Content in Polymorphonuclear and Mononuclear Cells from Patients with Allergic Rhinitis and/or Asthma

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We previously have found that monocytes from patients with allergic rhinitis and/or asthma produce less PGE_2 than cells from normal subjects in response to a histamine-induced lymphokine. In order to investigate this observation further, we measured the fatty acid content in the total phospholipids derived from the plasma, red cells, buffy coat cells, neutrophils, monocytes and lymphocytes of 27 allergic patients and 21 normal controls. There were no substantial differences between atopics and normals in the fatty acid analyses carried out for plasma and red cells. However, linoleic acid (18:2n-6) levels were elevated significantly in the buffy coat fraction, while arachidonic acid (20:4n-6) levels were reduced. Measurement of fatty acid levels after fractionation of the buffy coat population into neutrophils and monocytes yielded similar elevations in 18:2n-6 and reduced 20:4n-6. In contrast, lymphocytes appeared to have the reverse pattern, i.e., significantly reduced 18:2n-6 and elevated 20:4n-6 levels. These data suggest that atopic leukocytes may have altered essential fatty acid metabolism.
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Histamine receptor-bearing T lymphocytes have been shown to modulate the function of both T and B cells (1). Suppression of lectin-induced lymphocyte proliferation by histamine-activated T cells in vitro involves the participation of monocytes in both the generation and the effector stages (2,3). Activation of histamine-induced suppressor T cells requires an additional signal besides the ligand. The latter has been identified as interleukin 1 (3). Furthermore, histamine-induced suppressor factor (HSF), a product of histamine-stimulated T cells, has as one of its targets a prostaglandin-producing cell. The latter cell has been identified as a monocyte, and HSF has been shown to augment the production of thromboxane B_2, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ (4).

Atopic subjects have been documented to have abnormal histamine-induced (but not concanavalin A-induced) suppressor T cell function. The latter observation correlated with decreased phenotypic expression of T cell histamine type II receptors, but not of T cell histamine type I receptors, when compared to nonatopic control (5). An analysis in vitro of the defect in this histamine-induced suppressor system in atopic subjects has revealed that their lymphocytes have diminished HSF production and even if this lymphokine is provided, their monocytes produce less prostaglandin E_2 (6). To further investigate the observation that monocytes from atopic subjects produce less prostaglandin E_2, we have examined the fatty acid content present in phospholipids derived from plasma, red blood cells and leukocytes from normal subjects and atopic patients. Our study reveals a reduction in the arachidonic acid content of phospholipids in the monocyte and neutrophil fractions obtained from atopic subjects, while arachidonic acid levels in atopic lymphocytes were elevated relative to control subjects.

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MATERIALS AND METHODS

Patients. The study population consisted of 27 patients with documented seasonal allergic rhinitis and/or asthma. The patients were asymptomatic at the time of study and had at least one symptomatic season as well as one or more positive immediate skin reactions which correlated with the clinical history. All patients had a positive family history for atopy. The control population was 21 age-matched adults with negative personal and family history of atopy. There were 15 males and six females in the control group and 15 males and 12 females in the atopic group. The age range in the atopic population was 20-55 yr with a mean of 30.4 yr and in the control population was 20-40 yr with a mean of 28.8 yr. Subjects in both groups were caucasian and had similar nutritional status and drug history (none were taking nonsteroidal anti-inflammatory agents or birth control drugs).

Cell preparation. Samples of plasma, red cell, buffy coat, lymphocytes, monocytes and neutrophils were prepared in the following manner. One hundred ml of venous blood were drawn into heparinized syringes. One aliquot was centrifuged at 400 × g to remove the cellular components and the plasma was carefully removed and frozen at −20 C until assay. Another aliquot was allowed to sediment in the presence of 10% Dextran (Dextran 70, Pharmacia, Piscataway, New Jersey) for 30 min at 37 C. The latter leukocyte-rich plasma was expressed into a test tube. The remaining red cells then were expressed into a separate test tube, washed 2-3 times in RPMI-1640 (K.C. Biologics, Lanexa, Kansas) and frozen at 20 C until assay. Buffy coat samples were obtained by sedimentation of the red cells with Dextran as above and the white cells were expressed, washed 2-3 times in RPMI-1640 and frozen at −20 C. The white cell fraction was subdivided further by initial separation on Hypaque gradients. The latter method depletes platelets from the mononuclear cell fraction. Mononuclear cells were obtained by centrifugation in Ficoll-hypaque gradients (lymphocyte separation media, Litton Bionetics, Charleston, South Carolina), washed twice in RPMI-1640 and further separated into monocyte

TABLE 1

Fatty Acid Concentrations in the Total Phospholipids of Red Cells, Plasma and Total White Cells from Controls and Atopic Patients with Allergic Rhinitis and/or Asthma

	Red blood cells		Plasma		Buffy coat	
Fatty acids	Control	Atopic	Control	Atopic	Control	Atopic
n-6						
18:2	15.7± 2.5	15.8±2.6	27.2±3.5	26.0±3.5	9.3±3.9	11.3±3.9*[a]
18:3	0.2± 0.7	0.6±0.7	0.2±0.3	0.2±0.2	0.8±0.6	0.4±0.3
20:3	2.1± 0.4	2.3±0.5	2.9±0.8	2.8±0.5	2.0±0.8	1.8±0.7
20:4	15.0± 4.1	16.5±4.1*	11.5±1.8	12.3±1.6	18.8±3.7	12.8±4.3***
22:4	2.6±41.0	2.7±0.8	0.4±0.3	0.5±0.2	2.6±0.9	2.1±0.9
22:5	0.2± 0.3	0.4±0.8	0.1±0.2	0.1±0.2	0.2±0.2	0.8±0.9
n-3						
18:3	0.1±0.1	0.3±0.3*	0.1±0.1	0.1±0.1	0.8±0.5	1.2±1.0
20:5	0.3±0.2	0.2±0.4	0.6±0.5	0.5±0.3	0.4±0.8	0.3±0.7
22:5	1.6±0.6	1.2±0.6	0.7±0.3	0.7±0.2	1.1±0.4	0.6±0.4*
22:6	4.5±2.0	4.4±1.8	4.0±1.0	3.1±0.7**	1.4±0.6	1.4±1.6
Others						
16:0	23.7±4.2	23.5±3.4	26.4±1.3	26.7±1.4	17.1±1.7	15.7±3.3
18:0	14.2±2.7	12.9±2.6	11.8±1.8	10.8±1.9	20:0+2.5	20.9±1.9
18:1	16.6±2.1	15.9±2.2	11.5±0.9	12.5±1.7*	23.6±4.8	21.5±1.8*
18:2/20:4	1.1	1.0	2.4	2.1	0.5	0.9

Results are expressed as mean ± SD in mg/100 mg total fatty acids present. Results were analyzed by Student's t-test.

[a]Significant differences from control are indicated by *p = 0.05, **p = 0.01 and ***p = 0.001.

and lymphocyte fractions by centrifugation on percoll gradients (percoll from Pharmacia). One hundred million mononuclear cells in 2 ml were layered onto gradients of 40%, 50%, 60% and 70% percoll (7). The tubes were centrifuged at 1500 × g for 10 min at room temperature. The monocyte and lymphocyte fractions were recovered from the 50% and 60-70% layers, respectively. Each cell fraction was washed twice in RPMI-1640, the supernatant was aspirated and the cell pellet was frozen at −20 C. Each lymphocyte and monocyte fraction contained greater than 90-95% enriched cells (as assessed by E-rosetting and nonspecific esterase staining). Neutrophils were recovered from the bottom of the tube containing Ficoll-hypaque, and any contaminating red cells were eliminated by osmotic lysis in ammonium chloride (0.16 M).

Fatty acid determination. Fatty acid levels were determined as described previously (8). BHT was added to samples during processing to prevent oxidation. Plasma samples were extracted with chloroform/methanol (2:1, v/v). The extract was filtered through sodium sulphate, evaporated to dryness and taken up in 0.5 ml chloroform/methanol. The lipid fractions were separated by thin layer chromatography (TLC) on silica gel plates (E. Merck, Darmstadt, West Germany). The phospholipid fraction was methylated using boron trifluoride/methanol. The phospholipid fraction contains the full range of unsaturated fatty acids and is believed to provide fatty acid precursors for prostaglandin synthesis. The resulting methyl esters were separated and measured using a Hewlett-Packard 5880 gas chromatograph with a 6 ft column packed with 10% silar on chromosorb WAW 106/230. The carrier was helium (30 ml/min). Oven temperature was programmed to rise from 165 C to 190 C and 2 C/min. Detector temperature was 220 C and injector temperature 200 C. Retention times and peak areas were computed automatically by a Hewlett-Packard Level 4 integrator. Peaks were identified by comparison with standard fatty acid methyl esters from NuChek Prep Inc. (Elysian, Minnesota).

Red and white blood cells were washed and lipids were extracted according to the procedure of Dodge and Phillips (9). High performance liquid chromatography (HPLC) grade reagents from Fisher Scientific Co. (Ottawa, Ontario) were used throughout. Total phospholipids were separated by TLC. The fatty acids in the phospholipids then were methylated and analyzed as described. Results from control and atopic patients were compared by Student's t-test.

RESULTS

The fatty acid analysis of plasma, red cells and buffy coat cells is shown in Table 1. There were few differences between the normal and atopic groups in terms of plasma and red cell fatty acid analyses. Arachidonic acid (20:4 n-6) was elevated in the atopics' red cells and 20:6n-3 was reduced in the atopics' plasma. In contrast, there were clear cut differences between the two groups in the buffy coat cell phospholipids. The main dietary essential fatty acid (EFA), linoleic acid (18:2n-6), was elevated, whereas its metabolites, especially arachidonic acid (20:4n-6), were significantly reduced. The ratio of 18:2/20:4, commonly used as an indicator of the rate of linoleic acid metabolism along the EFA pathway, was elevated substantially in the atopic population.

When the buffy coat preparation was subdivided in polymorphonuclear leukocytes, lymphocytes and monocytes, major differences emerged between the subpopulations of cells (Table 2). In the polymorphonuclear leukocyte and monocyte populations, the differences between controls

TABLE 2

Fatty Acid Concentrations in the Total Phospholipids of Separated Polymorphs, Monocytes and Lymphocytes from Controls and Atopic Patients with Allergic Rhinitis and/or Asthma

	Polymorphonuclear Leukocytes		Monocytes		Lymphocytes	
Fatty acids	Control	Atopic	Control	Atopic	Control	Atopic
n-6						
18:2	12.4±3.1	13.2±3.1	14.2±4.7	17.1±5.9*[a]	18.4±4.8	11.7±3.5**
18:3	0.1±0.1	0.1±0.2	0.1±0.1*	0.1±0.1*	---	
20:3	1.7±0.8	1.1±09.5	1.4±0.6	1.3±0.0	1.8±1.2	2.3±1.1
20:4	11.2±4.4	9.6±2.8*	15.4±4.0	13.1±4.0*	13.7±6.0	16.2±4.1*
22:4	2.0±1.3	1.3±0.8	1.0±0.8	1.0±0.8	1.0±0.9	1.3±1.1
22:5	0.3±0.3	0.3±0.4	1.2±1.2	2.2±2.0	1.3±1.7	0.5±0.6
n-3						
18:3	1.3±0.6	1.4±0.2	2.6±2.6	1.1±1.2	1.1±1.2	0.8±1.0
20:5	0.2±0.1	0.2±0.2	0.1±0.1	0.0±0.0	0.4±0.7	0.0±0.1
22:5	0.7±0.4	0.7±0.4	0.6±0.7	0.5±0.7	0.9±0.7	0.9±0.8
22:6	1.1±0.8	1.2±0.6	1.0±0.9	0.6±1.0	1.2±0.9	1.5±0.9
Others						
16:0	17.9±2.6	19.7±2.4	18.6±2.0	20.3±3.9	19.2±3.6	22.6±3.5*
18:0	15.7±4.3	15.3±4.4	16.6±3.0	16.2±5.2	16.1±4.4	19.0±3.3*
18:1	29.3±4.3	28.8±3.3	22.4±3.1	22.3±2.6	21.1±4.6	18.6±3.7
18:2/20:4	1.1	1.4	0.9	1.3	1.3	0.7

Results are expressed as mean ± SD in mg/100 mg total fatty acids present. Results were analyzed by Student's t-test.

[a]Significant differences from control are indicated by *p = 0.05 and **p = 0.01.

and atopics were reflective of the total white cell population. Linoleic acid concentrations were elevated and its metabolites, including arachidonic acid, were reduced. In contrast, the changes in the lymphocyte population were diametrically opposite, with low levels of linoleic acid and elevated concentrations of arachidonic acid found.

DISCUSSION

These results are consistent with our previous data, which suggest that atopy is associated with a reduced ability of the monocytes to make PGE_2 (6). In the total white cell fraction and in the separated neutrophils and monocytes, the concentrations of the PGE_2 precursor, arachidonic acid, were reduced. A reduced level of arachidonic acid might be caused by excessive formation of 2-series PGs, or might itself be a cause of reduced formation of PGs. We suspect the latter is the case because of the concomitant changes in linoleic acid. If the low level of arachidonate were caused by excessive consumption, one would expect to see increased conversion of linoleic acid through to arachidonic, with reduced concentrations of linoleic acid as well. In fact, the levels of linoleic acid were above normal, suggesting there may be reduced conversion of linoleic acid to arachidonate in these cells. This would lead to a reduced availability of the precursor for the 2-series PG synthesis.

In contrast to the other white cell fractions, the atopics' lymphocytes had increased concentrations of arachidonic acid strikingly and reduced amounts of linoleic, suggesting enhanced metabolism of 18:2 to 20:4. We have no explanation for this finding. Further studies are necessary to clarify the differences between the arachidonic acid content in lymphocytes vs neutrophils and monocytes.

We are aware that the results from the buffy coat fraction cannot be correlated fully with the results from the separated fractions. The intact buffy coat preparation may contain other cells (e.g., platelets) not found in the separated fractions, and this requires further study.

The analysis of fatty acids in separated lymphocytes, monocytes and neutrophils was repeated on two separate occasions. The differences between fatty acid composition of lymphocytes and neutrophils from normal and atopic subjects failed to show any consistent trend. However, on both occasions, linoleic acid levels were elevated significantly and arachidonic acid levels were reduced significantly in the monocyte fraction. This is the white cell fraction that has been shown to generate reduced amounts of PGE_2 in atopic individuals (6). When the phospholipids were separated into phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine/phosphatidylinositol (PS/PI), PE and the PS/PI fractions both were found to contain reduced levels of arachidonic acid in the atopic patients (unpublished data). This reduced availability of arachidonic acid may relate to the reduced PGE_2 production. Although the reduction in arachidonic acid was relatively small, animals fed an EFA-deficient diet have been shown to have very low PGE production, in spite of little or no change in tissue arachidonic acid levels (10,11). Thus, PGE production appears related to only a small fraction of the total arachidonic acid present. This fraction seems to be supplied by dietary linoleic acid directly, and changes in it can produce drastic changes in PG production. The precise nature of this arachidonic acid component has not yet been identified.

It was noted in 1937 that patients with atopic eczema had reduced levels of plasma EFAs and an apparent inability to convert dietary linoleic acid to arachidonic acid (12). It recently has been shown that both plasma and red cells from patients with atopic eczema have elevated concentrations of linoleic acid and low levels of its metabolites, including arachidonic acid (13,14). The latter pat-

tern is similar to that observed in this study in the buffy coat fraction, monocytes and neutrophils. It therefore seems possible that atopic disorders are associated with abnormalities of EFA metabolism and consequently with changes in PG production. The EFA changes would explain the reduced ability of some cell lines in atopics to produce PGs (6) and also possibly the sensitivity of many atopics to the side effects of PG synthesis-inhibiting nonsteroidal anti-inflammatory drugs. Someone with an already lowered PG synthesis would be expected to be unusually susceptible to these agents. Atopic disorders may differ in the tissues in which these EFA/PG abnormalities are expressed. In atopic eczema, the abnormalities may be generalized and expressed in the plasma, whereas in allergic rhinitis or asthma, as in this study, they may be confined to specific cell types. Dietary correction of the EFA abnormality in eczema was associated with clinical improvement (15). It may be possible to devise nutritional strategies helpful in other forms of atopy.

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Measurement of the Incorporation of Orally Administered Arachidonic Acid into Tissue Lipids

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The applicability of a stable isotope method to monitor the mixing of dietary arachidonic acid with endogenous arachidonic acid in tissue lipids was evaluated. Rats were fed octadeuterated arachidonic acid during a 20-day period, and the entry of the dietary acid into lipid esters of various tissues was examined by gas chromatography-mass spectrometric (GC-MS) analysis of their fatty acids. The rats were maintained on a fat-free diet from weaning until 63 days old to enhance the ratio of the dietary acid to endogenous arachidonate. Three separate forms of eicosatetraenoic acid in the tissue lipids could be distinguished by GC-MS: octadeuterated arachidonic acid (recent dietary origin), unlabeled arachidonic acid (maternal origin) and unlabeled 4,7,10,13-eicosatetraenoic acid (originating from palmitoleic acid).

The total eicosatetraenoic acid in the tissue lipids contained about 90% arachidonate from recent dietary origin in lung, kidney, heart and fat, 70% in muscle and liver and 27% in brain. The n-7 isomer of eicosatetraenoic acid was estimated to make up 6% or less of the total eicosatetraenoic acid in lung, kidney, brain, muscle and heart tissue lipids, but it comprised around 15% of the total eicosatetraenoic acid in liver. The unlabeled arachidonic acid of maternal origin thus comprised only about 10% of the eicosatetraenoic acid in all tissues examined except muscle and brain, where it was 24% and 70% of the eicosatetraenoic acid, respectively.

The relative amounts of the three forms of eicosatetraenoic acid are consistent with a limited access of dietary arachidonate to the brain tissue and with a competition between the dietary n-6 isomer and the endogenous n-7 isomer for esterification in the liver. Because most muscle mass would have formed after weaning, the high proportion of maternal arachidonate in the muscle lipids suggested that maternal arachidonate may have been displaced from other tissues to muscle, from which it equilibrated slowly with dietary arachidonate acid. The combination of deuterated arachidonic acid and GC-MS analysis thus furnished more detailed information about the composition and origin of eicosatetraenoic acid in tissue lipid esters than that previously available from radiotracer studies or GC-MS analyses alone.
Lipids 21, 21-25 (1986).

Arachidonic acid is the immediate precursor fatty acid for the biosynthesis of the more prevalent eicosanoids. Its metabolic disposition in relation to other polyunsaturated acids was studied extensively twenty years ago (1-3). Mead (1) noted its turnover relative to acids in triglycerides. However, recent evidence (4) of a very rapid generation of eicosanoids from dietary arachidonate reopened the question of the relative rates of arachidonate entry from dietary fats and tissue phospholipids into the nonesterified pool of eicosanoid precursors. Details of the incorporation of arachidonic acid from the diet into tissue lipids and of its subsequent mobilization from the lipids of different tissues thus are of considerable importance in understanding the overall physiological control of eicosanoid biosynthesis. We describe a method using octadeuterated arachidonic acid and GC-MS analysis that should prove useful in providing a quantitative description of the mixing of dietary arachidonate with endogenous arachidonate among the various internal pools.

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MATERIALS AND METHODS

To accentuate the uptake of dietary arachidonate, the endogenous pools of arachidonate were lowered by providing pregnant rats with a diet deficient in essential fatty acids one week prior to full term and continuing the diet after birth throughout the period of nursing.

Three male Sprague-Dawley rats born and weaned under these conditions then were maintained on a fat-free diet (ICN Nutritional Biochemicals, Cleveland, Ohio) for 63 days until they reached a body weight of 180-200 g (4). They then underwent a 20-day regimen (4) of intermittent supplementation via oral intubation with a total of 265 mg of deuterium-labeled arachidonic acid (Hoffman-La Roche Inc., Nutley, New Jersey). GC-MS analysis of the deuterium-labeled arachidonic acid indicated that 98.9% of it was octadeuterated. At the end of this period the rats were killed, various tissues were removed, and the tissue lipids were extracted as described previously (4).

Transesterification of the dried lipid extracts was accomplished by heating the lipids at 80 C in H_2SO_4/methanol (1:9, v/v) for two hr. The mixture then was shaken with equal volumes of pentane and water. The organic layer, containing the fatty acid methyl esters, was dried under a stream of nitrogen and the residue dissolved in ethyl acetate for analysis by GC-MS on a Finnigan model 4510 mass spectrometer. The gas chromatographic separations were done isothermally either on a 6 ft × $^1/_4$ in. glass column packed with 10% Silar 5CP on 80/100 mesh Gas-Chrom Q (Applied Science Co., State College, Pennsylvania) at a temperature of 230 C, or on a 30 m capillary column coated with SP-2330 (Supelco Inc., Bellefonte, Pennsylvania) at a temperature of 220 C. The injector and jet separator zones were kept at a temperature 20 C higher than the column. When chemical ionization was used, methane was the reagent gas and the ionizer energy was 70 eV. The mass range of m/e 50 to m/e 350 was scanned in two sec. Identification of the component fatty acid methyl esters in the mixtures was based on their mass spectra. Under the chemical ionization conditions used, the base peak in the spectra of all fatty acid methyl esters was the M+1 ion. The M-31 ($M-OCH_3$) ions also were prominent in the spectra. The relative areas of the peaks corresponding to the major tissue fatty acids were determined by cutting out the peaks from a photocopy of the total ion current profile (overlapping peaks were separated at the

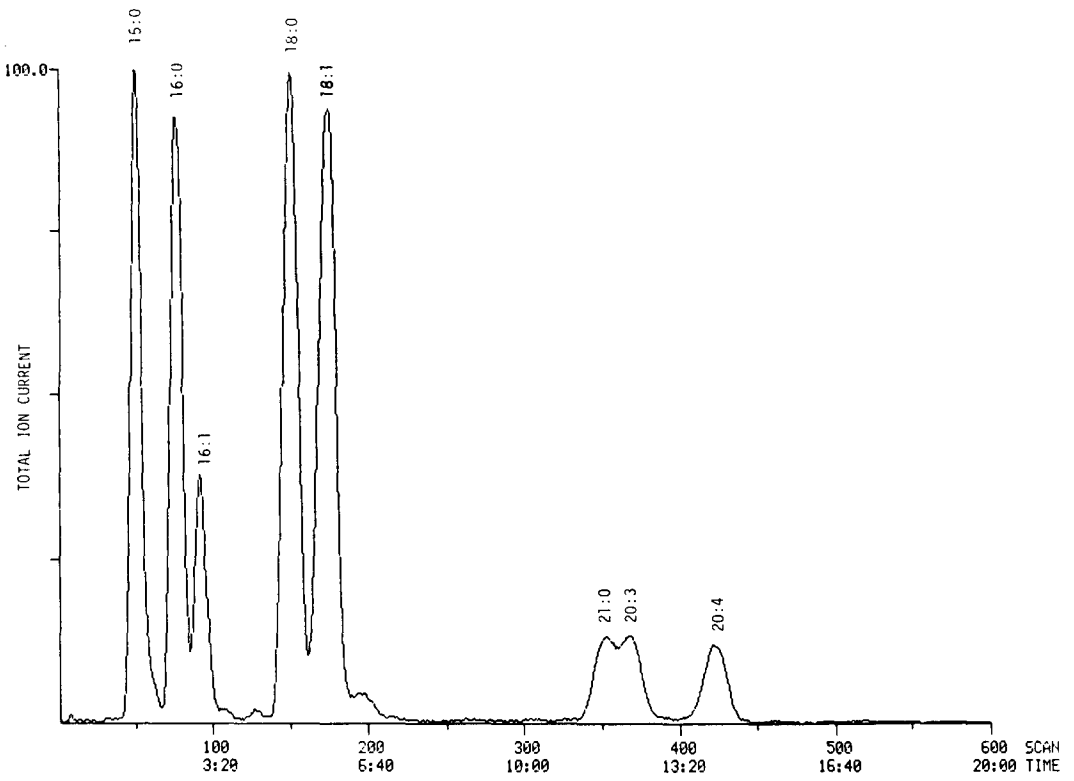

FIG. 1. Profile of fatty acid methyl esters from heart lipids of EFA-deficient rat after $^2H_8$-20:4 feeding. Fatty acid methyl esters obtained by transesterification of lipids from the heart tissue of rat 2 were analyzed by chemical ionization GC-MS at a column temperature of 230 C as described in Materials and Methods. The total ion current response of the mass spectrometer is shown as a function of the scan number and of the elution time (min). Full scale response was 111,000 ions.

minimum point between them) and weighing them on an analytical balance.

RESULTS AND DISCUSSION

The studies described in this report focus on the mixing of dietary and endogenous pools of arachidonate. The overall pattern of esterified fatty acids from the different tissues of one of the rats after the arachidonate feeding regimen was checked using the elution profile of the total ion current obtained during GC-MS analysis and represented an expected pattern. An example from the heart lipids of rat 2 is shown in Figure 1. Large peaks corresponding to the added internal standards, 15:0 (scan 51) and 21:0 (scan 353), are apparent. The major fatty acids esterified in the heart lipids were 16:0 (scan 77, 20 mol percent), 16:1 (scan 92, 8 mol percent), 18:0 (scan 151, 29 mol percent), 18:1 (scan 175, 30 mol percent), 20:3 (scan 368, 5 mol percent) and 20:4 (scan 423, 5 mol percent). The relatively small peak at scan 195 had an elution time consistent with 19:0 and was assigned to a 19:1 fatty acid methyl ester based on the base ion at m/e 311. Only small amounts (<20% of the base ion) of ions were observed in scan 195 at m/e 295 and m/e 293 (M+1 for 18:2 and 18:3) reflecting the absence of these polyunsaturated acids in the diet.

The overall fatty acid profile obtained for the total lipid in each tissue from rat 2 (Table 1) was similar from tissue to tissue, with 16:0, 16:1, 18:0 and 18:1 comprising more than 80% of the total fatty acids. Liver and adipose tissue did not contain a significant proportion of 16:1, perhaps reflecting a rapid metabolic processing of 16:1 in these tissues. All tissues except adipose had a significant proportion of 20:3, the classic indicator of a fatty acid-deficient diet (1). The proportion of 20:3 ranged from 1 mol percent for muscle lipids to 5 mol percent for heart lipid. The 2.9 mol percent of 20:3 in brain lipids in this study was similar to the reported accumulation of about 3 to 4 mol percent 20:3 in brain lipids of fatty acid-deficient rats (2).

Deuterated arachidonate in the tissue eicosatetraenoate. A peak corresponding to arachidonic acid was found in all tissues examined in all three rats, although it was in very small proportions in adipose and muscle. The proportion of the arachidonic acid among the total esters ranged from well below 1 mol percent in adipose and muscle lipids to almost 7 mol percent in kidney lipids.

The relative amounts of unlabeled (maternal) arachidonic acid and deuterated (dietary) arachidonic acid in the tissue lipids were determined by examination of the ions at m/e 319 (M+1 for the unlabeled 20:4 methyl ester) and m/e 327 (M+1 for the $^2H_8$–20:4 methyl esters). The signal intensities of these two ions were summed over the chromatographic peak corresponding to 20:4 methyl ester. The relative amounts of ions at m/e 327 and m/e 319 were used to determine the percentages of deuterated arachi-

TABLE 1

Fatty Acid Composition of Tissue Lipid Esters of a Rat After Feeding Deuterated Arachidonic Acid

	Weight percent								
Tissue	14:0	16:0	16:1	18:0	18:1	19:1[a]	20:1	20:3	20:4
Liver	--	25.0	tr	21.7	42.1	3.9	--	4.6	2.6
Lung	1.2	44.2	14.4	12.5	26.0	--	--	1.2	0.5
Kidney	1.2	26.2	13.4	16.5	30.5	1.8	--	3.7	6.7
Brain	--	27.4	5.1	29.7	24.6	3.4	1.7	2.9	5.1
Muscle	--	37.1	17.1	7.6	33.3	3.8	--	1.0	tr
Heart	--	20.3	8.0	29.4	29.9	1.6	--	5.3	5.3
Fat	--	20.3	tr	6.7	66.7	--	--	tr	tr

[a]Tentative assignment; base ion, m/e 311.

TABLE 2

Analysis of Eicosatetraenoic Acid in Tissue Lipids

	Percentage of total eicosatetraenoic acid as $^2H_3$–20:4n-6				Estimated percentages of unlabeled 20:4[a]	
	Rat 1	Rat 2	Rat 3	Average	20:4n-6	20:4n-7
Lung	79.9(n=1)	90.4±1.4(n=3)	92.4±4.2(n=2)	87.6±6.7	10	2
Liver	65.1	72.2±3.7(n=3)	77.0 (n=1)	71.4±6.0	15	15
Kidney	87.0	87.8±2.2(n=2)	84.6±4.2(n=2)	86.5±1.7	8	5
Brain	22.8	23.5±0.0(n=3)	33.5 (n=1)	26.6±6.0	70	nd
Muscle	60.5	73.2±3.0(n=3)	74.1 (n=1)	69.3±7.6	24	6
Heart	88.2	91.2±1.9(n=3)	90.9 (n=1)	90.1±1.7	7	3
Fat	79.0	86.0 (n=1)	90.3 (n=1)	85.1±5.7	--	
Blood	--	--	79.4 (n=1)			
Plasma	--	--	82.6 (n=1)			

[a]Distribution of unlabeled 20:4 estimated from the data shown in Fig. 3, as described in the text. nd, Not detected.

donic acid in the lipids of various tissues (Table 2). The agreement between replicate determinations of the proportion of labeled arachidonate in a given lipid sample from a single rat was within 5%, and the values for the percentage of deuterated (dietary) arachidonate in the eicosatetraenoate of a given tissue was similar among the different rats (Table 2). The deuterated arachidonate made up 85–90% of total eicosatetraenoate in the lipids of lung, kidney, heart and fat. However, only about 70% of the eicosatetraenoate in the liver and muscle lipids was deuterated, and only one-fourth of it in brain was in the deuterated form.

When pure standards were analyzed by GC-MS in the electron impact mode on a polar capillary column of SP-2330 (Fig. 2), the pure deuterated arachidonate methyl ester (m/e 326, M^+) eluted slightly before unlabeled arachidonate methyl ester (m/e 318, M^+). The values for the equivalent chain length (ECL) on the capillary column were 22.55 for $^2H_8$–20:4n-6 and 22.67 for 20:4n-6. Selected ion monitoring permitted individual detection of the two peaks even though the total ion current exhibited a single broad peak. The chemical ionization mass fragmentogram patterns for m/e 319 and m/e 327 in the fatty acid methyl esters of heart, muscle, brain, kidney and lung lipids (Fig. 3) confirmed that the octadeuterated 20:4 giving rise to the m/e 327 ion eluted slightly before the unlabeled 20:4 that gave rise to the ion at m/e 319. Further confirmation was obtained when the same elution

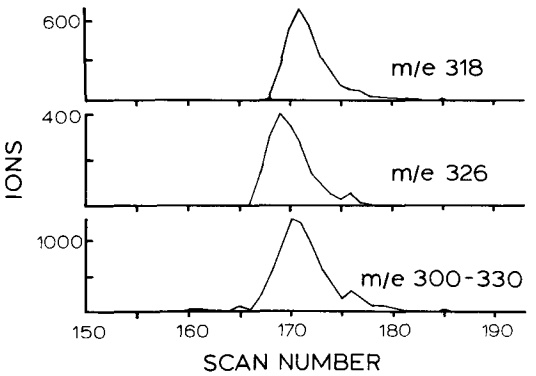

FIG. 2. GC-MS separation of standard unlabeled and deuterated arachidonic acid methyl esters. A mixture of 150 ng of unlabeled and 100 ng of octadeuterated arachidonate methyl esters was analyzed by GC-MS on a 30 m capillary column coated with SP-2330 at 220 C. Electron impact ionization at 70 eV was used and the mass range of m/e 300 to m/e 330 was scanned in two sec. Portions of the mass fragmentograms for the molecular ions of unlabeled (m/e 318) and deuterated (m/e 326) arachidonate methyl esters are shown, giving the number of ions detected in each mass range as a function of the scan number.

pattern was seen for the M-31 ions at m/e 295/287 (results not shown). The different scan numbers and elution times for 20:4 in Figures 1 to 3 are the result of changes in the chromatographic conditions for the different experiments, and did not affect the ECL values obtained.

The presence of an arachidonate isomer. In all cases

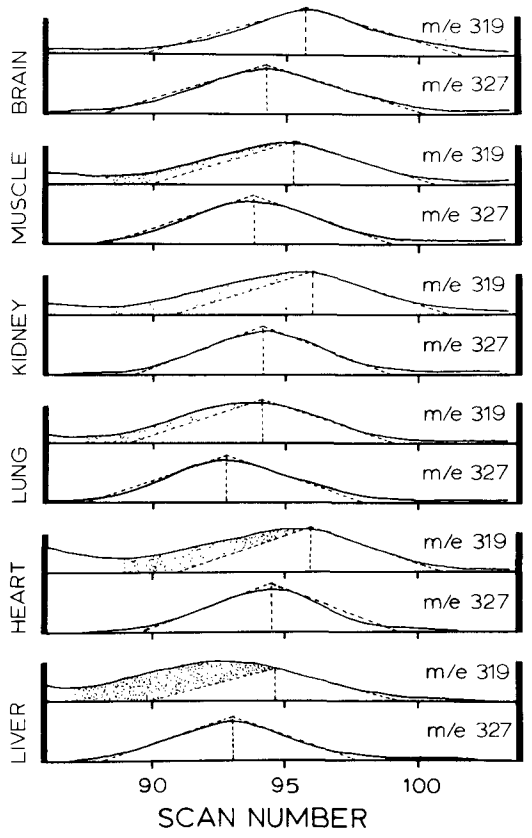

FIG. 3. Chemical ionization GC-MS analysis of labeled and unlabeled eicosatetraenoic acids from tissue lipids. Fatty methyl ester mixtures derived from tissue lipids were examined by GC-MS at a column temperature of 250 C as described in the text. A portion of the mass fragmentograms for the molecular ions of labeled (m/e 327) and unlabeled (m/e 319) eicosatetraenoic acid is shown for each tissue, with the shaded area indicating the part of the unlabeled eicosatetraenoic acid peak assigned to 20:4n-7 by the procedure described in the text. The vertical axis in each case indicates the intensity of ion current detected at the indicated m/e value.

using the chromatographic conditions in Figure 3, the peaks for the octadeutero isomer were symmetrical with a span of five scans to either side of the midpoint. This indicated that the 20:4 was chromatographing in the columns in a predictable manner without distortion of the peak geometry. This same geometry occurred for the peak for the unlabeled eicosatetraenoate of brain, with a consistent displacement of two scans later than the peak for the dietary octadeutero form. However, with the esters from the other tissues, the compound responsible for the ions at m/e 319 (M+1) and 287 (M−31) eluted in a considerably broader peak than the corresponding peaks for the octadeuterated acid (m/e 327 and 295) in the same chromatographic run. This indicated that considerable amounts of another isomer of arachidonic acid, eluting before the n-6 isomer, were present in the unlabeled eicosatetraenoic acid of the liver lipids. This is likely the 4,7,10,13-isomer (20:4n-7), formed from palmitoleic acid (16:1n-7), since 20:4n-7 has been reported in the lipids of rats on a diet deficient in essential fatty acids (3,5). The assignment of the n-7 structure to the isomer of eicosatetraenoic acid eluted earlier is supported by the observation (6) that the ECL value of a polyunsaturated fatty acid decreases as the distance between the methyl end of the molecule and the nearest double bond increases. Thus the n-7 isomer of 20:4 would be expected to elute before 20:4n-6 in the same manner as 18:2n-7 elutes before 18:2 n-6 and 20:3n-9 elutes before 20:3n-6 (7). A rough estimate of the relative amounts of the n-7 and n-6 isomers of the endogenous unlabeled 20:4 was obtained by assigning a symmetrical portion of the endogenous 20:4 peak (m/e 319) to arachidonate and the remainder to the n-7 isomer. Integration of the corresponding areas in the mass fragmentograms shown in Figure 3 provided the estimates presented in the last two columns in Table 2.

Incorporation and redistribution of eicosatetraenoates. The incorporation of dietary $^{2}H_{8}$–20:4 into the eicosatetraenoate of tissue lipids appeared limited by two separate factors: impeded access to the tissue and the presence of competing fatty acids. In brain tissue, most of the eicosatetraenoate from maternal origins (20:4n-6) appeared to be retained, with only a 27% penetration by the dietary eicosatetraenoate (indicated by the octadeuterated isomer) and undetectable amounts of the n-7 isomer. In this tissue, there appeared to be an impeded access of the external fatty acids to the tissue (or perhaps a much decreased turnover of 20:4), associated with the retention of large amounts of arachidonate of earlier origins. This tissue probably reached a significant portion of cell growth and lipid mass prior to weaning while maternal sources were dominant. Although we could not detect the n-7 isomer in brain lipids, it may have been present at less than 5% of the undeuterated 20:4, or less than 3.5% in overall 20:4. In the liver, which had the largest amounts of the isomeric 20:4n-7 (in amounts equal to maternal arachidonate), the penetration of deuterated dietary eicosatetraenoate was less than in other tissues. It may have been limited in part by competition from the endogenous polyunsaturated fatty acids (20:3n-9 and 20:4n-7) continuously synthesized from carbohydrate of the fat-free diet.

The considerable variation in the proportion of maternal 20:4n-6 in the tissues outside the brain, from 7% in heart to 24% in muscle (Table 2), suggested that the deuterated 20:4n-6 from the diet was not equilibrated yet with tissue arachidonate. Apparently, during the 20 days of intermittent 20:4 feeding, re-equilibration of the dietary and maternal acids among the tissues had not occurred yet. Kidney and heart tissue appeared to have replaced their 20:4 of maternal origin with the dietary 20:4. In contrast, muscle appeared to have acquired 20:4 by a redistribution process as the tissue developed (with similar ratios of n-7 isomer to maternal n-6) and was not rapidly exchanged with the dietary acid. Skeletal muscle may have acquired the maternal 20:4 during growth by a slow redistribution promoted by the endogenously formed 20:3 n-9 and 20:4n-7 in the developing rat. This hypothesis implies a selective acquisition by muscle of the displaced 20:4n-6, whereas other tissues such as kidney and heart would have released it in exchange for the other polyunsaturated acids, 20:3n-6 and 20:4n-7.

Extensive studies of the action of dietary polyunsaturated fatty acids upon tissue composition (e.g., 9,10) were reported just prior to the recognition of arachidonate as a precursor of prostaglandins. These studies showed that increased intake of polyunsaturated fatty acids developed

increased steady state levels of the 20- and 22-carbon elongated, desaturated products in tissues. Dietary linoleate increased the amount of 20:4 in liver lipids, but dietary arachidonate was about 10 times more effective (9). The n-7 isomer of 20:4 was not resolved in these studies in which the amounts of 20:4 and 20:3n-9 were reported to accumulate to different extents in liver, testes and heart (10). The studies used feeding periods of 59 and 100 days in accord with sound nutritional design. Later understanding of prostaglandin biosynthesis made apparent the importance of the kinetics of regulating the fatty acid supply (8), and investigators emphasized that the mobilization of arachidonate from tissue phospholipids is a physiologically controlled process. That control depends upon the turnover of the esterified pools of arachidonate stored in tissues. The use of a chemically identical but isotopically distinct form of arachidonate provides helpful evidence on the turnover that mixes endogenous and exogenous arachidonate forms.

The present results with eicosatetraenoate indicate that turnover of arachidonate occurred at relatively different speeds in different tissues. As more attention is given to the sources of substrates and inhibitors of eicosanoid biosynthesis, we expect that the dynamics of distribution and redistribution of these materials will have greater importance. The kinetics of turnover and mixing of dietary polyunsaturated fatty acids among the various tissue acids (and redistributing the endogenous ones) can have an important influence upon our interpretations of the availability of eicosanoid precursors in these tissues.

ACKNOWLEDGMENTS

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Papers from the **H. W. Kircher Memorial Symposium** on

Chemistry Biosynthesis and Function of Sterols

presented at the 76th AOCS Annual Meeting in Philadelphia, Pennsylvania, May 1985

In Remembrance of Henry Kircher

Wolfgang Sucrow
University of Paderborn, Paderborn, West Germany

This sterol symposium is dedicated to the memory of Henry Kircher, who died on January 5, 1984. Professor Kircher organized a number of sterol symposia in the past years, three of them in New Orleans and one in Philadelphia. Henry's sterol symposia were highlights for the small but worldwide community of sterol chemists. I am sure he felt pleasure in organizing these meetings. As speakers he selected well-known scientists as well as young colleagues with sometimes only a few, but promising and significant, papers. Henry picked them up from the literature. He did not just read dutifully as do most of us; he enjoyed studying the literature of a wide array of scientific interest and therefore was able to make a good choice of papers and people for such meetings. Henry also was an associate editor of *Lipids*.

It seemed to me Henry Kircher enjoyed most things he did more than other people do. Of course, there were some he did not like, and as a consequence avoided doing them. One thing he certainly enjoyed very much was working in the lab. I could see that during his sabbatical stay in Berlin and Paderborn in 1975/76. He worked in the lab with dedication and efficiency. He used to say that professors are lucky because the government pays them just for doing their hobbies.

Those of you who knew Henry also will remember his sense of humor, which was a very distinct aspect of his character. His humor could not be called sensitive; actually it was rather harsh, but full of plain truth. Of course, truth is not always welcome. Asked for his opinion, Henry might say, "Shall I be honest or polite?"

Henry had a lot of interests outside chemistry. In his curriculum vitae for the Deutsche Forschungsgemeinschaft he mentioned music, reading, talking, traveling, fishing, boating and hiking. Except for fishing and boating, I had ample occasions to realize the intensity with which Henry devoted himself to each of them.

Henry Winfried Kircher was born on April 7, 1925 in Frankfurt, Germany. His parents emigrated to the United States when he was four. He was educated in primary and secondary schools in Chicago. After his military service from 1946 to 1949, he attended Northwestern University where he was graduated with a B.Sc. For postgraduate studies he changed to the University of Chicago, where he earned a Ph.D. in 1953 under the supervision of Professor Weldon G. Brown. His work was about "The Reduction of Diketene and Structurally Related Compounds with Lithium Aluminum Hydride." In this same year (1953), he was employed as a research chemist by Rayonier Inc. at Shelton, Washington, where he worked for five years on different aspects of polysaccharide chemistry and Prins reactions of angelica lactones. In 1958 Henry decided to start an academic career and entered the Department of Agricultural Biochemistry of the University of Arizona as an assistant professor. He became an associate professor in 1962 and a full professor in 1966.

In Tucson, Henry began working on topics related to agriculture and biochemistry. One main topic concerned fatty acids—mainly the reactions and influence of sterculic acid on the nutrition of poultry—and the activity of lipases. From about 1965 the other topic began to evolve; it may be described as the ecological interdependence of desert cacti, insects, yeasts and sterols. Insects living on cacti eat the cactus sterols and modify them for their own use. In the context of this research program, quite a number of insects and plants were investigated for their sterol and triterpene contents. This work did not remain solely analytical, as a number of sterols which could not be isolated in a pure state had to be synthesized. It was the challenge of sterol synthesis that formally brought Henry and me close together.

Henry Kircher spent two sabbaticals on leave from Tucson, one in 1965/66 as a Visiting Scientist at the US Department of Agriculture Southern Regional Research Laboratory, New Orleans, and the other in 1975/76 as a guest professor in our lab at the Technische Universität Berlin and at the University of Paderborn through the Deutsche Forschungsgemeinschaft. He had planned another sabbatical for this year.

The results of Henry Kircher's scientific work are laid down in at least 70 papers. I believe that through these papers Henry will go on being with us, with those who knew him personally and with all those others who look into the literature seeking advice for analytic or synthetic problems in sterol chemistry. It is an honor and a pleasure to introduce the papers of this symposium. I am glad to say that all of the authors of this symposium are distinguished scientists in the sense of Henry Kircher's standards. He would be happy to be with us today.

Oxysterols: Chemical Synthesis, Biosynthesis and Biological Activities

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As a class of compounds, oxysterols have demonstrated a wide variety of biological properties. Due to the general interest in these compounds, new methods of chemical synthesis have been developed to provide them for biological investigation. The specific inhibition by oxysterols of cholesterol biosynthesis in mammalian cells has been shown to result primarily from a decrease in cellular levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity. Recent evidence suggests these cellular responses may be mediated by an oxysterol binding protein found in the cytosol of many lines of cultured cells. In certain instances, oxysterols have been shown to be produced in biological systems. These results support the supposition that oxysterols may regulate sterol biosynthesis at the cellular level. Included herein are the inhibitory effects of 9α, 11α-epoxycholest-7-en-3β-ol cholest-8-en-3β-ol-7-one and cholest-8-en-3β-ol-11-one on HMG-CoA reductase activity and their relative affinities for a cytosolic binding protein.
***Lipids 21,* 27-30 (1986).**

Steroids bearing a second oxygen function in addition to that at carbon-3 have demonstrated a variety of diverse biological activities (1-3). Some of these include cytotoxicity, atherogenicity, carcinogenicity, mutagenicity, hypocholesterolemia and effects on specific enzymes. In addition, several oxysterols have been isolated from drugs used in folk medicine for the treatment of cancer (4-6). Other studies have shown that certain oxysterols have significant activity in the inhibition of DNA synthesis in cultured cells (7,8). A number of oxygenated derivatives of cholesterol and sterol intermediates in cholesterol biosynthesis have been found to be potent inhibitors of sterol biosynthesis in animal cells in culture (2,3,9). The specific inhibition of cholesterol biosynthesis in mammalian cells by oxygenated derivatives of cholesterol and lanosterol has been shown in many cases to decrease cellular levels of HMG-CoA reductase activity. This response has been attributed to a decreased rate of HMG-CoA reductase synthesis (9-11) and in some instances to an increase in enzyme degradation (9,11).

A large number of oxysterols have been evaluated for their abilities to repress HMG-CoA reductase activity in cultured mammalian cells (2,3,12). In general, potency has been found to vary over a wide range depending on the structural features of the oxysterol. As a general trend, inhibitory activity increases as the distance between carbon-3 and the second oxygen function becomes greater. Steroids with oxygen groups in ring D and the side chain have been shown to have the greatest activity. An intact side chain is a requirement for potent activity; a decrease in the length of the (iso-octyl) side chain results in decreased activity (13). Other noticeable trends indicate a relationship between inhibitory activity and the extent to which the second oxygen function is sterically hindered. In general, axial hydroxyl groups are more hindered and possess lower activities than the less hindered equatorial conformation (2,12,13). Steric hindrance from other parts of the steroid molecule also can result in diminished activity (i.e., effect of carbon-14 alkyl substituents on the carbon-15 hydroxyl group) (14). It has been suggested that oxygen functions in conformationally flexible positions such as those in ring D and in the side chain produce more inhibitory steroids due to increased effective hydrogen bonding or hydrophilic interactions with receptor molecules (3).

These observations suggest a regulatory mechanism which, by analogy to steroid hormone receptors and bacterial induction-repression systems, requires a binding protein to recognize oxysterols and mediate subsequent cellular events. Evidence for the existence of a specific cytosolic receptor protein for oxysterols has been presented (12,15). After the activities of a number of sterols were evaluated, a good correlation was found between the actions of certain oxysterols on HMG-CoA reductase in L cells and their affinity for a oxysterol binding protein (12).

Recently it has been shown that under certain conditions biological systems can be induced to produce oxygenated derivatives of cholesterol and lanosterol (16,17). These results add support to the hypothesis that oxysterols may be natural regulators of cholesterol biosynthesis in the intact cell (18). It has been suggested that such regulatory oxysterols may arise endogenously from cellular cholesterol by either nonenzymic or controlled enzymic oxidation of cholesterol or from the analogous oxidation of biosynthetic precursors of cholesterol (e.g., lanosterol) (3,18,19). An alternate and more interesting pathway requiring the formation of endogenous oxysterols as by-products of cholesterol biosynthesis has been described (16,17,20-23). Compounds such as 24,25-epoxylanost-8-en-3β-ol and 24,25-epoxycholest-5-en-3β-ol may be derived from squalene-2,3,22,23-dioxide (SDO). With the use of inhibitors of 2,3-oxidosqualene cyclase, an increased concentration of SDO can be induced. Upon removal of these inhibitors (4,4,10-trimethyl-*trans*-decal-3β-ol [21] or 3β - [2 - (diethyl-amino)ethoxy] androst -5 -en -17 - one [U18666A] [23]), SDO appears efficiently metabolized to polar products (21,23), presumably 24,25-epoxylanost-8-en-3β-ol and 24(*s*),25-epoxy-cholest-5-en-3β-ol (16,20,22). The metabolism of SDO was associated with a suppression of HMG-CoA reductase (17,23), indicating that cyclized derivatives of SDO have biological effects similar to oxysterols. The immediate cyclization product of SDO, 24,25-epoxylanost-8-en-3β-ol, and two related oxylanosterol analogs, lanost-8-ene-3β-25-diol and lanost-8-ene-25-ol-3-one, have been prepared by chemical synthesis (Fig. 1) and were found to be strong inhibitors of HMG-CoA reductase activity in cultured rat intestinal epithelial cells (24). These oxysterols caused a 50% inhibition of reductase activity at 0.85×10^{-7} M, 1.67×10^{-7} M, and 4.18×10^{-7} M, respectively.

Among the more interesting biological properties of certain oxysterols are their abilities to act as hypocholes-

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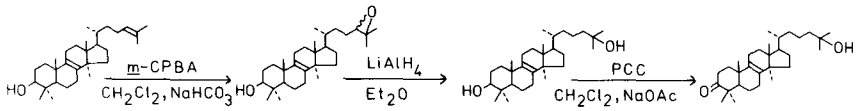

FIG. 1. Chemical synthesis of 24,25-epoxylanost-8-en-3β-ol, lanost-8-ene-3β-25-diol and lanost-8-ene-25-ol-3-one.

terolemic agents. Cholest-8(14)-en-3β-ol-15-one (25-28) and its palmitate and hemisuccinate esters (29), cholest-8(14)-en-3,15-dione (30) and 9α-fluorocholest-8(14)-en-3β-ol-15-one (31) have demonstrated sustained hypocholesterolemic activity in intact animals. The related unsaturated keto steroid cholest-4-en-3-one also is reported to possess similar activity (32). Recently two other oxysterols, cholest-8-en-3β-ol-7-one and cholest-8-en-3β-ol-11-one, were found to have significant but not sustained hypocholesterolemic activity when fed to rats at a level of 0.15% in the diet (Parish, E.J., Nanduri, V.B.B., Seikel, J.M., Kohl, H.H., and Nusbaum, K.E., unpublished results). The action of these two oxysterols as inhibitors of HMG-CoA reductase and their affinity for a cytosolic binding protein are shown in Table 1.

Due to the potential importance and general interest in oxysterols as a class of compounds, new methods of chemical synthesis have been developed to provide them for biological investigations. Other approaches to chemical synthesis have included the streamlining of existing or "classical" methods of synthesis.

A number of 15-oxygenated sterols have been found to be potent inhibitors of HMG-CoA reductase and to actively suppress sterol synthesis in animal cells in culture. In addition, several 15-keto sterols and their derivatives have demonstrated significant hypocholesterolemic action when administered to intact animals (2,27,28). These results stimulated an extensive synthesis program of new 15-oxygenated sterols and intermediates salient in their preparation. Many of these compounds have been prepared from 3β-benzoyloxy-14,15α-epoxycholest-7-ene (33,34), a key intermediate in the synthesis of a large number of these compounds (2). A new approach to the synthesis of 15-oxysterols involving the chromium (VI) oxidation of the 8,14-diene system was utilized in a novel synthesis of the hypocholesterolemic agent 9α-fluorocholest-8(14)-en-3α-ol-15-one (35).

Several steroids with hypocholesterolemic properties have in common the α,β-unsaturated ketone functionality. New methodology has been developed to prepare these compounds from the corresponding allylic alcohol by selective oxidation using a pyridinium chlorochromate-amine reagent system (36-40). The unsaturated keto sterols cholest-8-en-3β-ol-7-one and cholest-8-en-3β-ol-11-one, cited previously for their hypocholesterolemic activity, have been prepared from the key intermediate 3α-benzoyloxy - 9α,11α - epoxycholest - 8 - ene by a modification of known synthetic methods (Fig. 2) (41-46).

Among the most frequently encountered oxysterols are those with a keto or hydroxyl function at carbon-7. These compounds have been found in animal tissues and foodstuffs (1) and certain folk medicines (4-6,47) and have been shown to be significant inhibitors of HMG-CoA reductase (2,12), sterol synthesis (2,48) and cell replication (49-51). Recently a synthetic sequence was developed for the synthesis of 4,4'-dimethyl-7-oxygenated sterols using commercial cholesterol as a starting material (52). The concentrations of those sterols required for 50% inhibition of HMG-CoA reductase activity were similar to those reported for the corresponding sterols devoid of the 4,4'-dimethyl functionality (Table 1).

In the lanosterol series, a simplified method for the preparation of 14α-hydroxymethyl derivatives of 24,25-dihydrolanosterol has been described (53). These compounds are proposed intermediates in the biosynthesis of cholesterol from lanosterol and were found to be potent inhibitors of HMG-CoA reductase and sterol diosynthesis (54). The previously described oxylanosterol derivatives 24,25-epoxylanost-8-en-3β-ol, lanost-8-ene-3β-25-diol and lanost-8-ene-25-ol-3-one have been prepared (Fig. 1) directly from commercial lanosterol (a mixture of lanosterol and 24,25-dihydrolanosterol) in a simplified synthesis. The main feature of this synthesis is the direct monoepoxidation of lanosterol with *m*-chloroperbenzoic acid to yield the 24,25-epoxide. This material was isolated by selectively removing the remaining commercial starting material during repetitive crystallization from methylene chloride-

TABLE 1

Oxysterol Repression of HMG-CoA Reductase and Relative Affinity for Oxysterol-Binding Protein

Sterol	Repression of HMG-CoA reductase[a]	Relative binding affinity[a]
3β-Hydroxy-4,4'-dimethylcholest-5-en-7-one	1.5	—
4,4'-Dimethylcholest-5-ene-3β,7α-diol	1.5	—
4,4'-Dimethylcholest-5-ene-3β,7β-diol	1.7	—
3β-Hydroxycholest-5-en-7-one	1.7	1.4
Cholest-5-ene-3β,7α-diol	2.5	1.1
Cholest-5-ene-3β,7β-diol	2.7	4.4
9α,11α-Epoxycholest-7-en-3β-ol	2.3	3.8
3β-Hydroxycholest-8-en-7-one	1.1	0.49
3β-Hydroxycholest-8-en-11-one	9.0	—

[a]The values given are the concentrations necessary for 50% response in each assay calculated as described in the Experimental section.

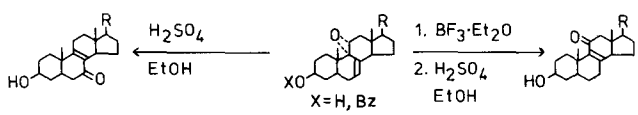

FIG. 2. Chemical synthesis of cholest-8-en-3β-ol-7-one and cholest-8-en-3β-ol-11-one.

methanol, followed by liquid chromatography of the remaining noncrystalline portion (24; Panini, Sexton, Parish and Rudney, submitted for publication). Epoxide ring opening with hydride resulted in the carbon-25 alcohol. Oxidation of the 3β-hydroxyl group with pyridinium chlorochromate resulted in the 3-keto derivative (55).

Research into the chemistry and biochemistry of oxysterols has resulted in major developments summarized briefly in this report. Further extension of the scope of this research is anticipated, especially in understanding the mechanism(s) of possible oxysterol regulation of mammalian cholesterol biosynthesis.

EXPERIMENTAL

The sterols 9α,11α-epoxycholest-8-en-3β-ol, cholest-8-en-3β-ol-7-one and cholest-8-en-3β-ol-11-one were prepared by chemical synthesis (to be presented elsewhere) and were found to have a purity of 98% or greater by gas liquid chromatography and/or thin layer chromatographic analyses (39).

The experimental procedures used in cell culture studies using mouse L cells (a subline of NCTC clone 929 mouse fibroblasts) and the determination of HMG-CoA reductase in cell homogenates have been described previously (12,52). The concentration of sterol in the medium that gave a 50% repression of HMG-CoA reductase after five hr of incubation was determined graphically from a plot of inhibitory activity (percentage of the control value) vs at least five concentrations of sterol. The assay of relative binding affinity of unlabeled sterols by competition with 25-hydroxy[^{3}H] cholesterol with the cytosolic binding protein has been described previously (12).

RESULTS AND DISCUSSION

The oxysterols 9α,11α-epoxycholest-7-en-3β-ol, 3β-hydroxycholest-8-en-7-one and 3β-hydroxycholest-8-en-11-one have been prepared by chemical synthesis using established methods (Fig. 2) (41-46). These compounds contain a diversity of structure in rings B and C; therefore it seemed worthwhile to examine them for their abilities to inhibit HMG-CoA reductase and their affinities for a cytosolic oxysterol-binding protein (Table 1, Fig. 3).

The epoxy sterol 9α,11α-epoxycholest-7-en-3β-ol was found to be a potent inhibitor of HMG-CoA reductase in L cells in culture and also exhibited moderate affinity of the oxysterol binding protein. This is the first example of a 9α,11α-epoxide to be examined in these systems. The keto sterol 3β-hydroxycholest-8-en-7-one shows excellent inhibition of reductase and a strong affinity for the oxysterol binding protein. Oxysterols with a ketone or hydroxyl function at carbon-7 are among the most frequently encountered. The results obtained from other carbon-7 oxysterols are presented in Table 1 for comparison (12,52). The oxysterol 3β-hydroxycholest-8-en-11-one was found to be a poor inhibitor of HMG-CoA reductase. This result was somewhat unexpected due to potent inhibition shown by another carbon-11 oxygenated sterol, cholest-7-ene-3β,11α-diol, which showed a 50% reduction of reductase activity at a 0.55 μM concentration (12). In addition, the keto sterol had no detectable affinity for the oxysterol binding protein, a property consistent with its poor ability to inhibit reductase activity.

The results described here are an addition to the knowledge of the correlation of sterol structure and the repression of HMG-CoA reductase activity and relative binding affinities for the cytosolic oxysterol-binding protein. The results of these and many other studies may aid in understanding the role of oxysterols in the regulation of sterol synthesis.

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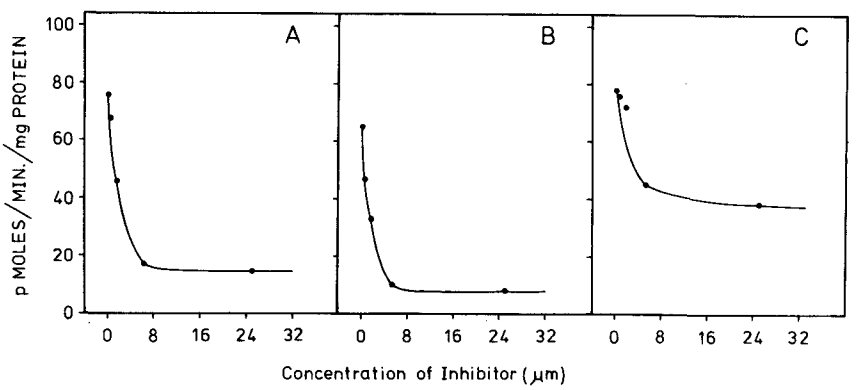

FIG. 3. Effects of oxysterols on the level of HMG-CoA reductase activity in L cells. A, 9α,11α-epoxycholest-7-en-3β-ol; B, 3β-hydroxycholest-8-en-7-one; C, 3β-hydroxycholest-8-en-11-one.

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The Squalene-2,3-Epoxide Cyclase as a Model for the Development of New Drugs

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The 2,3-oxido squalene (SO) cyclases represent a group of enzymes which convert SO into polycyclic triterpenoids such as lanosterol, cycloartenol, cucurbitadienol and β-amyrin. Taking into account the postulated model of the enzymatic cyclization of SO, we have investigated the possibility of designing compounds that would be selective and potent inhibitors of SO cyclases. Due to the fundamental role of sterols in animal, higher plant and fungal tissues, these inhibitors might behave as very selective (ipocholesterolemic, antifungal or phytotoxic) drugs.

Our first approach was the synthesis and biological evaluation of 2-aza-2,3-dihydrosqualene and its derivatives which, being protonated at physiological pH, would present some similarities to the C-2 carbon ion generated by the opening of the oxirane ring of SO. Microsomes from different sources (germinated pea cotyledons, maize seedlings, rat liver and yeasts) were utilized to determine the inhibition values (I_{50}: concentration of inhibitor producing 50% inhibition at a given substrate concentration).

From the results obtained so far we conclude that 2-aza-2-dihydrosqualene and its derivatives strongly inhibited the cyclases, the site of the enzyme responsible for binding to the inhibitor is quite sensitive to the steric hindrance, and the degree of the inhibitory activity is greater in higher plants than in rat liver or fungi.
Lipids 21, 31–38 (1986).

The inhibitors of 2,3-oxidosqualene cyclase (EC 5.4.99.7), a key enzyme in sterol biosynthesis (1,2) could provide new prospects in the area of antifungal, ipocholesterolemic and phytotoxic drugs.

Sterol inhibitors tested so far include: (i) drugs that block HMG-CoA reductase, such as compactin (3–5); (ii) compounds that inhibit the last stage in cholesterol biosynthesis, such as triparanol or 20,25-diazacholesterol (6–9), and (iii) antifungal agents that interfere with the sterol 14α-demethylase enzyme system during the biosynthesis of ergosterol (10–12). All these substances present certain deleterious side effects which make their practical clinical use difficult. For example, long-term administration of compactine and its derivatives leads to unexpected effects connected with the blockage of DNA synthesis, cell growth and cell division (13–16). These findings are not surprising considering that these compounds are potent inhibitors of the synthesis of mevalonic acid which, independently of its well-known function as a cholesterol precursor, plays an essential role in DNA replication (13,17).

In addition, the clinical use of 20,25-diazacholesterol or triparanol disclosed many disadvantages including accumulation of desmosterol (24-dehydrocholesterol) in serum, liver and plaques (7) and introduction of myotonia in mammals (18–20).

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Many substituted imidazoles and triazoles (e.g., myconazole) with broad spectrum activity against most pathogenic yeasts, fungi and gram-positive bacteria are known at low concentration to inhibit C-14 demethylation of lanosterol (21–22), which is related to loss of a membrane function. However, since the effect on ergosterol synthesis was observed at drug concentration several orders of magnitude lower than that required for any appreciable effect on growth, it was suggested that myconazole could affect some other physiological target present in fungi membrane (23). Moreover, findings that myconazole selectively affects cytochrome P-450 in yeast microsomes could suggest a more gradual mechanism for this type of drug (24).

Inhibitors of 2,3-oxidosqualene cyclase, compared with the sterol inhibitors seen previously, potentially could present further advantages as a model for designing new drugs. They could greatly affect membrane structure and function in higher plants or pathogenic fungi by producing an accumulation of acyclic precursors that could not imitate the role of the sterols in membranes. This is not always the case when the target of sterol inhibition follows lanosterol formation. For example, treatment of a suspension of bramble cells with tridemorph, a systemic fungicide used in the control of powdery mildews (25) and which caused a dramatic accumulation of 9β,19-cyclopropyl sterols (more than 90% of total sterols), slightly reduces growth, suggesting that 9β,19-cyclopropyl sterols could substitute, at least partially, in the functions normally ascribed to Δ^5-sterols (26). In this context, 2,3-oxidosqualene cyclase inhibitors could present a potential interest as phytotoxic or antifungal agents.

In mammals, an enzymic target lies after the synthesis of mevalonic acid and before lanosterol demethylation; thus these kinds of sterol inhibitors could be more specific ipocholesterolemic agents, as they do not interfere with the physiological steps connected with cell division. For the same reason, they do not cause abnormal accumulation of steroid precursors, which alone may contribute to the development of atherosclerotic tissues.

Evidence exists in the literature that the biosynthesis of sterols can be inhibited in vivo and in vitro at the 2,3-oxidosqualene cyclization step by a variety of compounds (27–36). However, no examples are known where the synthesis of these inhibitors has been made on a rational basis using as a model the mechanism of enzyme cyclization of 2,3-oxidosqualene in different tissues. In a few cases kinetic measurements were made with the enzyme.

Taking advantage of our experience studying the mechanisms of different 2,3-oxidosqualene cyclases in higher plants (37–43), we were able to design new inhibitors of 2,3-oxidosqualene cyclase which could mimic the high-energy intermediates along the reaction pathway. In addition, we also have tested both inhibitory activity and

kinetics of new compounds in microsomes from rat liver, yeast and higher plants.

MATERIALS AND METHODS

Microsomes. The enzymatic work has been performed with microsomes prepared from cell-free extracts from pea (*Pisum sativum,* Papillionaceae) cotyledons (38,58), maize embryos (37), rat liver (64) and *Saccharomyces cerevisiae* (Balliano, G., unpublished results).

Enzymatic assays and action of inhibitors. The 2,3-oxidosqualene β-amyrin, cycloartenol and lanosterol cyclases activities have been measured as described previously (57–64).

The experimental details concerning the determination of the inhibition curves and the measurements of the kinetic parameters of the inhibition curves were discussed in a recent report (64). Analytical methods for the purification and identification of the various cyclization products have been discussed elsewhere (37–42).

Inhibitors and authentic materials. Most of the inhibitors and [3-^{3}H]-R,S-2,3-oxidosqualene have been synthesized by us as described elsewhere (44,57,64). AMO 1618 (2-isopropil-4-dimethylamino-5-methylphenyl-2-piperidinecarboxilate methyl chloride), NDI (*N*-dodecylimidazole) and U18666A (3β-[2-(diethylamino)ethoxy]androst-5-en-17-one hydrochloride) were provided by BASF, Rhône-Poulenc and Upjohn Co., respectively. Chloroquine was purchased from Sigma. 2,3-Epiminosqualene was synthesized according to the method of Avruch and Oehlschlager (45).

RESULTS AND DISCUSSION

The enzymatic model. The current view of the mechanism of 2,3-oxidosqualene (SO) (**1**) cyclization is shown in Figure 1; it bears out many of the predictions of the biogenetic isoprene rules (46–48).

Two fundamental types of the cyclase enzyme can be envisaged: the cyclase responsible for the cyclization of chair-boat-chair-boat (cbcb) folded squalene 2,3-oxide giving lanostane-type tetracyclic triterpenoids such as lanosterol (**2**), cycloartenol (**3**), parkeol (**4**) and cucurbitadienol (**5**).

The mechanistic pathway involves the intermediacy of the protosterol cation (**6**), which then is stabilized by methyl and hydrogen migration yielding a lanostane C-9 carbonium ion (**7**), which is bound to the enzyme by a suitable nucleophilic group (49–50). Withdrawal of the enzyme, together with the elimination of C-11-β, C-8-β and C-19-β protons would give parkeol (**4**), lanosterol (**2**) or cycloartenol (**3**). Similarly, removal of the enzyme could allow further methyl and hydrogen migration (C10→C9; H5→H10), and the abstraction of C-6 proton will give 10α-cucurbita-5,24-dien-3β-ol (**5**), which could be considered the general precursor of cucurbitacins (41–42).

These types of cyclases are similar in their mechanistic aspect. In fact, the basic group B, required to remove C-11, C-19 and C-6 protons from the transition state (**7**), might occupy an identical regiospecific position on the active site of the different cyclases (40–42). The second group of SO cyclases, which includes α- and β-amyrin synthetase, is involved in the cyclization of cccb folded squalene 2,3-oxide (**1**). Following Ruzicka's hypothesis

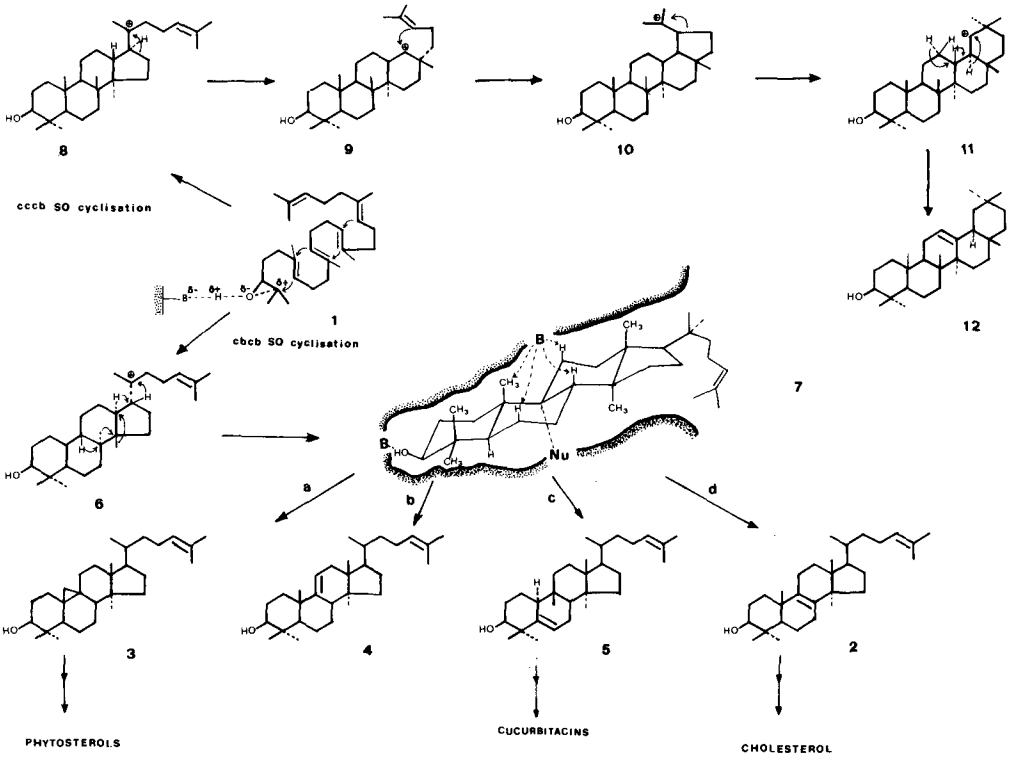


FIG. 1. Cyclization of 2,3-oxidosqualene in animal, higher plant and fungal tissues.

(47,48), the cyclization of squalene 2,3-epoxide leads to the dammarenyl cation (**8**), which undergoes a complex rearrangement through the ions baccarenyl (**9**)→lupenyl (**10**)→omolupenyl (**11**) to give β-amyrin (**12**). The complex mechanism, responsible for the elaboration of the E ring during the biosynthesis of pentacyclic triterpenes, strongly depends on the thermodynamic stability of the intermediate ions (**9**) and (**10**), whereas an enzymic control would be needed to direct from (**11**) the 1,2-*trans*-diaxial hydrogen shift and the final elimination of the 12α proton (38).

At first sight two different regiospecific sites for the enzyme could be envisaged for the β-amyrin synthetase, one which is responsible for the cyclization of SO and of the final rearrangement, and the other interested in the elaboration of the E ring.

From these results, two suggestions can be drawn: (i) the two cyclase enzymes could be very similar in the region of the enzyme (site **a**) responsible for the recognition and cyclization of the substrate; and (ii) the dammarenyl and the protosteryl ions (**8**) or (**6**) could be stabilized by an electron-bearing residual of the active site of the enzyme, leading to a transient interaction of (**8**) or (**6**) with the enzyme.

*Attempt to mimic the C-20 carbonium ion (**6**) or (**8**).* As shown in Figure 1, the mechanism of 2,3-oxidosqualene cyclase involves different carbocationic intermediates, which could be transiently stabilized by a suitable active site of the enzymes. These biosynthetic intermediates could be compared with the enzyme-bound high energy intermediates (HEI) (51–55).

Taking this fact into account, we applied the above strategy to the rational design of inhibitors of *S*-adenosylmethyonine (SAM) cycloartenol C-24 methyl transferase and 2,3-oxidosqualene β-amyrin cyclase (55–57). The general device used in these studies was the synthesis of analogues of carbocation HEIs by replacing the carbon atom bearing the positive charge with a positively charged nitrogen atom (protonated amine). The results are that this type of inhibitor may have a much higher affinity for the active site of the enzyme than the traditional ground state analogous inhibitors (51–52).

With these considerations in mind, we thought it possible to mimic the C-20 carbonium ion of (**6**) or (**8**) by replacing the C-20 with a suitable nitrogen atom. The resulting 20-azadammaran-3β-ol or 20-aza-protosterol could behave as a selective inhibitor of SO β-amyrin synthetase or SO cycloartenol (a lanosterol) synthetase, respectively.

Dipterocarpol (**13**) was the most advantageous available material. Therefore we started with the synthesis of 20-azadammaran-3β-ol (**14a**) as a specific inhibitor of SO-β-amyrin synthetase. Dipterocarpol (**13**) first was degraded to 17-oxo-13β-octanordammaran-3β-yl acetate (**15**); to the latter then was reconstructed the sidechain-bearing C-20 nitrogen with known chirality at C-17, giving the two epimeric 20-azadammarenols (**14a**) or (**14b**) (Fig. 2).

The inhibition activity of each amine was determined by incubation of microsomes from germinating peas in the presence of [3-^{3}H]SO and various amounts of the inhibitors. The results obtained were discouraging: (**14a**) was a very poor inhibitor of 2,3-oxidosqualene-β-amyrin synthetase (58). On the other hand, the formation of C-20 carbonium ion (**8**) during the biosynthesis of β-amyrin was largely as shown by other authors (59–62).

We tentatively explained this apparent contradiction by postulating that, in an enzymic model, two different steps were involved in the complex mechanism responsible for the cyclization of SO to the final triterpenes: (i) recognition of the substrate and its cyclization to the C-20 ion (**8**) or (**6**) and (ii) rearrangement of the ions (**8**) or (**6**) to give lanosterol, cycloartenol or β-amyrin.

If this is the case because of the continuous dynamic evolution of the cyclase conformation during the cyclization step and rearrangements, only those compounds which can bind to the conformational ground state of the enzyme (recognition) or take advantage of the favorable binding interaction occurring in the transition state complex, related to the opening of the oxirane ring of SO, could behave as SO-cyclase inhibitors. According to this hypothesis, one could suggest that the active site of the enzyme reaches certain conformations, complementary to C-20 ion intermediate (**8**), only after undergoing a specific sequence of catalytic events and hence conformational changes, which in the absence of catalysis cannot be reached spontaneously or, if so, only slowly (63). Along this line we found also that azadecalines in which the C-8 was replaced by an amino group in order to mimic the carbocation HEI occurring after B ring formation were ineffective in blocking cyclase in higher plants (pea seedlings, maize) or in rat liver (64).

These mechanistic hypotheses are seriously limited by the fact that 20-azadammarane (**14a**) has been tested only in higher plant microsomes which show SO β-amyrin or cycloartenol synthetase activity. Further experiments could be carried out in different tissues with 3β-dammarenol synthetizing ability.

Analogs of C-2 carbonium ion HEI. (a) Inhibition by 2-aza-2,3-dihydrosqualene and derivatives. The failure to inhibit the C-20 carbonium ion (**8**) involved in the biosynthesis of β-amyrin focused our attention on the first transient state connected with the opening of the oxirane ring of squalene 2,3-oxide (**1**). Such a mechanism implies polarization at the C-2 oxygen bond leading to a charge deficiency at C-2 of the squalene epoxide molecule (Fig. 1).

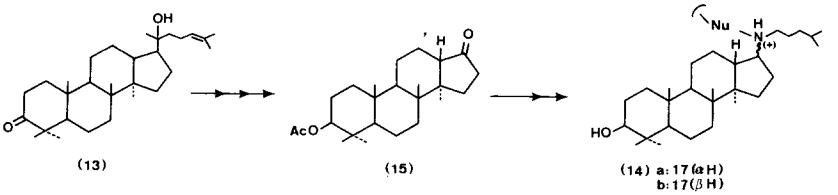
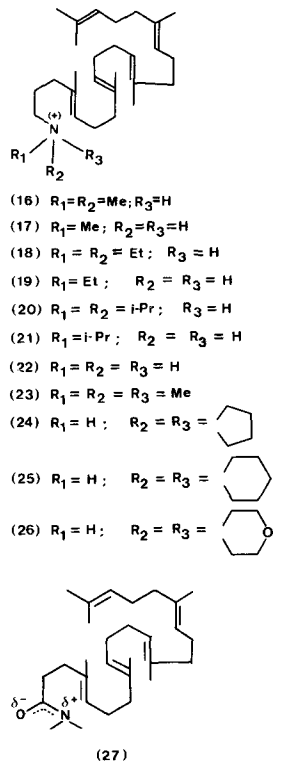
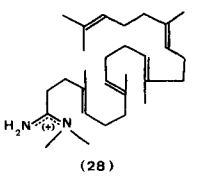
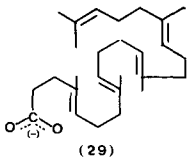


FIG. 2. Synthesis of 20-azadammaran-3α-ol (14a) from dipterocarpol (13).




(28)


(29)

(27)

FIG. 3. 2-Aza-2,3-dihydrosqualene and derivatives.

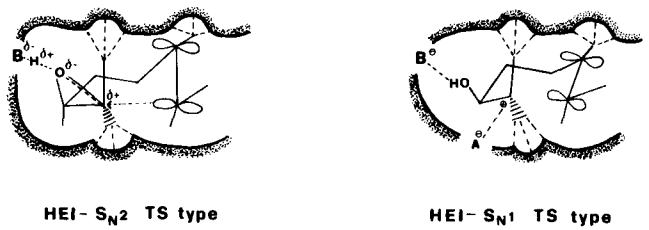


FIG. 4. SN_1 or SN_2 character of TS formed from the opening of oxirane ring of SO.

TABLE 1

Inhibition of 2,3-Oxidosqualene Cyclases by 2-Aza-2,3-dihydrosqualene (16) and Derivatives

Compounds	$I_{50}(\mu M)$		
	Rat liver	Pea seedlings	*S. cerevisiae*
16	7.5	1.3	10
17	ND	3.0	ND
18	3.2	0.55	14
19	ND	3.0	ND
20	100	120	ND
21	ND	48	ND
22	62	32	50
23	5.1	1.1	ND
24	>100(85)[a]	ND	ND
25	3.5	ND	ND
26	8.0	ND	ND
27	40	>100(75)[a]	ND
28	ND	1.5	ND

ND, not determined.

[a]The number in brackets represents the residual activity (%) at I=0.1 mM.

We have tried to mimic this transient carbocationic HEI by designing molecules such as 2-aza-2,3-dihydrosqualene (**16**) and a series of related aza compounds (**17**–**28**) (Fig. 3) and testing their effects in vitro on 2,3-oxidosqualene lanosterol cyclase (rat liver microsomes and *Saccharomyces cerevisiae*), 2,3-oxidosqualene β-amyrine cyclase (pea seedling microsomes) and on 2,3-oxidosqualene cycloartenol cyclase (maize seedling microsomes) (Table 1).

As expected according to our hypothesis, the inhibition of the cyclases tested so far in this series is due to positively charged species and is very sensitive to the basicity of the azasqualene derivatives.

Among the molecules tested, *N,N*-dimethyl (**16**) and *N,N*-diethyl (**18**) compounds were the most powerful, the inhibition being particularly strong for the pea enzyme.

To check whether the neutral or the protonated form of the amine was the inhibitory species, derivative (**23**) containing a quaternary amine was tested. For both animal and plant cyclases, (**23**) was as strong an inhibitor as the tertiary amine (**16**). On the other hand, a compound characterized by a delocalization of a negative charge such as 1,1′,2-*tris-nor*-squalene-3-carboxylic acid (**29**) did not inhibit the cyclase (pea seedlings). When the *N*-substituents were bulkier than ethyl, the inhibition strongly decreased, indicating incidence of steric problems. Similarly, when the primary amine (**22**) was tested, the lack of the methyl substituents resulted also in a decreased affinity of the enzyme. These results show that inhibition of the 2,3-oxidosqualene cyclase is controlled by steric factors at the *N*-2 center.

We tried also to modulate the delocalization of the positive charge in the azasqualene skeleton through the synthesis of squalene amide (**27**) and squalene amidine (**28**). The results showed that (**27**) was a poor inhibitor of the cyclase, by contrast, the more basic squalene dimethyl amidine (**28**) was an excellent inhibitor of pea seedling cyclase.

In order to correlate basicity with the steric problems at the *N*-2 center, we also tested some azasqualene derivatives bearing a five or six-membered ring. In this context, in rat liver microsomes the squalene pyrrolidine (**24**), which could be considered a cyclized *N,N*-diethylazasqualene, is much more active than the corresponding squalene pyperidine (**25**). By contrast, the more basic squalene morpholine (**26**) retained most of the inhibitory activity presented by (**18**). The total results related to the *N*-alkylazasqualene derivatives (**16**–**28**) provide better insight into the nature of the HEI involved in the opening of the oxirane ring of squalene 2,3-epoxide (**1**).

According to Van Tamelen (46,65), the oxirane ring opening of SO (**1**) can be viewed as an SN_2-like reaction with a high degree of the neighboring π-bond participation (66). However, the importance of a trisubstituted center at C-2 of the molecule for its enzymic transformation also suggests an appreciable degree of SN_1 character in the A-ring closure (67) (Fig. 4). Indeed, the paramount role of the positively charged species in the inhibition of the cyclases tested so far, together with the sensitivity to the substituent nature at the nitrogen atom analogous to substrate specificity of the mammalian cyclase (68), seems to be more favorable, at least in higher plants, to the SN_1 character of the HEI rather than an SN_2 HEI transition state.

(b) Inhibition by 2-aza-2,3-dihydrosqualene *N*-oxides. We also tried to mimic the dipoles involved in the enzymic opening of 2,3-oxidosqualene through the synthesis of a series of *N*-oxide derivatives of azasqualene (**30–35**) (Fig. 5). These compounds, because of their strong dipolar moments, could present some structural and electronic similarities to the SN_2-like transition state (Fig. 4). In Table 2 are indicated the comparative results obtained with the azasqualene *N*-oxides and derivatives (**30–35**) in our in vitro systems (microsomes from peas, rat liver and yeast).

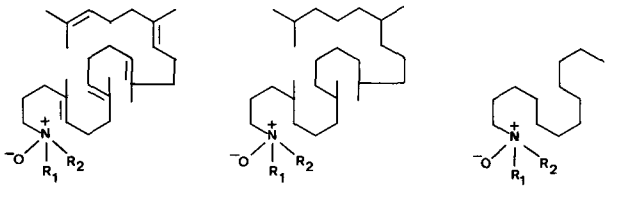
The data obtained so far show that these compounds are systematically more active than the parent tertiary amines in higher plants and rat liver cyclases. When the azasqualene *N*-oxides were tested on microsomes from yeast, almost the same inhibition was observed for the tertiary amines and the corresponding *N*-oxides.

In higher plants and animals, two structural features seem to contribute to inhibition: (i) the parent *N,N*-diethylamine is more inhibitory than the corresponding *N,N*-dimethyl derivative, and (ii) in the *N,N*-dialkyl series, the length of the alkyl chain and the presence of double bonds appear to be important. These favorable features both are present in *N,N*-diethylazasqualene *N*-oxide (**31**), the most potent inhibitor of this group.

When the *N*-oxide function is linked to a different skeleton, a net reduction of the inhibitory activity was observed (compare the activity of U18666A *N*-oxide [**36**] with the parent amine).

To explain such experimental results, one could envisage the involvement of an SN_2-like transition state with a strong dipolar character in the C-2-oxygen bond (Fig. 4). It should be observed that *N*-oxides, which are globally neutral, present some similarities to the ammonium cations (charge distribution, binding affinity for water) (69–71).

(c) Inhibition by tertiary alkylamines with different skeletons. The aim of our work also was to obtain specific inhibition activity in the cyclases from higher plants, animals or yeast. For this reason we decided to modify the azasqualene amines and related *N*-oxide derivatives: (i) (Fig. 6) by saturating the double bond system of the squalene carrier (**38,39**); (ii) by reducing the length of the saturated alkyl chain (**40,41**), and (iii) by decreasing the lipophylic character of the azasqualene or azasqualane derivatives (see the bis-azasqualene and bis-azasqualane series [**42–47**]). We also decided to test the activity of some *N,N*-dialkyl-3-phenylpropyl amines (**48–49**) (Fig. 7) or *N,N*-dialkyl-2-phenoxyethyl amines (**50–51**) bearing an aryl residue which could imitate the B-cycle formed during



(30) $R_1 = R_2 = Me$ (31) $R_1 = R_2 = Et$ (32) $R_1 = R_2 = Me$ (33) $R_1 = R_2 = Et$ (34) $R_1 = R_2 = Me$ (35) $R_1 = R_2 = Et$

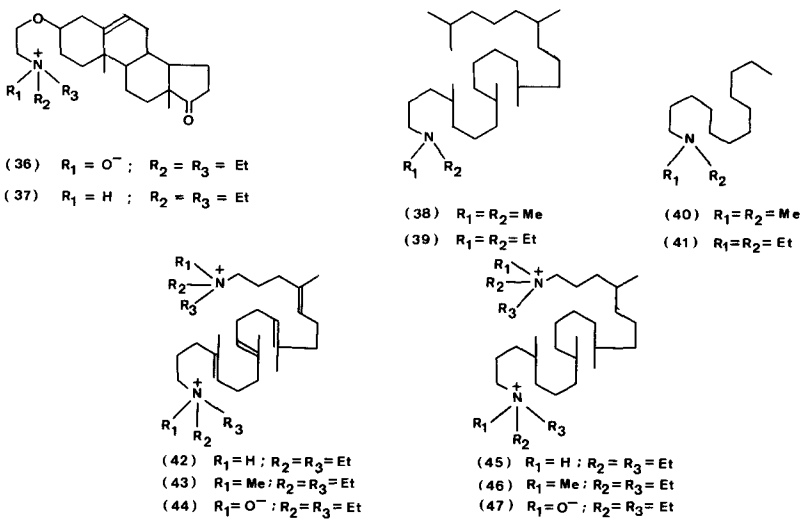
FIG. 5. 2-Aza-2,3-dihydrosqualene *N*-oxide and other saturated long chain *N*-oxide derivatives.

TABLE 2

Inhibition of 2,3-Oxidosqualene Cyclases by Aza-Dihydrosqualene-*N*-oxides (30,31), Azasqualane-*N*-oxides (32,33), Long Chain Tertiary Amine *N*-Oxides (34,35), U18666A (37) and the *N*-Oxide Derivative (36)

Compounds	$I_{50}(\mu M)$		
	Rat liver	Pea seedlings	*S. cerevisiae*
30	3.7	0.3	16
31	1.5	0.15	14
32	8.0	1.0	ND
33	3.5	0.5	ND
34	3.6	0.3	40
35	3.2	0.3	32
36	7.0	1.0	ND
37	1.9	0.2	0.1

ND, not determined.



(36) $R_1 = O^-$; $R_2 = R_3 = Et$ (37) $R_1 = H$; $R_2 = R_3 = Et$ (38) $R_1 = R_2 = Me$ (39) $R_1 = R_2 = Et$ (40) $R_1 = R_2 = Me$ (41) $R_1 = R_2 = Et$ (42) $R_1 = H$; $R_2 = R_3 = Et$ (43) $R_1 = Me$; $R_2 = R_3 = Et$ (44) $R_1 = O^-$; $R_2 = R_3 = Et$ (45) $R_1 = H$; $R_2 = R_3 = Et$ (46) $R_1 = Me$; $R_2 = R_3 = Et$ (47) $R_1 = O^-$; $R_2 = R_3 = Et$

FIG. 6. U18666A, azasqualane derivatives, *N,N*-dialkyldodecilamines, bis-azasqualene and bis-azasqualane series.

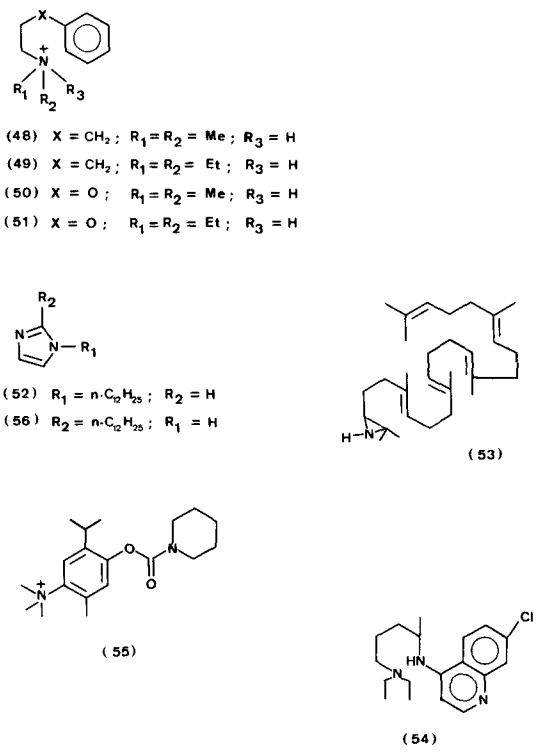


FIG. 7. *N,N*-dialkyl-3-phenylpropylamines, *N,N*-dialkyl-2-phenoxyethylamines and compounds known in the literature.

TABLE 3

Inhibition of 2,3-Oxidosqualene Cyclases by Azasqualanes (38,39) and Long Chain Tertiary Amines (40,41)

Compounds	$I_{50}(\mu M)$		
	Rat liver	Pea seedlings	*S. cerevisiae*
38	21	22	100
39	18	1.4	35
40	>100(60)[a]	50	70
41	2.0	0.3	40

[a]The number in brackets represents the residual activity (%) at I=0.1 mM.

the cyclization mechanism. From Table 3 we can see that, inside a homogeneous group, saturation of the squalene skeleton, which gives a more lipophylic character, leads to diminished inhibitory activity in all types of 2,3-oxidosqualene cyclase (animals, higher plants and yeast). Results also showed that the difference between the activity of azasqualene and *N,N*-dialkyldodecyl derivatives is more pronounced in *N,N*-dimethyl than *N,N*-diethyl or *N,N*-diethyl *N*-oxide derivatives. In any case, the inhibitory efficacy against the different cyclases decreases in the aryl or aryloxy series, the single exception being the *N,N*-diethyl derivatives (**49**) or (**51**) in pea microsomes (Table 5).

By comparing the results obtained with the different classes of lipophylic compounds, it can be concluded that the squalene skeleton is the most favorable carrier for the tertiary amines or relative *N*-oxides and that its activity decreased in the following order: squalene > dodecyl > squalane > 3-phenylpropyl > 2-phenoxyethyl.

TABLE 4

Inhibition of 2,3-Oxidosqualene Cyclases by the Bis-Azasqualene and Bis-Azasqualane Compounds

Compounds	$I_{50}(\mu M)$		
	Rat liver	Pea seedlings	*S. cerevisiae*
42	3.0	ND	6.0
43	6.5	ND	4.0
44	1.5	0.15	4.0
45	18	ND	10
46	17	ND	ND
47	3.1	ND	ND

ND, not determined.

TABLE 5

Inhibition of 2,3-Oxidosqualene Cyclases by 3-Phenylpropyl Amines (48,49) and 2-Phenoxyethyl Amines (50,51)

Compounds	$I_{50}(\mu M)$	
	Rat liver	Pea seedlings
48	>100(66)[a]	>100(90)
49	ND	13
50	—[b]	—
51	ND	40

ND, not determined.
[a]The number in brackets represents the residual activity (%) at I=0.1 mM.
[b]No inhibition at I=0.1 mM.

More spectacular results were obtained when the squalene skeleton was not changed drastically, but made more polar by introducing an additional *N,N*-dialkylgroup (bis-*N,N*-diethylazasqualene or squalane [**42**] or [**45**]), a trialkylammonium group (as in [**43**]) or an *N,N*-dialkyl amine-*N*-oxide group (as in the bis-*N,N*-diethylazasqualene *N*-oxide or squalane [**44**] and [**47**]). The results showed that in yeast the cyclase is very sensitive to the increasing polarity of the carrier system; thus the bis-*N,N*-diethylazasqualene ammonium derivative (**43**) and the bis-*N,N*-diethylazasqualene *N*-oxide (**44**), which are less active than the corresponding mono derivatives (**23**) or (**31**) in a cyclase system from rat liver, are the most active compounds in microsomes obtained from *S. cerevisiae* (Table 4).

From the results obtained, the following conclusions could be drawn: (i) In yeast, a limiting factor for inhibitory activity seems to be a pronounced lipophylic character of the carrier linked to the tertiary amine. Indeed, when a second hydrophylic group such as an *N,N*-dialkyl group or a trialkylammonium or an *N*-oxide was introduced in the last isoprenic unit of the azasqualenes, the inhibitory activity improved. This trend does not seem to be due to the amphiphylic character of (**43**) or (**44**), as the activity of 2,3-oxidosqualene cyclase is not affected by detergent (72). On the other hand, a perturbation of the microenvironment of the membrane-associated yeast cyclase by molecules such as (**43**) or (**44**) cannot be excluded totally. (ii) In relation to the point of the less or more SN_2-like high energy transition state (Fig. 4), one could argue that

yeast cyclase inhibitors take advantage of the positive charge present in derivatives (**43**) or (**44**).

(d) Comparative inhibition of known compounds. Besides the azasqualenes, we also have tested in our in vitro systems compounds shown in the literature to inhibit the 2,3-oxidosqualene cyclase, such as U18666A (**37**), *N*-dodecylimidazole (**52**), 2,3-epiminosqualene (**53**), chloroquine (**54**) and AMO 1618 (**55**) (27–36) (Fig. 7).

All these compounds are characterized by the presence of a basic group (which could be protonated at physiological pH) linked to a less or more hydrophobic carrier such as a steroidic nucleus, a saturated alkyl chain, an aromatic polycyclic ring or a squalene skeleton.

U18666A (**37**), NDI (**52**) and 2,3-epiminosqualene (**53**) are equally active in higher plant, animal and yeast cyclases (Tables 2 and 6). Nevertheless, the mechanism of action of the various compounds could be very different. For example, considering the poor basicity of the imidazole ring, the mode of action of NDI could not derive from the competitive binding of (**52**) to the catalytic site of the enzyme leading to the protonated NDI derivative, but must derive instead from its peculiar structure. In fact, the (**56**) shift of the alkyl chain to position 2 of the imidazole ring totally eliminated the inhibitory activity of NDI. In relation to this point, we note that NDI (but not 2-alkyl imidazole) is able to inhibit the cytochrome P-450 monooxygenase (73-75).

U18666A (**37**) is quite an extraordinary compound, as it is the most active inhibitor of yeast cyclase and also is very effective in the case of pea and rat liver cyclases. However, in marked contrast to the azasqualene derivatives, the *N*-oxide derivative of U18666A is much less efficient in lowering the activity of both plant and animal cyclases.

Finally, both chloroquine (**54**) and AMO 1618 (**55**) were described in the literature as affecting the biosynthesis of sterols at the cyclization step (34–36). When these compounds were tested in our in vitro systems, a complete lack of activity was observed. In fact, inhibitory activity of (**54**) or (**55**) was documented only in whole cell systems or cell-free extracts converting precursor (mevalonate, squalene) into 2,3-oxidosqualene. Consequently, one cannot exclude that AMO 1618 or chloroquine interfere with certain binding or activating soluble proteins.

Kinetic measurements. For animal and plant cyclases, molecules such as 2-aza-2,3-dihydrosqualene (**16**), the corresponding *N*-oxide derivative (**30**), 2,3-epiminosqualene (**53**) and *N*-dodecylimidazole (**52**) consistently proved to be noncompetitive inhibitors. Ki is comparable to the corresponding I_{50} (Fig. 8).

These results underline the inherent limitations of the kinetic analysis derived from isotropic solutions when dealing with complex anisotropic systems. A detailed discussion of this subject appeared in a recent report (64).

TABLE 6

Inhibition of 2,3-Oxidosqualene Cyclases by Imidazole Derivatives (52) and (56), 2,3-Dihydro-2,3-epiminosqualene (53), AMO 1618 (55) and Chloroquine (54)

Compounds	$I_{50}(\mu M)$		
	Rat liver	Pea seedlings	*S. cerevisiae*
52	3.9	0.4	ND
53	0.4	0.2	10
54	—[a]	—	ND
55	—	—	ND
56	—	—	ND

ND, not determined.

[a]No inhibition at I=0.1 mM.

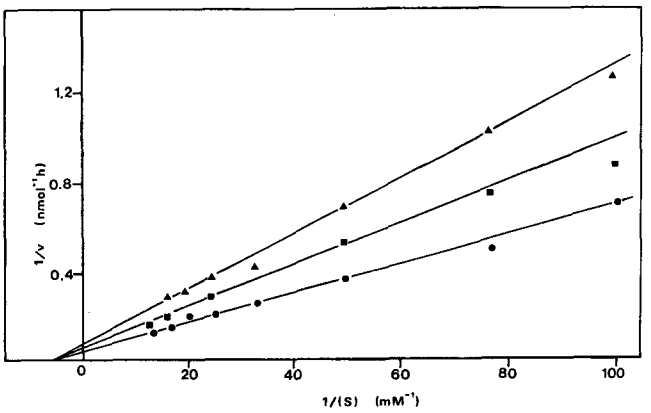


FIG. 8. Inhibition of rat liver 2,3-oxidosqualenelanosterol cyclase by 2-aza-2,3-dihydrosqualene (16). Conditions: pH 7.4, 37 C. The concentrations of (16) are (— ● —) none, (— ■ —) 5μM and (— ▲ —) 10 μM. Protein concentration: 1.95 mg microsomal protein/ml.

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Sterols of Cucurbitaceae: The Configurations at C-24 of 24-Alkyl-Δ^5-, Δ^7- and Δ^8-Sterols

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The major sterols of the seeds of *Benincasa cerifera, Cucumis sativus, Cucurbita maxima, C. pepo* and *Trichosanthes japonica* and of the mature plant tissues (leaves and stems) of *Citrullus battich, Cucumis sativus* and *Gynostemma pentaphyllum* of the family Cucurbitaceae were 24-ethyl-Δ^7-sterols which were accompanied by small amounts of saturated and Δ^5- and Δ^8-sterols. The 24-ethyl-$\Delta^{7,22}$-, $\Delta^{7,25(27)}$- and $\Delta^{7,22,25(27)}$-sterols constituted the predominant sterols for the seed materials, whereas the 24-ethyl-Δ^7- and $\Delta^{7,22}$-sterols were the major ones for the mature plant tissues. The configurations of C-24 of the alkylsterols were examined by high resolution ^{1}H NMR and ^{13}C NMR spectroscopy. Most of the 24-methyl- and 24-ethylsterols examined which lack a $\Delta^{25(27)}$-bond (i.e., 24-methyl-, 24-methyl-Δ^{22}-, 24-ethyl- and 24-ethyl-Δ^{22}-sterols) were shown to occur as the C-24 epimeric mixtures in which the 24α-epimers predominated in most cases. The 24-ethylsterols which possess a $\Delta^{25(27)}$-bond (i.e., 24-ethyl-$\Delta^{25(27)}$- and 24-ethyl-$\Delta^{7,22,25(27)}$-sterols) were, on the other hand, composed of only 24β-epimers. The Δ^8-sterols identified and characterized were four 24-ethyl-sterols: 24α- and 24β-ethyl-5α-cholesta-8,22-dien-3β-ol, 24β-ethyl-5α-cholesta-8,25(27)-dien-3β-ol and 24β-ethyl-5α-cholesta-8,22,25(27)-trien-3β-ol. This seems to be the first case of the detection of Δ^8-sterols lacking a 4-methyl group in higher plants, and among the four Δ^8-sterols the latter two are considered to be new sterols. The probable biogenetic role of the Δ^8-sterols and the possible biosynthetic pathways leading to the 24α- and 24β-alkylsterols in Cucurbitaceae are discussed.

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The great majority of higher plants contain sterols bearing a Δ^5-bond with a 24α-alkyl substituted side chain (24*R* if a saturated side chain, 24*S* if the Δ^{22} derivative) represented by sitosterol (24α-**2f**) (1,2). Some higher plants, including members of the family Cucurbitaceae, however, contain predominantly 24-alkyl-Δ^7-sterols (2–9). The major sterols present in the seeds of Cucurbitaceae are 24-ethyl-5α-cholesta-7,22-dien-3βol (**3g**) (all sterols possessing a Δ^{22}-bond described here have a *trans* [*E*]-configuration at C-22), 24-ethyl-5α-cholesta-7,25(27)-dien-3β-ol (**3jj**) and 24-ethyl-5α-cholesta-7,22,25(27)-trien3-β-ol (**3k**) (4–9) (Though experimentally unproven in this study, the 25[27] rather than 25[26] designation of the Δ^{25}-bond was made here for natural 25-dehydrosterols according to the work of Nes et al. [2,10].) The configurations at C-24 of the 24-ethyl-Δ^7-sterols from the seeds of *Cucurbita* species have been demonstrated by high resolution ^{1}H NMR spectroscopy (10–13) to be 24α for **3g**, which lacks a $\Delta^{25(27)}$-bond, and 24β for **3j** and **3k**, which contain $\Delta^{25(27)}$-bonds. The 24β-ethyl stereochemistry for **3j** and **3k** is consistent with the observation on the 24β-ethyl-Δ^5-sterols with a $\Delta^{25(27)}$-bond system isolated from the other families of higher plants (10,14,15). Our recent studies, mainly by ^{13}C NMR spectroscopy, on the stereochemistry at C-24 of the 24-ethyl-Δ^7-sterols lacking a $\Delta^{25(27)}$-bond isolated from some species of Cucurbitaceae (i.e., *Lagenaria* [16,17], *Luffa* [17], *Citrullus* [18], *Cucumis* [19], and *Trichosanthes* [20]) have demonstrated the occurrence of 24β-epimers or the co-occurrence of 24α- and 24β-epimers. The 24-ethyl-$\Delta^{7,25(27)}$- (**3j**) and $\Delta^{7,22,25(27)}$-sterols (**3k**) from these Cucurbitaceae species have been shown to be 24β-epimers (18, 19), consistent with the observations cited above (10–13).

In this study, we have examined by high resolution ^{1}H NMR and ^{13}C NMR spectroscopy the configuration at C-24 of the 24-alkylsterols isolated from additional species of the family Cucurbitaceae. We have identified and characterized four Δ^8-sterols: 24α- and 24β-ethyl-5α-cholesta-8,22-dien-3β-ol (**4g**), 24β-ethyl-5α-cholesta-8,25(27)-dien-3β-ol (**4j**) and 24β-ethyl-5α-cholesta-8,22,25(27)-trien-3βol (**4k**). The latter two are considered to be new sterols, while the former two so far have been detected only in a marine sponge (21).

EXPERIMENTAL

Crystallizations were performed in acetone/methanol (MeOH). Melting points (mp) taken on a heat block were uncorrected. Preparative thin layer chromatography (TLC) on silica gel (0.5 mm thick) was developed three times using *n*-hexane/ethyl acetate (6:1, v/v). Argentic (silver nitrate/silica gel; 1:4, w/w) preparative TLC (0.5 mm thick silica gel) of steryl acetate was developed four times with CCl_4/CH_2Cl_2 (5:1, v/v). Preparative high performance liquid chromatography (HPLC) was carried out on a Partisil 5 ODS-2 (25 cm x 10 mm id) (Whatman, Clifton, New Jersey) column with MeOH as a mobile phase (flow rate, 4 ml/min) using a UV detector monitoring at 210 nm. Gas liquid chromatograpy (GLC) was performed with a Shimadzu GC-4CM instrument on a SCOT OV-17 glass capillary column (30 m x 0.3 mm id, column temp 260 C) under the conditions already described (22). Rc values on the argentic TLC and relative retention time (RRT) values on the HPLC and GLC of steryl acetate were taken relative to cholesterol (**2a**, cholest-5-en-3β-ol) acetate. Mass spectra (EI-MS, 70 eV) were taken on a Shimadzu LKB-9000 gas chromatograph-mass spectrometer (GC-MS) (> m/z 100, 2% OV-17, 2 m x 3 mm id glass column); high resolution EI-MS (70 eV) were recorded on a Hitachi M-80A double focusing GC-MS instrument by a direct injection. ^{1}H and ^{13}C NMR spectra were recorded on a Hitachi R-250 (250 MHz for ^{1}H; 62.9 MHz for ^{13}C), a JNM FX-100 (Japan Electron Optics Laboratory Co., Tokyo, Japan) (100 MHz for ^{1}H; 25.0 MHz for ^{13}C) or a JNM FX-400 (400 MHz for ^{1}H) instrument in a $CDCl_3$ solution, with tetramethylsilane (TMS) as internal standard. The FT ^{13}C NMR were measured at ca. 0.2 M solutions under the following conditions: frequency 25.0 MHz

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(62.9 MHz); spectral width, 200 ppm (250 ppm); pulse width, 6 μsec (10 μsec); acquisition time, 2.5 sec (2.0 sec); number of data points, 8192 (16000); probe temp, ca. 30 C.

Materials. The seeds of *Benincase cerifera* Savi (wax gourd), *Cucumis sativus* L. (cucumber), *Cucurbita maxima* Duschesne (squash) and *C. pepo* L. (pumpkin) were supplied by Sakata Seeds Co. (Yokohama, Japan). The seeds of *Trichosanthes japonica* Regel (*T. kirilowii* Maxim. var. *japonica* Kitamura) and the dried leaves and stems (aerial part) of *Gynostemma pentaphyllum* Makino were purchased from Kinokuniya Kan-yaku Kyoku Co. (Tokyo, Japan). The leaves and stems of *C. sativus* and *Citrullus battich* Forskål (watermelon) were collected at a local farm. The following authentic sterols were used in this study: 24ξ-methyl-5α-cholestan-3β-ol (24-methylcholestanol, 1c); 24ξ-ethylcholestanol (**1f**) and 24ξ-ethyl-22-dehydrocholestanol (**1g**) (23); cholesterol (**2a**) and 24-dehydrocholesterol (desmosterol, **2b**) (22); a mixture of 24α- (campesterol) and 24β-methylcholesterols (**2c**) (24); 24α- and 24β- (brassicasterol) 24-methyl-22-dehydrocholesterols (**2d**) (25); 24-methylenecholesterol (**2e**) (23); 24α- (sitosterol) and 24β- (clionasterol) ethylcholesterols (**2f**) (24); 24α-ethyl-22-dehydrocholesterol (stigmasterol, **2g**) (23); 24*Z*-ethylidenecholesterol (isofucosterol, **2i**) (23); 24β-ethyl-25(27)-dehydrocholesterol (cleroasterol, **2j**) and 22-dehydroclerosterol (**2k**) (22); 24α- and 24β-methyl-5α-cholest-7-en-3β-ol (24-methyllathosterols, **3c**) (24); 24α- (stellasterol) and 24β-methyl-22-dehydrolathosterols (**3d**) (26); 24-methylenelathosterol (**3e**) (22); 24α- (22-dihydrospinasterol) and 24β- (22-dihydrochondrillasterol) ethyllathosterols (**3f**) and spinasterol (**3g**) (18); chondrillasterol (**3g**) (16); 24β-ethyl-25(27)-dehydrolathosterol (**3j**) and 24β-ethyl-22,25(27)-bisdehydrolathosterol (**3k**) (18); 24*Z*-ethylidenelathosterol (avenasterol, **3i**) and 24-ethyl-24(25)-dehydrolathosterol (peposterol, **3h**) (20).

Extraction and fractionation of sterol mixture. Dried and ground seeds were extracted with CH_2Cl_2 using a Soxhlet extractor, and the unsaponifiable lipid was obtained from the extract by alkaline hydrolysis (27). Chopped and dried leaves and stems (aerial part) were extracted with hot MeOH. To remove the phospholipid, the MeOH extract was treated with cold acetone (28), and the unsaponifiable lipid was obtained from the acetone soluble lipid by alkaline hydrolysis. The unsaponifiable lipid was chromatographed on a silica gel column (*n*-hexane, *n*-hexane/diethyl ether and then *n*-hexane/ethyl acetate as eluant) to provide the sterol mixture. The elution was monitored by TLC on the Merck precoated silica gel. The sterol mixture was separated by preparative TLC into two fractions, A and B; fraction A from the faster moving zone (Rf = 0.33) consisted mainly of Δ^7-sterols accompanied by saturated sterols, and fraction B from the slower moving zone (Rf = 0.31) was composed mainly of Δ^7-sterols accompanied by Δ^8-sterols (24). The two fractions were acetylated (pyridine-Ac_2O, room temp) and the resulting acetyl derivatives were fractionated by argentic TLC. In some cases where a fraction still consisted of a mixture, a further fractionation was carried out by HPLC.

RESULTS

Sterols from the seed and aerial part plant materials were identified as acetates based on comparison of the GLC, argentic TLC and GC-MS data with those of authentic compounds. For some isolated compounds, the identification was substantiated by the mp, HPLC and NMR data. Table 1 lists the molecular ion peaks, RRT in GLC and HPLC, Rc in argentic TLC and mp of the acetyl derivatives of the identified and newly characterized sterols. The specific structures of the sterols are reproduced in Figure 1. Table 2 shows the sterol content in the dried seed and aerial part plant materials of the Cucurbitaceae, together with the percentage of sterol components, determined based on GLC, argentic TLC and HPLC data.

TABLE 1

Molecular Ion (M^+) and Chromatographic and Mp Data of Some Sterols from Cucurbitaceae

Ring system	Sterol	Data for the acetyl derivatives				
		M^+	RRT[a]		Rc[a]	Mp[b]
		(m/z)	GLC	HPLC	Argentic TLC	(C)
Δ^0(1)	**1c**	444	1.33		1.18	
	1f	458	1.66		1.18	
	1g	456	1.46		1.18	
Δ^5(2)	**2a**	368[c]	1.00	1.00	1.00	
	2b	366[c]	1.21		0.60	
	2c	382[c]	1.31	1.07	1.00	134–136
	2d	380[c]	1.14		0.65	
	2e	380[c]	1.25		0.18	
	2f	396[c]	1.63	1.18	1.00	135–137
	2g	394[c]	1.43	1.04	0.96	143–145
	2i	454	1.81		0.53	
	2j	394[c]	1.64	0.86	0.36	123–125
	2k	452	1.52	0.80	0.14	146–150
Δ^7(3)	**3c**	442	1.55	1.07	1.00	149–153
	3d (24α)	440	1.36	0.86	0.63	169–172
	3d (24β)	440	1.36	0.91	0.63	169–171
	3e	440	1.61	0.80	0.16	142–145
	3f	456	1.94	1.18	1.00	158–161
	3g	454	1.70	1.04	0.95	180–182
	3h	454	2.31		0.74	
	3i (24*E*)	454	2.04		0.53	
	3i (24*Z*)	454	2.15		0.53	
	3j	454	1.95	0.88	0.41	160–163
	3k	452	1.80	0.76	0.14	175–178
Δ^8(4)	**4g**	454	1.54	0.95	0.89	151–153
	4j	454	1.74	0.80	0.40	133–135
	4k	452	1.63	0.69	0.18	152–154

[a]RRT and Rc were expressed relative to **2a** acetate.

[b]Determined for the steryl acetates isolated from *Cucumis sativus* seeds with one exception. **3e** was isolated from *Gynostemma pentaphyllum* aerial part.

[c]M^+-HOAc.

Identification of uncommon sterols. The steryl acetate (RRT = 1.46 in GLC) detected, accompanied by **1c** and **1f** acetates in the least polar fraction on the argentic TLC of the acetylated sterol B fraction from *Cucumis sativus* seeds, showed the following: M^+ at m/z 456 (rel int 21%) in MS, together with the other ions at m/z 441 (2%), 413 (4%, M^+-C_3H_7, allylic cleavage of the terminal isopropyl group), 353 (34%, M^+-C_3H_7-HOAc), 344 (21%, M^+-C_8H_{16}, C-20–C-22 vinylic cleavage and a 1H transfer), 315 (24%, M^+-$C_{10}H_{19}$[side chain]-2H), 257 (74%, M^+-$C_{10}H_{19}$-HOAc) and 107 (base peak), which were consistent with the sterol

TABLE 2

Sterol Content and Sterol Compositions (%) of Some Cucurbitaceae

Ring system	Sterol	Seeds					Aerial part		
		Benincase cerifera	*Cucumis sativus*[a]	*Cucurbita maxima*	*Cucurbita pepo*	*Trichosanthes japonica*	*Citrullus battich*	*Cucumis sativus*[b]	*Gynostemma pentaphyllum*
Contents of sterol mixture (mg/100 g of dried plant materials)		38	63	52	83	83	33	63	43
Δ^5(2)	**2a**	tr[c]	tr	tr	tr	0.1	0.3	0.1	0.9
	2b		tr	tr				tr	tr
	2c	1.6	0.4	0.6	2.2	0.3	tr	0.2	0.6
	2d	tr	tr			tr	tr	tr	tr
	2e			1.1	2.7	tr		tr	tr
	2f	7.0	3.5	1.0	4.4	6.9	1.4	1.1	2.8
	2g	1.5	1.5	0.6	0.3	0.7		0.3	tr
	2i (24*Z*)		tr		0.1	tr	tr	tr	tr
	2j	tr	0.3	0.7	1.6	0.2	0.9	0.3	1.0
	2k		0.3		0.1			tr	
Δ^7(3)	**3c**	1.8	1.3	0.4	0.4	1.7	tr	3.1	2.3
	3d	0.5	0.6			0.2	0.6	0.6	4.4
	3e	2.1	tr	tr	0.9	tr	0.9	0.4	0.9
	3f	6.9	2.3	1.9	6.1	0.2	34.3	36.1	0.6
	3g	17.4	11.3	27.1	22.2	34.3	31.2	43.9	61.9
	3h	tr	tr			tr	0.1	tr	
	3i (24*E*)	1.9	0.3	1.7	0.8	tr	2.4	0.9	
	3i (24*Z*)	5.0	2.1	10.9	9.7	tr	8.6	1.0	
	3j	37.1	18.3	17.1	25.4	11.0	12.1	9.3	3.2
	3k	12.4	54.9	32.1	17.6	35.8	1.7	tr	0.8
Δ^8(4)	**4g**	0.7	0.9	0.2	0.7	1.1	0.5	0.2	0.6
	4j	0.8	0.3	0.4	0.2	0.4	0.2	tr	0.1
	4k	0.2	1.2	0.5	0.1	2.4	0.3	0.2	
Others, unidentified		3.1	0.5	3.7	4.5	4.7	4.5	2.3	19.9

[a]Trace amounts of **1c**, **1f** and **1g** also were present.

[b]Trace amounts of **1c** and **1f** also were present.

[c]tr = Trace.

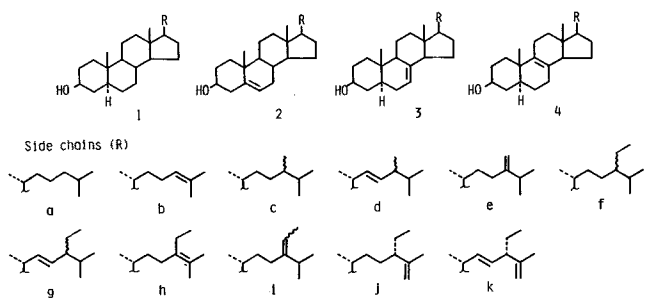

FIG. 1. Sterols of Cucurbitaceae. All C-22–C-23 double bonds are *trans*.

with a saturated nucleus and a 24-ethyl-Δ^{22} side chain (29). The MS data and the chromatographic mobilities were identical with those of authentic **1g** (23), and hence, the steryl acetate was identified as 24ξ-ethyl-22-dehydrocholestanol (**1g**) acetate.

The MS of the steryl acetate (RRT = 1.21 in GLC) isolated from the acetylated sterol A fraction from *Cucurbita pepo* seeds showed the following fragmentation ions: at m/z 366 (base peak, M^+-HOAc), 351 (30%, M^+-Me-HOAc), 253 (52%, M^+-C_8H_{15}[side chain]-2H-HOAc), 245 (22%) and 213 (12%), which indicated that the sterol has a monounsaturated nucleus and a C_8-monounsaturated side chain (29). The absence of M^+ in the MS suggests that the nucleus double bond is located at C-5. The ^{1}H NMR (100 MHz) spectrum had the signals at δ 0.68 (3H, s, 18-H_3), 1.01 (3H, s, 19-H_3), 0.93 (3H, d, 21-H_3, J = 6.9 Hz), 1.60 (3H, s, 27-H_3), 1.68 (3H, s, 26-H_3), 2.03 (3H, s, 3β-OCOMe), 4.64 (1H, m, 3α-H), 5.06 (1H, t, 24-H, J = 7.2 Hz) and 5.40 (1H, m, 6-H). These spectral data and the chromatographic data were indistinguishable from those of authentic **2b** acetate. Hence, this acetate was identified as desmosterol (**2b**) acetate.

The steryl acetate (RRT = 2.04 in GLC) in the acetylated sterol B fraction from *C. sativus* seeds showed M^+ at m/z 454 (2%) with other ions at m/z 439 (4%, M^+-Me), 379 (3%, M^+-Me-HOAc), 356 (37%, M^+-C_7H_{14} [cleavage at C-22–C-23 with 1H transfer due to McLafferty type rearrangement)], 313 (100%, M^+-$C_{10}H_{19}$[side chain]-2H), 296 (7%, M^+-C_7H_{14}-HOAc), 255 (14%), 253 (15%), 227 (10%) and 213 (21%). The MS were consistent with the acetate of the Δ^7-sterol with a 24-ethyl-$\Delta^{24(28)}$- or a $\Delta^{24(25)}$-side chain (29,30). Taking into account the GLC mobility, this was identified as isoavenasterol (24*E*-**3i**) acetate. The steryl acetate (RRT = 2.31 in GLC) in the same fraction as above had almost indistinguishable MS with that of 24*E*-

TABLE 3

Methyl Group ^{1}H NMR Chemical Shifts[a] ($CDCl_3$) of 24-Methyl- and 24-Ethylsterols

Sterol	Substituent at C-3β	Origin[b]		18-H_3 (s)	19-H_3 (s)	21-H_3 (d,J~6.5)	26-H_3 (d,J~6.8)	27-H_3 (d,J~6.8)	28-H_3 (d,J~6.8)	29-H_3 (d,J~7.3)
2c[c,e]	OAc	B	(24α)	0.677	1.017	0.910	0.850	0.801	0.773	—
			(24β)	0.677	1.017	0.917	0.856	0.781	0.773	—
3c[e]	OAc	B	(24α)	0.529	0.809	0.911	0.844	0.791	0.777	—
			(24β)	0.529	0.809	0.923	0.856	0.783	0.777	—
3c (ref. 24)	OH	A	(24α)	0.536	0.800	0.916	0.852	0.804	0.775	—
3c (ref. 24)	OH	A	(24β)	0.532	0.796	0.924	0.857	0.784	0.778	—
3d	OAc	B	(24α)	0.543	0.813	1.008	0.839	0.822	0.912	—
3d	OAc	B	(24β)	0.543	0.812	1.016	0.837	0.821	0.914	—
2f[e] (ref. 19)	OH	B	(24α)	0.680	1.009	0.921	0.833	0.815	—	0.844
			(24β)	0.680	1.009	0.925	0.833	0.815	—	0.854
2f (ref. 19)	OH	A	(24α)	0.680	1.009	0.921	0.835	0.813	—	0.844
2f (ref. 19)	OH	A	(24β)	0.680	1.009	0.925	0.833	0.813	—	0.854
3f[e](ref. 19)	OH	B	(24α)	0.535	0.796	0.925	0.833	0.813	—	0.844
			(24β)	0.535	0.796	0.930	0.833	0.813	—	0.854
3f (ref. 19)	OH	A	(24α)	0.536	0.796	0.926	0.837	0.815	—	0.846
3f (ref. 19)	OH	A	(24β)	0.535	0.796	0.931	0.832	0.811	—	0.855
2g[e]	OAc	B	(24α)	0.675	1.020	1.021	0.846	0.796	—	0.804
			(24β)	0.675	1.020	1.021	0.846	0.790	—	0.810
2g (ref. 24)	OH	A	(24α)	0.699	1.012	1.021	0.846	0.797	—	0.805
2g (ref. 24)	OH	A	(24β)	0.697	1.011	1.025	0.844	0.791	—	0.811
3g[c,e]	OAc	B	(24α)	0.548	0.812	1.028	0.846	0.798	—	0.805
			(24β)	0.548	0.812	1.028	0.846	0.792	—	0.811
3g[c]	OAc	C	(24β)	0.548	0.815	1.029	0.845	0.792	—	0.813
3g[e]	OAc	D	(24α)	0.548	0.813	1.027	0.850	0.800	—	0.806
			(24β)	0.548	0.813	1.027	0.845	0.792	—	0.813
3g	OAc	A	(24α)	0.548	0.813	1.027	0.850	0.800	—	0.806
4g[e]	OAc	B	(24α)	0.621	0.964	1.032	0.850	0.801	—	0.805
			(24β)	0.621	0.964	1.032	0.845	0.792	—	0.813
4g(ref. 21)[d]	OH	A	(24α)	0.624	0.951	1.028	0.847	0.797	—	0.805
4g(ref. 21)[d]	OH	A	(24β)	0.622	0.949	1.029	0.841	0.790	—	0.810
2j[c]	OAc	B	(24β)	0.668	1.015	0.904	1.561(s)	4.643(s,1H) 4.732(s,1H)	—	0.800
3j[c]	OAc	B	(24β)	0.523	0.805	0.907	1.561(s)	4.645(s,1H) 4.731(s,1H)	—	0.800
4j[c]	OAc	B	(24β)	0.599	0.959	0.912	1.562(s)	4.645(s,1H) 4.729(s,1H)	—	0.800
2k[c]	OAc	B	(24β)	0.690	1.017	1.008	1.651(s)	4.696(s,2H)	—	0.832
3k[c]	OAc	B	(24β)	0.541	0.810	1.015	1.651(s)	4.699(s,2H)	—	0.833
4k[c]	OAc	B	(24β)	0.617	0.961	1.019	1.650(s)	4.699(s,2H)	—	0.833
4k	OAc	B	(24β)	0.617	0.962	1.020	1.650(s)	4.697(s,2H)	—	0.834

[a]Chemical shifts given in δ values from TMS; coupling constants in Hz; determined at 400 MHz unless otherwise specified.

[b]A, authentic sterol; B, *Cucumis sativus* seeds; C, *Trichosanthes japonica* seeds; D, *Benincasa cerifera* seeds.

[c]Determined at 250 MHz.

[d]Determined at 360 MHz.

[e]Mixture of C-24 epimers.

3i acetate, i.e., m/z 454 (3%, M^+), 439 (6%), 379 (5%), 356 (28%), 313 (100%), 296 (8%), 255 (15%), 253 (22%), 227 (8%) and 213 (21%). However, since the RRT in GLC was consistent with that of **3h** acetate, this was identified as peposterol (**3h**) acetate (10,20).

Characterization of the 24-ethyl-Δ^8-sterols. High resolution MS of the steryl acetate (RRT = 1.52 in GLC) isolated from the acetylated sterol B fraction from *C. sativus* seeds showed M^+ at m/z 454.3782 (35%, $C_{31}H_{50}O_2$, calcd. 454.3807), with the following fragmentation ions: at m/z 439.3782 (11%, M^+-Me), 411.3239 (5%, M^+-C_3H_7), 379.3348 (6%, M^+-Me-HOAc), 315.2289 (19%, M^+-$C_{10}H_{19}$ [side chain]), 313.2167 (24%, M^+-$C_{10}H_{19}$-2H), 288.2075 (24%), 255.2076 (37%), 229.1966 (45%), 213.1663 (15%) and 81.0711 (100%), which suggested that the sterol has a monounsaturated nucleus and a monounsaturated C_{10}-side chain. The ion M^+-C_3H_7 is characteristic for the Δ^{22}-unsaturation (29). The ^{1}H NMR (400 MHz) (Table 3) showed 18-H_3 and 19-H_3 singlets at δ 0.622 and 0.964, respectively, with no ring-olefinic signal, suggesting a Δ^8-sterol (21). The 26-H_3, 27-H_3 and 29-H_3 signals were consistent with those of **2g** and **3g** acetates which possess a 24-ethyl-Δ^{22} side chain; hence, this acetate was identified as 24-ethyl-5α-cholesta-8,22-dien-3β-ol (**4g**) acetate. The ^{1}H NMR (Table 3) further showed that the sterol consisted of the 24α- and 24β-epimers, as will be discussed later.

The steryl acetate (RRT = 1.74 in GLC) isolated from the same fraction as above showed M^+ in high-resolution MS at m/z 454.3773 (89%, $C_{31}H_{50}O_2$), with the fragmentation ions at m/z 439,3545 (34%, M^+-Me), 394.3555 (8%, M^+-HOAc), 379.3343 (10%, M^+-Me-HOAc), 341.2445 (6%, M^+-C_7H_{14}-Me), 313.2166 (21%, M^+-$C_{10}H_{19}$[side chain]-2H), 299.1958 (4%, M^+-C_7H_{14}-Me-HOAc), 288.2122 (6%), 273.1856 (6%), 255.2067 (12%, M^+-$C_{10}H_{19}$-HOAc), 229.1974 (17%), 227.1822 (27%), 213.1655 (27%) and 55.0565 (100%). This suggested that the sterol is a 24-ethylsterol with two double bonds, one in the nucleus and the other in the side chain. The fragment ions at m/z 341 and 299 were consistent with the unsaturation at either $\Delta^{24(28)}$ or at $\Delta^{25(27)}$ in the side chain (31). The singlets at δ 0.599 (18-H_3) and 0.959 (19-H_3) in the ^{1}H NMR (Table 3) coupled with no ring-olefinic signal suggested a Δ^8-sterol (21). The 26-H_3, 27-H_3 and 29-H_3 signals were in agreement with those of **2j** and **3j** acetates, which bear a 24β-ethyl-$\Delta^{25(27)}$-side chain. Hence, the steryl acetate was regarded to have a structure of 24β-ethyl-5α-cholesta-8,25(27)-dien-3β-ol (**4j**) acetate.

The high resolution MS of the steryl acetate (RRT = 1.63 in GLC) isolated from the same fraction as above of *C. sativus* seeds showed M^+ at m/z 452.3659 (51%, $C_{31}H_{48}O_2$, calcd. 452.3652) with the following fragments: at m/z 437.3410 (9%, M^+-Me), 392.3483 (5%, M^+-HOAc), 377.3238 (9%, M^+-Me-HOAc), 368.2655 (8%, M^+-C_6H_{12}), 353.2504 (3%, M^+-C_6H_{12}-Me), 315.2286 (20%, M^+-$C_{10}H_{17}$ [side chain]), 313.2133 (22%, M^+-$C_{10}H_{17}$-2H), 299.2002 (19%), 288.2086 (13%), 255.2069 (43%), 229.1991 (34%), 213.1644 (19%) and 81.0686 (100%), indicating the presence of a monounsaturated nucleus and a diunsaturated C_{10}-side chain. The fragments at m/z 368 and 353 arising from the bond cleavage at C-23–C-24 with ^{1}H loss suggested the C-25 unsaturation (31). The ^{1}H NMR gave 18-H_3 and 19-H_3 singlets at δ 0.617 and 0.961, respectively, while signals due to ring-olefinic protons were absent, suggesting the sterol was a Δ^8-sterol (21). Since the signals due to 26-H_3, 27-H_3 and 29-H_3 are in accord with those of **2k** and **3k** acetates which have a 24β-ethyl-$\Delta^{22,25(27)}$ side chain (Table 3), the structure 24β-ethyl-5α-cholesta-8,22,25(27)-trien-3β-ol (**4k**) acetate was assigned. These Δ^8-sterols are considered to be natural products and not artifacts produced from Δ^7-sterols during the course of isolation, because the chemical isomerization of a Δ^7-sterol under certain conditions would afford the $\Delta^{8(14)}$-isomer rather than the Δ^8-isomer (18).

Identification of C-24 alkyl epimers of 24-alkylsterols and estimation of the relative proportions of the C-24 epimers by ^{1}H and ^{13}C NMR spectroscopy. High resolution ^{1}H NMR spectroscopy was used to determine the configurations at C-24 of the 24-alkylsterols isolated from the Cucurbitaceae plant materials. The ^{1}H signal assignment was performed by comparison with the ^{1}H NMR data of authentic and known reference compounds (11,19, 21,24,32–34). The relative proportions of diastereoisomers of C-24 epimeric mixtures were estimated primarily on the basis of comparison of the spectra with those of the mixtures of corresponding and relevant alkylsterols in known proportions cited in the literature (34). ^{13}C NMR has been proven a useful tool for quantitative analysis of an epimeric mixture of 24-alkylsterols (17,18,20,35,36); if sufficient amounts of isolated sterols were available, the mixture examined further by ^{13}C NMR for those sterols, in which the signal assignments were made by comparison with those of literature data. Relative proportions of diastereoisomers in mixtures were estimated by averaging the relative intensity of each pair of ^{13}C resonances for which the chemical shift differed appreciably between epimers. This quantification by the ^{13}C NMR was based on the consideration that the spin-lattice relaxation times of corresponding carbons in the two epimeric side chains might be quite similar (35). Attempted determination of

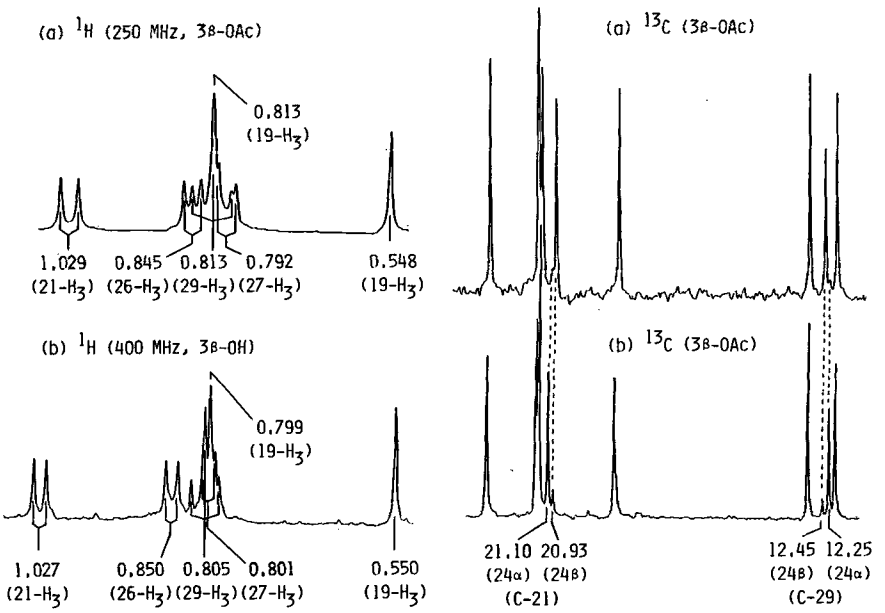

FIG. 2. Partial ^{1}H NMR and ^{13}C NMR (62.9 MHz) spectra (in $CDCl_3$) of 24-ethyl-Δ7,22-sterols. (a) Isolated from *Trichosanthes japonica* seeds. (b) Isolated from *Cucurbita maxima* seeds. Chemical shifts given in δ values from TMS.

TABLE 4

^{13}C NMR Chemical Shifts[a] ($CDCl_3$) of Some 24-Ethylsterols

Carbon	2f-OAc[b] (62.9 MHz) (24α) (24β)	2f-OAc[c] (25.0 MHz) (24α) (24β)	3f-OAc[b] (25.0 MHz) (24α) (24β)	3f-OAc[c] (25.0 MHz) (24α) (24β)	2g-AOc[b] (62.9 MHz) (24α) (24β)	2g-OH[d] (22.5 Mhz) (24α) (24β)	3g-OAc[b] (62.9 MHz) (24α) (24β)	3g-OH[d] (22.5 MHz) (24α) (24β)
C-1	36.99	36.9	36.9	36.8 36.8	37.00	37.3 37.4	36.84	37.2 37.2
C-2	27.78	27.6	27.5	27.5 27.5	27.78	31.8 31.8	27.49	31.6 31.7
C-3	73.96	73.7	73.4	73.5 73.5	73.93	71.8 71.8	73.37	71.1 71.1
C-4	38.12	37.9	33.8	33.8 33.8	38.12	42.3 42.3	33.82	38.1 38.1
C-5	139.60	139.4	40.0	40.1 40.1	139.60	140.8 139.9	40.04	40.4 40.4
C-6	122.59	122.4	29.5	29.5 29.5	122.58	121.7 121.7	29.53	29.7 29.7
C-7	31.88	31.8	117.2	117.2 117.2	31.88	32.0 32.0	117.27	117.5 117.5
C-8	31.88	31.8	139.4	139.5 139.5	31.88	32.0 32.0	139.38	139.6 139.6
C-9	50.03	49.9	49.3	49.3 49.3	50.06	50.2 50.2	49.27	49.6 49.6
C-10	36.59	36.4	34.2	34.2 34.2	36.61	36.6 36.6	34.18	34.3 34.3
C-11	21.03	20.9	21.4	21.5 21.5	21.02	21.3 21.3	21.48	21.6 21.6
C-12	39.72	39.7	39.5	39.5 39.5	39.63	39.8 39.8	39.42	39.6 39.6
C-13	42.31	42.3	43.3	43.4 43.3	42.21	42.3 42.3	43.22	43.3 43.3
C-14	56.67	56.6	55.0	55.0 55.0	56.77	56.9 56.9	55.05	55.2 55.2
C-15	24.30	24.3	23.0	23.0 23.0	24.36	24.4 24.4	23.00	23.1 23.1
C-16	28.23	28.2	27.9	28.0 27.9	28.91 28.80	28.9 28.8	28.49 28.39	28.5 28.4
C-17	56.01	56.0	56.1	56.1 56.0	55.93	56.1 56.2	55.87	56.0 56.0
C-18	11.87	11.8	11.9	11.9 11.9	12.05	12.2 12.2	12.05	12.1 12.1
C-19	19.31	19.4	12.9	13.0 13.0	19.31	19.4 19.4	12.92	13.1 13.1
*Me*CO	21.43	21.1	21.4	21.5 21.5	21.42	— —	21.48	— —
Me*C*O	170.46	170.0	170.5	170.7 170.7	170.40	— —	170.41	— —
C-20	36.15 36.27	36.0	36.6 36.4	36.6 36.4	40.48	40.4 40.3	40.83	40.8 40.8
C-21	18.79 18.84	18.8	18.9	18.9 18.9	21.08 20.93	21.1 20.9	21.10 20.93	21.1 20.9
C-22	33.91	33.9	33.8	33.8 33.9	138.24	138.2 138.1	138.09	138.2 138.1
C-23	26.10 26.39	26.0	26.2 26.5	26.2 26.5	129.24	129.4 129.5	129.38	129.5 129.6
C-24	45.84 46.07	45.8	45.9 46.1	45.9 46.0	51.22	51.3 51.3	51.24	51.3 51.3
C-25	29.16 28.95	29.1	29.2 29.0	29.1 28.9	31.88	32.0 32.0	31.86	32.0 32.0
C-26	19.81 18.99	19.8	19.8 18.9	19.8 19.0	21.22 18.99	21.3 19.0	21.39 18.99	21.3 19.0
C-27	19.04 19.60	19.0	19.1 19.6	19.1 19.6	18.99 21.22	19.0 21.3	19.02 21.39	19.0 21.3
C-28	23.08 23.02	23.0	23.1	23.0 23.0	25.40	25.5 25.5	25.42	25.5 25.5
C-29	11.99 12.32	11.9	12.0 12.3	12.0 12.3	12.25 12.42	12.1 12.3	12.25 12.45	12.3 12.5

[a]Chemical shifts given in δ values.

[b]Isolated from *Cucumis sativus* seeds; mixtures of C-24 epimers.

[c]Authentic sterol.

[d]Authentic sterol (ref. 17).

the relative proportion at 25.0 MHz of the mixtures (I and II) of spinasterol (24α-**3g**; isolated from spinach seeds [18]) and chondrillasterol (24β-**3g**; isolated from *Trichosanthes japonica* seeds which contain a small amount of the 24α-epimer [see Fig. 2]) acetates in known proportions (24α: 24β for I = 70:30; for II = 30:70) afforded good results showing 73:27 for I and 34:66 for II.

Tables 3 and 4 list the ^{1}H and ^{13}C NMR data, respectively, of authentic sterols and the 24-alkylsterols isolated from certain Cucurbitaceae plant materials indicated, with the configurational assignment at C-24. The sterols isolated from other plant materials had NMR data almost indistinguishable from those of the corresponding sterols listed in the tables.

Figure 2 showed the partial ^{1}H and ^{13}C NMR spectra of the 24-ethyl-$\Delta^{7,22}$-sterols (**3g**) isolated from the seeds of *Trichosanthes japonica* and *Cucurbita maxima*. Based on the ^{1}H NMR data, the sterols from both cucurbits were assigned as single epimers, i.e., 24β-epimer for the *T. japonica* sterol and 24α-epimer for the *C. maxima* sterol. However, the ^{13}C NMR of both sterols clearly displayed the small signals arising from the opposite epimer in addition to those from the major epimer, which suggests the coexistence of both diastereoisomers. Thus, the 24β-epimer was the major component accompanied by a minor proportion of its 24α-epimer of **3g** from *T. japonica*, while the sterol from *C. maxima* was composed predominantly of the 24α-epimer accompanied by a minor 24β-epimer, which was ambiguous from the ^{1}H NMR data.

The relative proportions of the C-24 epimers of the 24-alkylsterols lacking a $\Delta^{25(27)}$-bond of the Cucurbitaceae examined by ^{1}H and ^{13}C NMR spectroscopy in this and our recent studies (16–20,37) are shown in Table 5. Proportions of the sterol (for which both ^{1}H and ^{13}C NMR data were available) were estimated on the basis of the ^{13}C NMR data. All of the 24-methylsterols with Δ^5-, Δ^7- and

$\Delta^{7,22}$-bonds were shown to be mixtures of 24α- and 24β-epimers in which the 24α-epimers were always predominant (with one exception being the 24β-epimer as the major component for **3c** from *Benincase cerifera* seeds). The co-occurrence of both 24α- and 24β-epimers was observed further in the Δ^5- (**2f**) and Δ^7- (**3f**) 24-ethylsterols. In most cases, the 24α-epimers were the major components. As with the 24-ethyl-Δ^{22}-sterols bearing Δ^5- (**2g**), Δ^7- (**3g**) and Δ^8- (**4g**) ring systems, although most of the sterols examined were found to be mixtures of C-24 epimers, some consisted of a single diastereoisomer, either 24α- or 24β-epimer. Chemical epimerization at C-24 on the above sterols during the course of isolation is unlikely because authentic **3g** isolated from spinach seeds (18) by the same method as that of this study was proved by ^{13}C NMR to be a single 24α-epimer.

24-Ethylcholesta-7,25(27)-dienol (**3j**) and 24-ethylcholesta-7,22,25(27)-trienol (**3k**) isolated from the seeds of *Citrullus battich* and *Lagenaria leucantha* var. *gourda* have been demonstrated to be 24β-epimers by hydrogenation followed by ^{13}C NMR comparison with authentic 24-ethyllathosterols (24α- and 24β-**3f**) (18). The ^{13}C NMR data of **3j** and **3k** acetates isolated in this study from the seeds of *Benincase cerifera*, *Cucumis sativus* and *Cucurbita maxima* and from the aerial part of *Citrullus battich* were consistent with those of authentic 24β-**3j** and 24β-**3k** acetates (18), respectively; hence these sterols were regarded to be 24β-epimers. Although ^{13}C NMR data was not available for the Δ^5- (**2j** and **2k**) and Δ^8- (**4j** and **4k**) isomers of **3j** and **3k** isolated from *C. sativus* seeds in this study, the ^{1}H NMR comparison (26-H_3, 27-H_3 and 29-H_3 signals) of the Δ^5- and Δ^8-isomers (Table 3) with **3j** and **3k** suggested unequivocally that these sterols also have a 24β-configuration. Thus, only the 24β-epimers of 24-ethylsterols possessing a $\Delta^{25(27)}$-bond, i.e., **2j**–**4j** and **2k**–**4k**, are present in the Cucurbitaceae examined.

DISCUSSION

The Cucurbitaceae plants investigated contained 24-ethyl-Δ^7-sterols as the most predominant sterols, consistent with previous observations (2–14,16–20). As for the minor sterols, we detected four 24-ethyl-Δ^8-sterols, i.e., 24α- and 24β- **4g**, **4j** and **4k**, accompanied by Δ^5- and ring-saturated sterols. The occurrence in nature of Δ^8-sterols lacking a 4-methyl group is quite rare; they so far have been detected only in some lower organisms, i.e., in fungi (1,2,38), bacteria (2) and in the marine sponge *Axinella cannabina* (21,39). Among the four cucurbitacious Δ^8-sterols, 24α- and 24β-**4g** have been identified recently in *A. cannabina* (21), whereas **4j** and **4k** are considered new sterols. The Δ^8-sterols play a role as biosynthetic intermediates for other sterols through the sequence $\Delta^8 \rightarrow \Delta^7 \rightarrow \Delta^{5,7} \rightarrow \Delta^5$ (1,2 40). Taking into account this fact and that the Δ^7- and Δ^5-sterols are the component sterols of Cucurbitaceae species, it is highly probable that the Δ^8-sterols detected here are metabolized (at the C-4, C-14 demethylated sterol level) into other sterols in the Cucurbitaceae. The other uncommon sterols identified in this study were desmosterol (**2b**) and 24-ethyl-22-dehydrocholestanol (**1g**). Sterol **2b** is the major sterol of some red algae (3) but has never been found in higher plants, and **1g** has previously been reported to occur only in a few plant species (23,41,42). While the sterols **2j** (13), **2k** (13), **3h** (10,20) and 24*E*-**3i** (30) have been detected in some Cucurbitaceae species, this study demonstrated the widespread occurrence of these sterols in the family Cucurbitaceae.

As has been shown in Table 5, the co-occurrence of C-24 epimers was demonstrated in the stereochemically examined type (Δ^5, Δ^7 and Δ^8) of 24-alkylsterols from most of the plant materials investigated. As for the 24-methylsterols, whereas the coexistence of C-24 epimers of 24-methyl-Δ^5-sterols (**2c**) in many higher plants (2,10,32, 43) and of 24-methyl-$\Delta^{5,22}$-sterol (**2d**) in some Cruciferae species (25,44) has been proven, this study seems to be the first demonstration of the presence of 24-methyl-Δ^7- (**3c**) and $\Delta^{7,22}$-sterols (**3d**) as C-24 epimeric mixtures in higher plants. The co-occurrence of C-24 epimers of 24-ethyl-Δ^5- (**2f**), Δ^7- (**3f**) and $\Delta^{7,22}$- (**3g**) sterols from some Cucurbitaceae has been shown recently (18–20). Our study has demonstrated further the coexistence of C-24 epimers of 24-ethyl-$\Delta^{5,22}$-sterol (**2g**) isolated from *C. sativus* seeds. Our detection of poriferasterol (24β-**2g**) in a higher plant seems to be the first such instance. Although high resolution ^{1}H NMR spectroscopy has shown that 24-ethylsterols, which lack a $\Delta^{25(27)}$-bond, isolated from some Cucurbitaceae (**3g** from *Cucurbita pepo* seeds [11], **3f** and **3g** from the pericarp and leaves of *C. pepo* [10] and from the roots of *Bryonia dioica* [9] and **2f**, **2g**, **3f** and **3g** from *C. maxima* seeds [12,13]) are composed only of the 24α-epimers, the co-occurrence of their 24β-epimers in minor proportions ($\lesssim$10%) cannot be dismissed, since it is hard to detect such minor components by only ^{1}H NMR (34), even at 400 MHz. In this study, all of the 24-ethylsterols possessing a $\Delta^{25(27)}$-bond, i.e., three 24-ethyl-$\Delta^{25(27)}$-sterols (**2j, 3j** and **4j**) and three 24-ethyl-$\Delta^{22,25(27)}$-sterols (**2k, 3k** and **4k**), isolated from the Cucurbitaceae were proven to be 24β-epimers, consistent with previous observations (2,11–13,18,19).

Based on the considerations of sterol biogenesis (2,10, 45–47) and the present detection of 24-alkylsterols, four possible biosynthetic pathways to 24α- and 24β-alkylsterols in Cucurbitaceae can be postulated, as has been shown in Figure 3. The 24β-ethyl pathway, through side chain **k**, and the 24α-ethyl pathway have been proposed already for Tracheophyte sterols (2,10,46). The presence of additional sterols with side chain **f**(24β) may suggest the presence of an alternative pathway, **j**$\rightarrow$**f**(24β)$\rightarrow$**g**(24β), which is one of the possible sequences to 24β-ethylsterols in algae (2,45). The 24α-methyl pathway leading to sterols with side chain **c**(24α) has been discussed previously (2,10,45, 46). The detection of further 24α-methylsterols with Δ^{22}-side chain (d) may be explained by the presence of a **c**(24α)$\rightarrow$**d**(24α) dehydrogenation route analogous to the 24α-ethyl pathway. The 24β-methylsterols with side chain **c**(24β) are considered to be formed via 24-methyl-$\Delta^{25(27)}$-sterols (2,45–47). The occurrence of the latter sterols, i.e., 24β-methyl-25(27)-dehydrocholesterol and its Δ^7-isomer, has been demonstrated recently in *Cucurbita maxima* seeds (12,13). The 24β-methylsterols with side chain **d**(24β) may arise from those with **c**(24β) by dehydrogenation analogous to the 24α-ethyl pathway.

The great majority of eukaryotes contain sterols bearing a Δ^5-bond, and most higher plants contain predominantly

TABLE 5

Relative Proportions of C-24 Epimers of Some 24-Alkylsterols from Cucurbitaceae Estimated by ^1H and ^{13}C NMR[a]

Cucurbitaceae	Side chain	24-Me (c)		24-Me-Δ^{22} (d)	24-Et (f)		24-Et-Δ^{22} (g)		
	Ring system	Δ^5 (2)	Δ^7 (3)	Δ^7 (3)	Δ^5 (2)	Δ^7 (3)	Δ^5 (2)	Δ^7 (3)	Δ^8 (4)
	Config. at C-24	α:β	α:β	α:β	α:β	α:β	α:β	α:β	α:β
Seeds									
Benincasa cerifera			2:8	8:2		6:4[b]	10:0[b]	6:4[b]	
Citrullus battich (ref. 18)								5:5[b]	
Cucumis sativus		8:2	7:3	8:2	9:1[b]	7:3[b]	8:2[b]	3:7[b]	4:6
Cucurbita maxima						7:3[b]		9:1[b]	10:0
Cucurbita pepo			8:2			9:1[b]		9:1[b]	
Lagenaria leucantha var. *gourda* (ref. 18)								5:5[b]	
Lagenaria siceraria (ref. 17)								0:10	
Luffa cylindrica (ref. 16)								0:10[b]	
Trichosanthes japonica					4:6[b]			1<9[b]	
Mature plant tissues									
Citrullus battich (aerial part)			7:3			9:1[b]		6:4[b]	
Cucumis sativus (aerial part)		7:3	8:2	8:2	9>1[b]	9>1[b]	10:0	7:3[b]	
Gynostemma pentaphyllum (aerial part)			8:2					1<9[b]	
Trichosanthes japonica (roots; ref. 20)						8:2[b]		8:2[b]	

[a]If isolated from *Trichosanthes cucumeroide* was shown to be a 24α-epimer (37). **3j** and **3k** isolated from the seeds of *B. cerifera*, *Citrullus battich* (18), *Cucumis sativus*, *Cucurbita maxima* and *L. leucantha* var. *gourda* (18) and from the aerial part of *C. battich* were shown to be 24β-epimers on the basis of the ^{13}C NMR data. **2j**, **2k**, **4j** and **4k** isolated from *C. sativus* seeds also were shown to be 24β-epimers on the basis of the ^1H NMR data.

[b]Estimated based on the ^{13}C NMR data. All others were based on the ^1H NMR data.

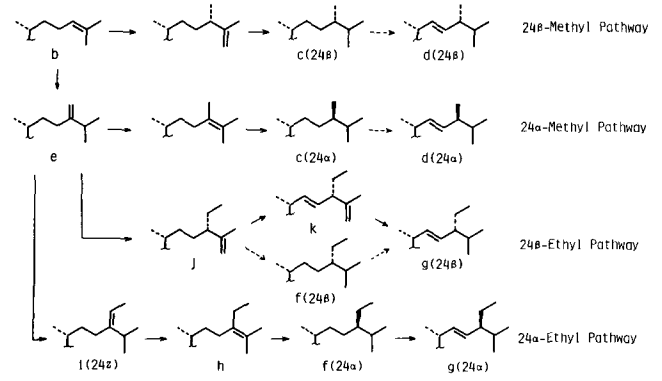

FIG. 3. Possible biogenetic sequences to 24α- and 24β-alkylsterols in Cucurbitaceae (cf. refs. 2, 10, 47-49). Dotted arrows denote that the routes have not been proposed thus far in higher plants.

24α-alkyl-Δ^5-sterols, e.g., sitosterol (24α-**2f**). It has been suggested that the plants of the family Cucurbitaceae may show an evolutionary transition in terms of sterols (2,13, 48). This suggestion is emphasized by the present study, which demonstrates the presence of significant amounts of 24β-alkylsterols in addition to 24α-alkylsterols accompanied by several uncommon sterols, including Δ^8-sterols.

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Sterol Composition During the Life Cycle of the Soybean and the Squash

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Sterol analyses were performed on soybeans and squash at intervals throughout the life cycle from seed to mature seed-bearing plant. The sterols of the soybean (24-methylcholesterol, stigmasterol and sitosterol) increased in quantity from that in the seed in each stage examined except for a pause or decrease prior to flowering and a decrease at senescence. Individual sterols remained in the same proportion to each other and changes in content were similar in roots and shoots. In the squash a much more complicated sterol mixture was found, composed primarily of C-7 unsaturated sterols characteristic of Cucurbitaceae. Sterol composition also increased during the life cycle except for approximately two wk in the preflowering to early flowering period. The data indicate low synthesis or high turnover of sterols (or both) in these plants in the weeks at or just prior to flowering.
Lipids 21, 48-51 (1986).

A great deal of analytical and biochemical work has been accomplished with plant sterols in the last decade. The variety of plant sterols in nature is greater than originally thought. Relatively new techniques of high performance liquid chromatography (HPLC) and capillary gas chromatography have helped to identify components of many previously unresolved mixtures, and many established or postulated biochemical pathways are available to explain the occurrence of the diverse array of compounds. However, remarkably little is known about the role of sterols in plants other than their well-known role as a component of cellular membranes (1). Sterols are known to occur in different quantities in various parts of the plant, and different sterols predominate in different parts of the life cycle of some plants (2-4). Even so, no detailed study has been conducted to determine qualitative and quantitative changes in sterol composition throughout the life cycle of a higher plant. Such a study should give clues to any roles of specific sterols as well as document any large conversion of sterols to cellular metabolites. The current study used the well-studied soybean with a typical tracheophyte sterol composition (3) and the squash with a complex and unusual mixture of sterols (4-6).

MATERIALS AND METHODS

Soybeans (*Glycine max* var. Essex) and squash (*Cucurbita maxima* var. Fordhook zucchini) were grown in the greenhouse and sterols were extracted from oven-dried material and isolated using standard methods (7). The sterol fraction was quantified by gas chromatography (GC) on a 30 m SE-30 capillary column and identified by this system assisted by GC-mass spectroscopy (GC-MS) on a DB-1 bonded methyl silicone column and a Finnigan-MAT model 4500 mass spectrometer as described previously (8). Relative retention times on the SE-30 capillary column were quite comparable to those reported for the SE-30 packed column (9). Cholesterol was used as the quantitative internal standard in the soybean and 7-cholesterol as the standard in the squash. Soybeans were planted on July 10, 1979, and samples were taken from stages of plant growth as follows: stage 1, seed; stage 2, young seedlings, 1 wk; stage 3, older seedlings with trifoliate leaves, 3 wk; stage 4, vegetative plants prior to flowering, 5 wk; stage 5, flowering plants, 7 wk; stage 6, plants with immature seeds, 11 wk, and stage 7, senescent plants with mature seeds, 16 wk.

Squash were planted on March 9, 1984, and in a replicate experiment on October 1, 1984, and samples were taken weekly for 12 wk. The 12-wk period produced all stages of the life cycle including the production of fruit with seeds, but senescent plants were not obtained at the end of this period.

RESULTS AND DISCUSSION

The principal sterols of whole soybean plants in all stages of the life cycle were 24-methylcholesterol, stigmasterol and sitosterol. These data are in accord with numerous reports on soybean seed sterols (10-13). The data reported here also agree with reports which analyzed sterol composition during germination (14) or in various plant parts (13,16). Cholesterol, 7-stigmastenol and β-amyrin also were found in small to negligible quantities. Sterol composition over the life cycle of the soybean is summarized in Figure 1. The sterol content of the soybean on a per-plant basis increases at each stage from germination until it declines at senescence, with the exception of the preflowering stage (stage 4) where a small decline occurs. This is seen in the data for roots as well as shoots. Fatty acid composition, which also was monitored, did not show a leveling-off or decrease when expressed in the same way. The sterol decline is from 114 μg per plant at stage 3 to 85 μg per plant at stage 4. The sterol decline is even greater in leaves during the same period, from 76 μg to 36 μg per plant. These reductions occur as the dry weight of the plants is increasing from 163 mg to 541 mg dry weight per plant. Figure 2 shows that at all stages in roots and shoots, the relative quantities of 24-methylcholesterol, stigmasterol and sitosterol remain essentially constant. When plotted in the more traditional way on the basis of dry weight (Fig. 3), the trend is toward a lower sterol composition with time, but the decline of sterol content in shoots at stage 4 is striking, reaching a level nearly as low as in senescent mature plants at stage 7.

A number of recent reports have demonstrated a sig-

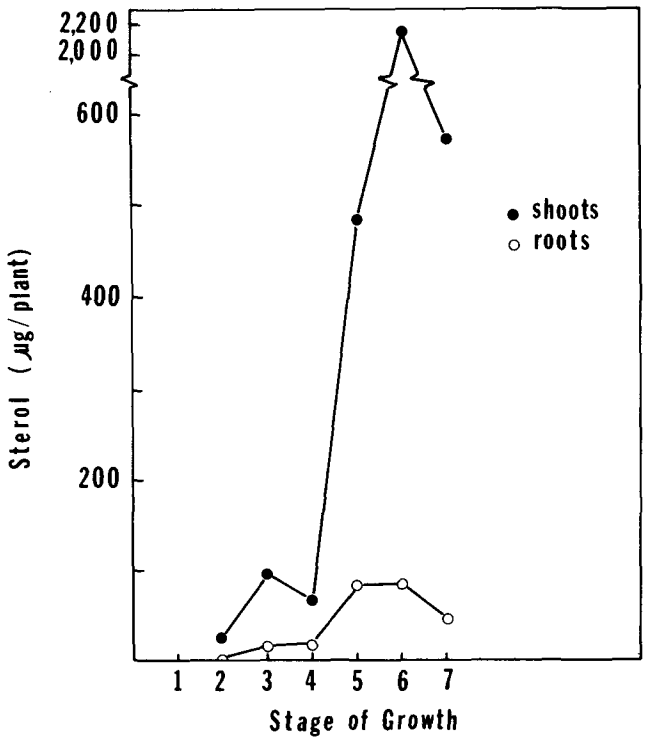
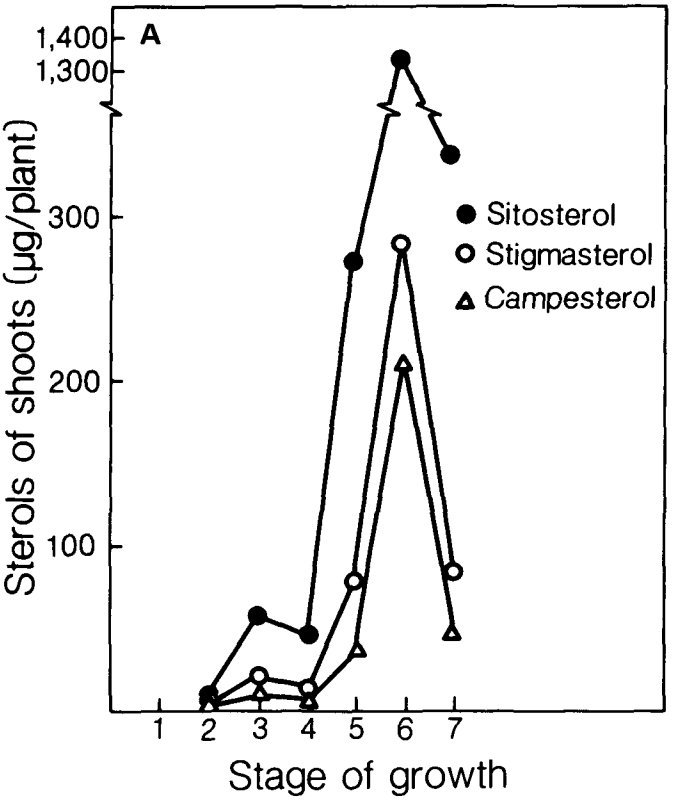


FIG. 1. Total sterol of soybean shoot and root (μg/plant).



nificantly different sterol composition in members of the Cucurbitaceae (2,5,6,17,18). Examination of five species of cucurbit seed was made in order to select one with a sterol mixture containing a good variety of sterols. Analysis of watermelon, muskmelon, pumpkin, squash and cucumber produced the data in Table 1. The major seed sterols of each species were 24β-ethylcholesta-7,22,25-trien-3β-ol, 24-ethylcholesta-7,22-dien-3β-ol and 24β-ethylcholesta-7,25-dien-3β-ol. Also present were other sterols with a monounsaturated nucleus at either the 7-, 5- or 8-position. Identifications and quantification of individual sterols will be reported in a future publication. The 24-ethyl-7,22-sterol was isolated by argentation chromatography and chromatographed on a long capillary column (19) to determine the ratio of 24α- to 24β-ethylsterol in each species. The percentage of 24-ethyl-7,22-sterol which is 24α (spinasterol) is 60%, squash; 30%, cucumber; 20%, muskmelon; 40%, watermelon, and 70%, pumpkin. Due to its low concentration in seed sterols, 24-ethylcholest-7-en-3β-ol was not examined for orientation at C-24. The squash was selected for examination of sterol composition over the life cycle for comparison with the soybean. In a 12-wk study (Fig. 4), the sterol composition per plant increased with time in both experiments at all stages, except during weeks 6-8 where a period of level composition occurred. During this time of unchanged sterol composition, however, the dry weight of the average plant increased from 350 mg to 1500 mg. This caused a strong reduction in the relative composition of sterols in the early flowering and preflowering periods in the midperiod of the 12-wk study (Fig. 5).

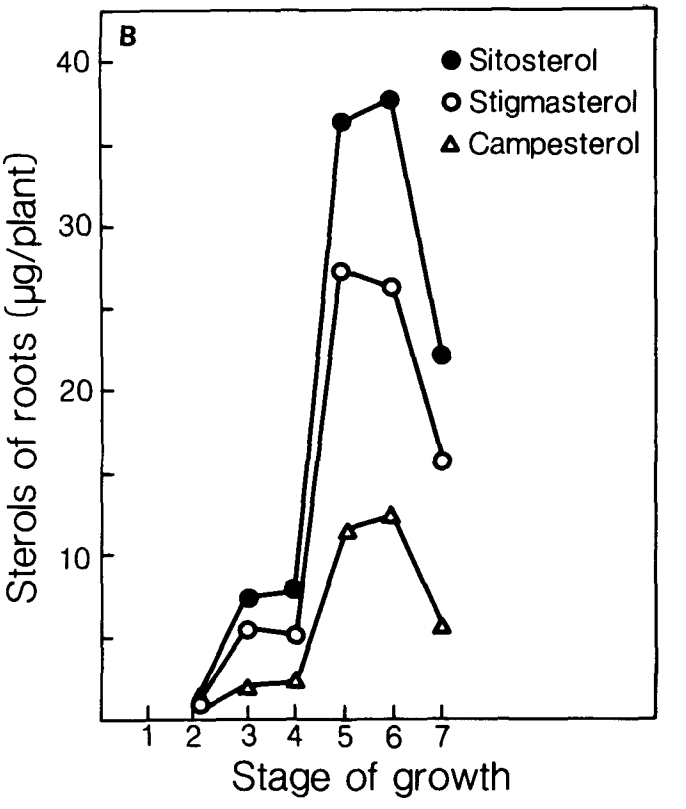


FIG. 2. A. Composition of major sterols of soybean shoots during the life cycle. B. Composition of major sterols of soybean roots during the life cycle.

TABLE 1

Individual Sterol Composition[a] in Five Cucurbitaceae Species

Plant	Double bond location					
	7,22	7	7,25	7,24(28)Z	7,22,25	Other sterols
Squash	23	4	18	12	39	5
Pumpkin	33	7	17	11	25	7
Cucumber	9	1	16	3	48	23
Watermelon	15	4	23	2	22	34
Muskmelon	9	2	19	--	43	27

[a]Percent of total sterol, each compound with a 24-ethylcholestanol skeleton with double bonds at the indicated positions.

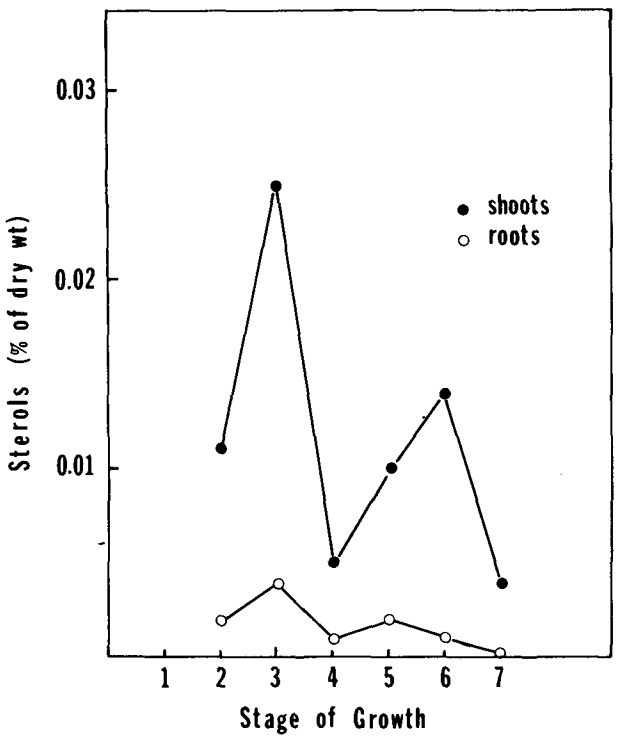


FIG. 3. Sterol composition of soybean shoots and roots during the life cycle (% dry weight).

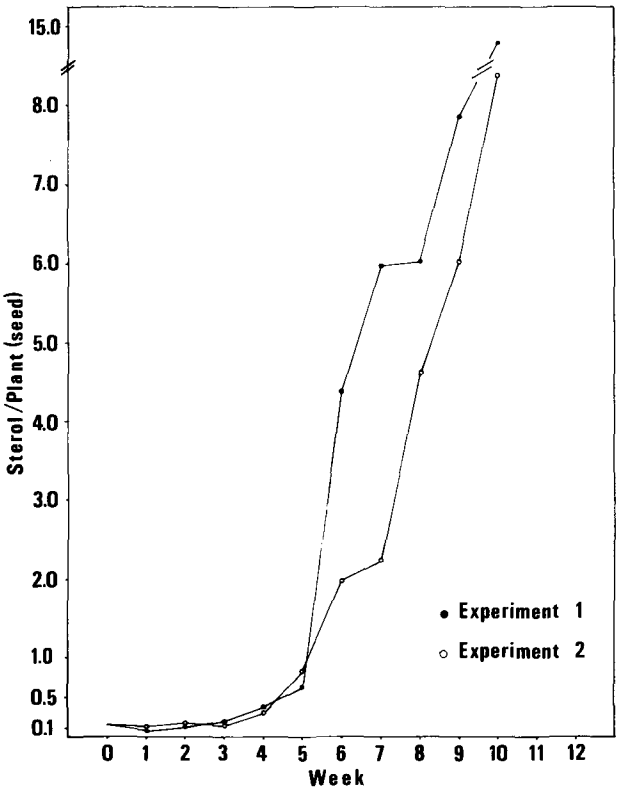


FIG. 4. Total sterol of squash during the life cycle (mg/plant).

The phenomenon of a leveling-off (per-plant basis) or a decline in the quantity of sterol at a time when total plant biomass is doubling or tripling suggests either (i) a total cessation of sterol synthesis at this time; (ii) a significant conversion of plant sterols to other compounds, balancing the rate of sterol synthesis, or (iii) both a reduction of sterol synthesis and an increased rate of sterol metabolism to nonsterols.

The analytical procedure described here does not include sterols in the glycoside or acetylated sterol glycoside form. Both are rarely of quantitative importance in plants since most plant sterols exist in the free form (1,15). For example, free sterols make up 95% of the total sterol in sorghum grain (20). Analysis of sterol glycoside composition in squash revealed that sterol glycoside composition was rather constant over the life cycle and that the sterol glycoside composition never exceeded 15% of total sterol. A rapid shift from sterol to cucurbitacin synthesis does not appear likely since cucurbitacin synthesis has been reported to decline soon after germination (21).

Sterols long have been considered membrane components (their primary role) and precursors for various kinds

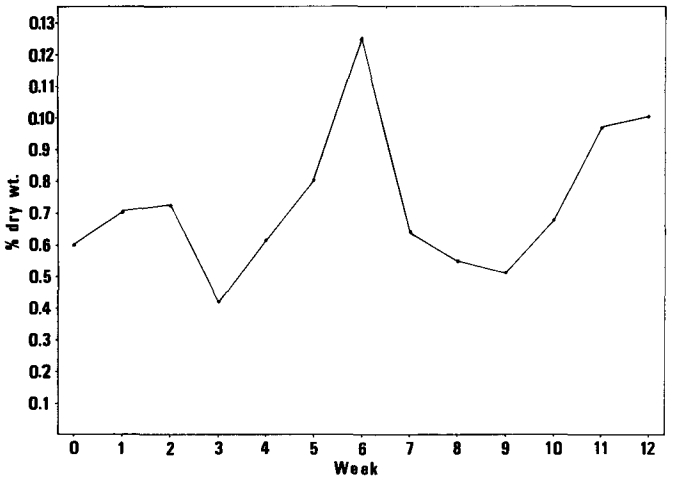


FIG. 5. Sterol composition of squash during its life cycle (% dry weight, average of two experiments).

of steroidal hormones (22,23). The hormonal role is well-established in animals (1,24), but although steroidal metabolites in plants with hormonal properties have been identified (25-27), none account for the quantities that our data suggest are likely. Steroids have been suggested as molecules involved in the flowering process (28), but efforts to establish a link between steroids and the flowering process have failed (29). The current data do not establish any such link. Changes in steroid composition which correlate to the flowering process have been reported previously (30,31), but these reports do not measure sterol content throughout the life cycle. The data presented here do provide evidence of major metabolic events concerning sterols and present the possibility of a sterol role in developmental processes of plants at or before flowering.

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Design of High Energy Intermediate Analogues to Study Sterol Biosynthesis in Higher Plants

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Several enzymes of plant sterol biosynthesis involve during their catalysis postulated or demonstrated carbocationic high energy intermediates (HEI). The aim of this study was to interfere with plant sterol biosynthesis by means of rationally designed species able to mimic these carbocationic HEI. It has been demonstrated previously that the design of transition state (TS) or HEI analogues could lead to powerful and specific inhibitors of enzymes. We applied this approach to the following target enzymes: 2,3-epoxy-2,3-dihydrosqualene cyclase, AdoMet-cycloartenol-C-24-methyltransferase (AdoMet CMT), cycloeucalenol-obtusifoliol isomerase (COI) and Δ^8-Δ^7-sterol isomerase. Very potent inhibitors have been obtained in the four cases. As an example, analogues of cycloartenol substituted at C-25 by a charged heteroatom (N, As, S) have been synthesized and shown to be able to mimic the C-25 carbocationic HEI involved in the reaction catalyzed by the AdoMet CMT. These compounds were shown to be very potent and specific inhibitors of this enzyme both in vitro ($Ki=2.10^{-8}$ M, $Ki/Km=10^{-3}$) and in vivo. The potent inhibitors described are powerful tools to control in vivo the sterol profile of plant cells and therefore to study the structural and functional roles of sterols in cell membranes. Moreover, these compounds constitute leader molecules of a new class of rationally designed inhibitors which could be of value in plant protection.

Lipids 21, 52-62 (1986).

Most eukaryotic cells contain sterols. In vascular plants, sitosterol (**1**) and stigmasterol (**2**) often constitute more than 80% of the total sterols (1). Sterols play at least two basic roles in living organisms. First, they are membrane constituents and as such are concentrated mainly in the plasma membrane (2) where they can regulate membrane fluidity (3). In addition, a very small fraction of cholesterol may be involved in a "metabolic" function (4). This last function still is not precisely defined, except when it appears that sterols are precursors of compounds having important physiological functions such as ecdysteroids in insects (5,6), antheridiol in some fungi (7) and brassinolids in plants (8). Very little is known about the structural role of 24-ethyl sterols such as **1** and **2** in higher plant membranes on one hand and about the metabolic role of sterols in eukaryotic organism membranes on the other. To increase our knowledge of these two aspects of sterol function, we planned to use sterol biosynthesis inhibitors (SBI). Following treatment of plant cells or whole plants by SBI, one would expect a decrease of the content of the two main sterols (**1** and **2**) and an accumulation of precursors. Several cases can be distinguished depending on the position of the enzymatic target of SBI in the biosynthetic scheme leading to higher plant sterols (Fig. 1). If the aimed enzyme is upstream from cycloartenol (**3**), the biosynthetic intermediates which would accumulate (e.g., 2,3-epoxy-2,3-dihydrosqualene [**4**]) are structurally very remote from the final Δ^5-sterols and would not be able to play the function normally ascribed to sterols in, for example, membranes. By contrast, if the targeted enzyme is downstream from cycloartenol, the accumulating intermediates are structurally more or less similar to the final sterols and could fill, at least partially, some functions normally devoted to sterols. In both cases interesting physiological consequences would be expected.

A possible reaction pathway corresponding to the transformation of a substrate (S) into a product (P) is summarized in the equation

$$E+S \rightleftarrows ES \rightarrow [ES]^{\ddagger} \rightarrow EI \rightarrow EP \rightleftarrows E+P$$

The concept of TS analogues is a successful one for the design of potent enzyme inhibitors (9,10). According to Bartlett and Marlowe (11), the rationale for this approach is the recognition that "additional binding interactions develop between an enzyme and the substrate, as their complex approaches its TS conformation. Such extra binding interactions, which are not available in the ground state complex, assist in lowering the energy of activation of the reaction and therefore, are a mechanism for enzymatic rate enhancement. TS analogue inhibitors seek to take advantage of these extra binding interactions by incorporating key structural elements of the unstable, TS form of the substrate in the stable structure of the inhibitor." The result is that TS analogue inhibitors may have a much higher affinity for the active site of the enzyme than traditional ground state analogue inhibitors (12,13). In many cases, the mechanism of the enzyme would involve enzyme-bound HEI (see equation), which often are better defined than TS. In such cases it is likely that the energy of the appropriate TS approximates that of the HEI. Therefore, the same strategy as that developed above for TS analogue inhibitors would apply for HEI analogue inhibitors.

From Figure 2, it is evident that several enzymes of

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Nomenclature: tridemorph (**52**), 4-(1,5,9-trimethyldecyl)-2,6-dimethyl morpholine; fenpropimorph (**51**), 4-[3-[4-tert-butylphenyl]-2-methyl]propyl-2,6-dimethylmorpholine; zymosterol (**12**), 5α-cholesta-8,24-dien-3β-ol; episterol (**41**), 5αergosta-7,24(28)-dien-3β-ol; fecosterol (**42**), 5α-ergosta-8,24(28)-dien-3βol; fucosterol (**38**), stigmasta-5,E-24(28)-dien-3β-ol; isofucosterol (**38a**), stigmasta-5-Z-24(28)-dien-3βol. Other steroids cited in the text have been drawn in Figure 1.

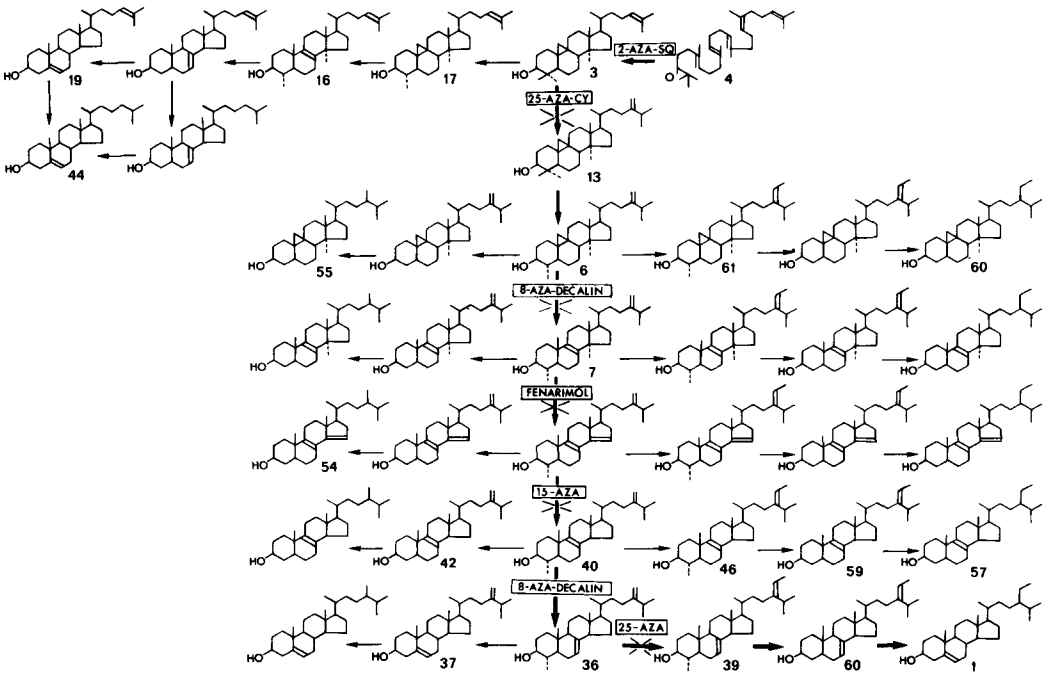


FIG. 1. Targets of the sterol biosynthesis inhibitors (SBI) used in the present study. 2-Aza-Sq: 2-aza-2,3-dihydrosqualene (9); 25-aza-cy: 25-azacycloartanol (15); 8-aza-decalin: N-benzyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol (48); fenarimol: α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidine methanol; 15-aza: 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol. The biosynthetic pathway operating in control plants is indicated with heavy arrows.

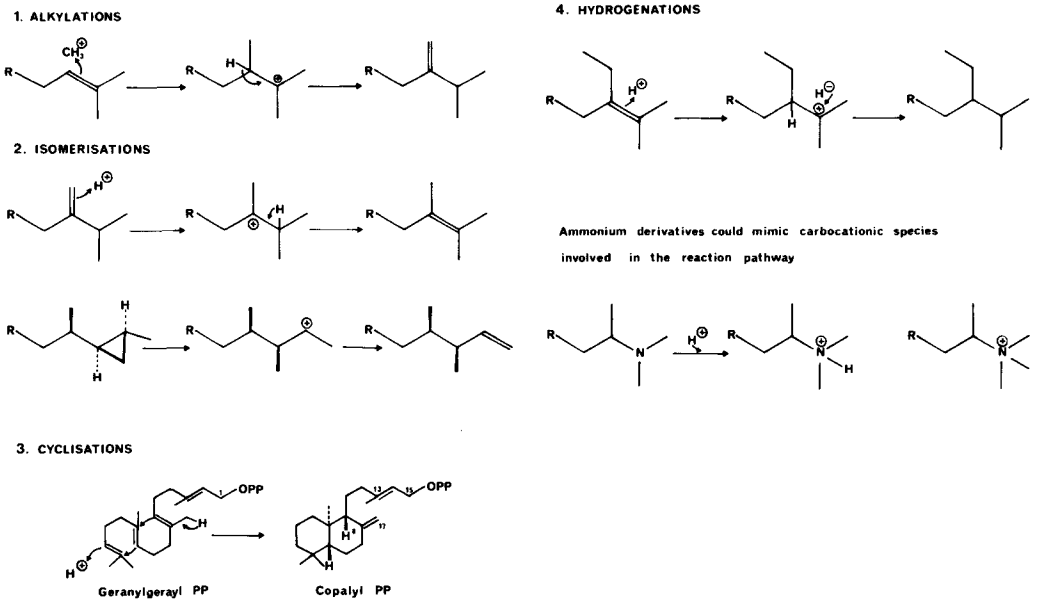


FIG. 2. Enzymic reactions belonging to sterol biosynthesis and involving demonstrated or postulated carbocationic intermediates. R: tetracyclic sterolic nucleus.

sterol biosynthesis catalyze reactions involving carbocationic intermediates; some of them, possibly stabilized transiently by the active site of the enzyme as a part of the enzyme mechanism, may be considered typical HEIs. This is the case especially for enzyme-catalyzed isomerizations, alkylations, hydrogenations and cyclizations listed in Figure 2. As shown also in Figure 2, a general strategy to mimic carbocationic HEI could be to replace the carbon atom bearing the positive charge with a nitrogen atom; the resulting tertiary amine having a pKa close to 10 is protonated at physiological (7.4) pH, and the resulting stable ammonium derivative displays a positive charge at a position identical to that occupied by the unstable HEI and could be considered a potential HEI analogue inhibitor. Such a strategy has been applied with success to design new inhibitors of targeted enzymes involved in plant sterol biosynthesis.

MATERIALS AND METHODS

Plant material, treatment of plants with SBI, enzymatic assays and inhibition of enzymes by SBI have been described previously (16–21).

Most of the inhibitors have been synthesized by ourselves as described elsewhere (16,18,19,21). The other inhibitors were provided as follows: **28,** Prof. Counsell (Ann Arbor, Michigan); **25–27,** Prof. Oehlschlager (Burnaby, British Columbia, Canada); **34,** Dr. Cenedella (Kirksville, Missouri); and **35,** Dr. Montavon (Basel, Switzerland). **36** and **39** were extracted from barley seeds. **40** was extracted from AY 9944-treated suspension cultures of bramble cells (14). **37** was extracted from a commercial pollen of unknown origin. Fecosterol was provided by D.H.R. Barton (Gif sur Yvette, France) and episterol by L.J. Goad (Liverpool, United Kingdom). **29** was purchased from Sigma (St. Louis, Missouri). **13** was given by Dr. Itoh (Tokyo, Japan) and **16** by Prof. Goutarel (Gif sur Yvette, France).

RESULTS

Inhibition of 2,3-epoxy-2,3-dihydrosqualene-β-amyrin (cycloartenol) cyclases [ESA(C)C]. The 2,3-epoxy-2,3-dihydrosqualene (**4**) cyclases represent a group of enzymes which convert **4** into polycyclic triterpenoids such as cycloartenol (**3**), lanosterol (**8**) or α and β-amyrin (**5**) (25) (Fig. 3). Enzymic cyclization of the all-*trans* **4** is believed to be triggered by a general acid-catalyzed epoxide ring opening assisted by the neighboring π-bonds (26). Such a mechanism implies the polarization of the C-2 oxygen bond leading to a charge deficiency at C-2 of **4** (Fig. 3). According to the strategy developed in the introduction, we have investigated the possibility of designing compounds which would be selective and potent inhibitors of the epoxysqualene cyclases. Accordingly, we have synthesized 2-aza-2,3-dihydrosqualene (**9**) and 2-aza-2,3-dihydrosqualene-N-oxide (**10**) as well as several derivatives (18,19, 27). The tertiary amines such as **9,** being protonated at physiological pH, could show some similarities with the transient HEI C-2 carbocation (**11**) that results from the protonation of the oxiran ring. The N-oxide (**10**), because of its strong dipolar moment (28), presents structural and electronic similarities with one possible TS (**4a**) involved in the first step (general acid-catalyzed oxiran ring opening) of the enzymatic cyclization of **4.** Accordingly **9** and **10** should behave as inhibitors of ESA(C)C. As shown elsewhere (18,19,24,27), this was indeed the case, as **9** and especially **10** were found to be powerful inhibitors of both plant and animal cyclases. This subject is developed in another paper of this symposium (24).

Inhibition of the S-Adenosyl-L-methionine-sterol-C-24-methyltransferase. The sterol-C-24-methyltransferase catalyzes the insertion of a methyl group into a Δ^{24} acceptor sterol. It has been demonstrated that the Δ^{24} sterol substrate for this enzyme in plants is cycloartenol (**3**) (29) whereas zymosterol (**12**) is the best substrate in yeast (30). **3,** when incubated in the presence of S-adenosyl-L-methionine (AdoMet) and the enzyme, is converted essentially into 24-methylene cycloartenol (**13**). A possible mechanism for this reaction is summarized in Figure 4. The C-methylation reaction can be considered in its initial step as a nucleophile attack of the Δ^{24} double bond of various sterols on the sulfonium methyl group of AdoMet (31–33). This reaction leads to the formation of an HEI (**14**) possessing a methyl (of unknown configuration in higher plants) at C-24 and a carbocation at C-25. After a hydride shift from C-24 to C-25, an elimination of a proton at C-28 occurs giving a 24-methylene sterol (33,34). The different steps of this reaction have been shown to proceed stereospecifically (31,32). In particular, it was shown that the stereochemistry at C-25 was not lost during the passage through the carbocationic HEI (**14**). One possibility to explain such stereochemical features would be the involvement of a transient interaction between the C-25 carbo-

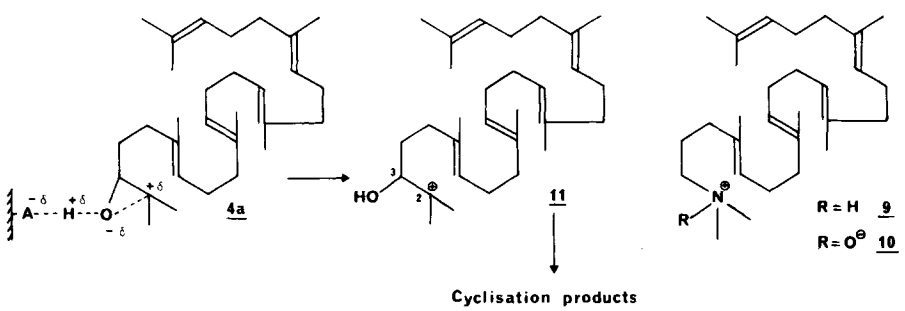


FIG. 3. 4a: Presumed transition state involved in the enzymic opening of the oxiran ring of 2,3-oxidosqualene (4).

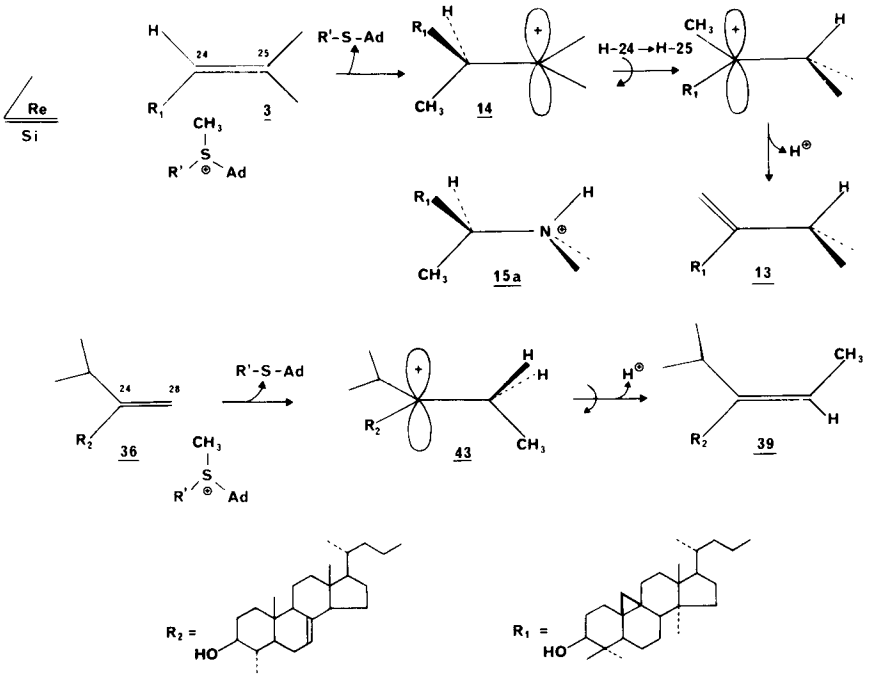


FIG. 4. A possible mechanism for the C-24 methylation of cycloartenol (3) by the AdoMet-3-C-24-methyltransferase and for the C-28 methylation of 24-methylene lopenol (36) by the AdoMet-36-C-28-methyltransferase. The C-24 methylation pathway is adapted from the work of Mihailovic (31). R′: $CH_2CH_2CH(NH_2)CO_2H$; Ado: adenosyl.

cationic HEI and the active site of the enzyme. With these considerations in mind, we thought it possible to mimic **14** by replacing the C-25 with a positively charged nitrogen atom (e.g., protonated amine) in the intermediate structure. In spite of the geometric differences existing between ammonium ions and carbocations, the two species present a charge analogy. Therefore, aza-steroids should be potential inhibitors of the methylation reaction in plants, as previously observed in yeast where 24-dihydro-25-azazymosterol has been shown to block in vivo and in vitro the C-24 methylation of zymosterol (**12**) in yeast (35). To ascertain this point in plant systems, microsomes obtained from maize embryos were incubated in the presence of the two substrates (AdoMet and **3**) of the C-24-methyltransferase and of various concentrations of 25-azacycloartenol (**15**). The data obtained showed that the AdoMet-cycloartenol-C-24-methyltransferase (AdoMet CMT) activity was reduced strongly ($I_{50} \pm 30$ nM) (I_{50}:concentration of inhibitor producing 50% inhibition at a given substrate concentration) (16).

Kinetic parameters of the inhibition. We first determined the K_M values for the two substrates (AdoMet and **3**) of the AdoMet CMT. The values found were $K_M^3 = 30$ μM and $K_M^{AdoMet} = 40$ μM. From the Lineweaver-Burk representations of the inhibition of the AdoMet CMT by **15**, it could be concluded that **15** is a noncompetitive inhibitor with respect to both **3** and AdoMet. The calculated Ki (30 nM) was three orders of magnitude lower than the K_M^3 (30 μM), attesting to the potency of **15** as an inhibitor (16).

Molecular parameters of the inhibition. (a) Substrate requirements of the AdoMet CMT. Before determining the molecular features involved in the inhibition of the AdoMet CMT by azasteroids, it was important to know the molecular features of the substrate that are involved in its ability to be methylated by the enzyme. For this purpose the compounds listed in Table 1 were used as potential substrates. The results show that cycloartenol was the best substrate of the series whereas lanosterol (**8**) was much less efficiently methylated. Surprisingly 31-nor-lanosterol (**16**) was as efficiently methylated as 31-nor-cycloartenol (**17**). This suggested that the differing capacities of 9β,19-cyclopropyl- and Δ^8-sterols to be methylated are fully expressed when the 4β-methyl is present, such as in **3** and **8.** When this methyl group is absent, such as in **16** and **17,** selectivity is no longer observed. When the 3β-hydroxyl group is engaged in an ester function such as in cycloartenyl acetate (**18**), the methylation capacity is abolished. This result gives evidence that the presence of a free hydroxyl group in the structure of the substrate is absolutely required for the methylation reaction.

(b) Influence of the charge of the nitrogen atom carried by the inhibitor molecule. Tertiary amines such as **15** have a pKa close to 10 and thus are protonated mainly at the pH (7.4) of the AdoMet CMT assay. The question which may be raised is whether the neutral or the protonated amine was the inhibitory species. To find out, the derivative possessing a quaternary ammonium function (**20**) was tested as an inhibitor. The results (Table 2) showed that **20** was as strongly inhibitory as **15,** giving evidence that the charged species possesses its own inhibitory power. However, this does not exclude that the neutral amine could display some inhibitory capacity. In that context the inhibitory power of electrostatically neutral isosteric ana-

TABLE 1

Substrate Requirements of the AdoMet-Cycloartenol-C-24-Methyltransferase (AdoMet CMT) and of the AdoMet-24-Methylenelophenol-C-28-Methyltransferase (AdoMet LMT) from Suspension Cultures of Bramble Cells

Substrate	Product expected	Radioactivity incorporated in the product (dpm)	
$\Delta^{24(25)}$-Sterols[a]			
Cycloartenol (**3**)	24-Methylene cycloartanol (**13**)	9,700	(100)[c]
Lanosterol (**8**)	24-Methylene-24-dihydro-lanosterol	850	(9)
31-Nor-cycloartenol (**17**)	Cycloeucalenol (**6**)	3,400	(35)
31-Nor-lanosterol (**16**)	Obtusifoliol (**7**)	3,300	(34)
Desmosterol (**19**)	24-Methylene cholesterol (**37**)	2,450	(25)
Cycloartenyl acetate (**18**)	13-Acetate	0	(0)
$\Delta^{24(28)}$-Sterols[b]			
24-Methylene lopenol (**36**)	24-Ethylidene lophenol (**39**)	3,650	(100)[d]
4α-Methyl fecosterol (**40**)	4α-Methyl-5α-stigmasta-8,Z-24(28)-dien-3β-ol (**46**)	2,950	(80)
Episterol (**41**)	5α-Stigmasta-7, Z-24(28)-dien-3β-ol (**60**)	2,100	(58)
Fecosterol (**42**)	5α-Stigmasta-8, Z-24(28)-dien-3β-ol (**59**)	360	(10)
24-Methylene cholesterol (**37**)	Isofucosterol (**38a**)	190	(5)
Cycloeucalenol (**6**)	Cyclofontumienol (**61**)	0	(0)

The incubation mixture contained 0.35 ml microsomes, 100 μM (0.1 μCi) [methyl-^{14}C]AdoMet and the substrates (100 μM) emulsified in aqueous Tween 80 (final concentration 0.1%). Total volume 0.5 m. Incubations were carried out at 30 C for 1 hr. Each value is the average of six experiments.

[a]Substrates of the AdoMet CMT.

[b]Substrates of AdoMet LMT.

[c]Percent of activity relative to the best substrate (**3**) of AdoMet CMT.

[d]Percent of activity relative to the best substrate (**36**) of AdoMet LMT.

TABLE 2

Inhibition of the AdoMet-Cycloartenol-C-24-Methyltransferase by Various Azasteroids Influence of the Charge of the Nitrogen Atom

Compounds		Ki (nM)
15	25-Azacycloartanol	30
21	24-Dihydrocycloartenol	No inhibition
20	25,26,27-Trisnor-24-trimethylammonium-cycloartanol	35
22	24-Dihydro-25-methyl-cycloartenol	200,000

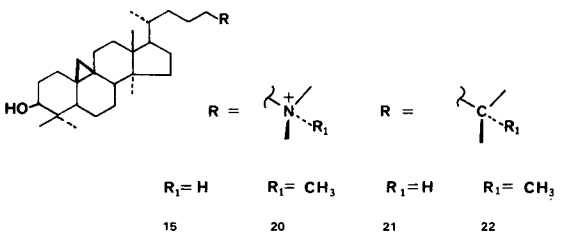
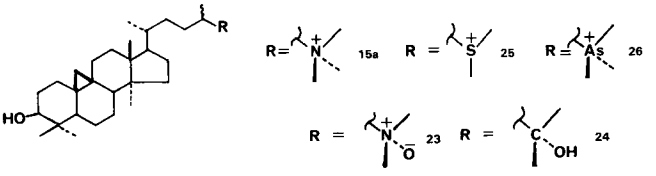


TABLE 3

Inhibition of the AdoMet-Cycloartenol-C-24-Methyltransferase by Various Azasteroids and Their N-Oxide Derivatives. Replacement of the N Atom by Other Heteroatoms (S, As)

Compounds		Ki (nM)
15a	(24-R)-24-Methyl-25,26,27-trisnor-24-trimethylammonium-cycloartanol iodide	35
25	(24-R,S)-24-Methyl-25,26,27-trisnor-24-dimethylsulfonium-cycloartanol iodide	50
26	(24-R,S)-24-Methyl-25,26,27-trisnor-24-trimethyl-arsonium-cycloartanol iodide	25
23	(24-R,S)-24-Methyl-25,26,27-trisnor-24-dimethylamine-N-oxide-cycloartanol	15
24	(24-R,S)-24-Methyl-25-hydroxy-cycloartanol	100,000



logues of **15** and **20** such as 24-dihydrocycloartenol (**21**) and 24-dihydro-25-methylcycloartenol (**22**) was looked for. The compounds **21** and **22** when assayed were found to have negligible inhibitory properties (Table 2). Compound **23** (a tertiary amine N-oxide) is globally neutral but presents a strong dipolar moment allowing it to be written formally with a positive charge centered on the nitrogen atom and a negative charge on the oxygen atom. The results showed that **23** was strongly inhibitory (Ki ± 15 nM) (Table 3). Here again, (24-R,S)-24-methyl-25-hydroxy-cycloartenol (**24**), which can be considered an electrostatically neutral isosteric analogue of **23**, was only slightly inhibitory (Table 3).

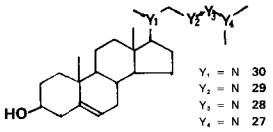
(c) Replacement of the nitrogen by other heteroatoms. To check the generality of our hypothesis, the 25-trimethylammonium group was replaced by substituents containing a cationic heteroatom, such as dimethylsulfonium (**25**) and trimethyl arsonium (**26**) groups. The results obtained (Table 3) gave evidence that **25** and **26** were inhibitors of the AdoMet CMT. In particular, **26** was remarkably potent. Thus, the carbocationic HEI (**15**) is well-mimicked by three heteroatomic cations capable of displaying a similar positive electrostatic field, independent of their hardness.

(d) Influence of the localization of the positive charge in the side chain. To check if the 25-nitrogen-substituted triterpenoids were the closest analogues to the postulated HEI (**14**), azasterols (**27–30**) possessing a nitrogen atom at various positions (25, 24, 23 and 20) in the side chain were tested. As shown in Table 4, 25-azacholesterol (**27**) was

TABLE 4

Inhibition of the AdoMet-Cycloartenol-C-24-Methyltransferase by Various Azasteroids. Influence of the Location of the Positive Charge

Compounds		Ki (nM)
27	25-Azacholesterol	45
28	24-Azacholesterol	180
29	23-Azacholesterol	90
30	20-Azacholesterol	11,000
31	Solanine	No inhibition



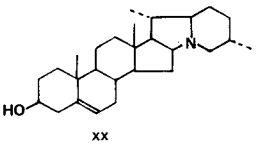


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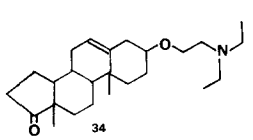
TABLE 5

Inhibition of the AdoMet-Cycloartenol-C-24-Methyltransferase by Various Azasteroids. Influence of the Tetracyclic Moiety

Compounds		Ki (nM)
15	25-Azacycloartanol	30
27	25-Azacholesterol	45
32	25-Azalanostenol	30
33	25-Azacycloartanyl acetate	30
34	U 16888 A	4,800
9	2-Dihydro-2-azasqualene	No inhibition
35	Ro 4-4445	No inhibition



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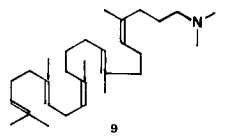

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the most active of the series, and **28** was four times less inhibitory than **27.** Surprisingly **29** was intermediary between **27** and **28.** Finally **30** was 200 times less potent than **27.** Solanine (**31**), a naturally occurring azasteroid, was shown not to be inhibitory at the highest concentration (100 μM) tested.

(e) Influence of the tetracyclic moiety of the 25-azasteroids. To evaluate the contribution of the inhibition of the uncharged part of the structure of the preceding analogues, we tested the tertiary amines (**32–35** and **9**). The first class of products considered (**32–34**) still contained a triterpenoidal skeleton. The results (Table 5) show that 25-azalanosterol (**32**) was as inhibitory as **15,** that the acetate of 25-aza-cycloartenol (**33**) was as active as the free alcohol (**15**) and that 25-azacholesterol (**27**) was significantly less active than **15.** The results showing that **15** is not a better inhibitor than **32** whereas cycloartenol is a much better substrate than lanosterol (Table 1) suggest that the typical conformation of cycloartenol no longer is required in the inhibitor structure. Likewise, the fact that the acetate of 25-azacycloartenol (**33**) is as inhibitory as **15** itself although cycloartenyl acetate was shown not to be a substrate for the AdoMet CMT (Table 1) showed that the free hydroxyl, which is absolutely required for the substrate to be transformed, is not required for the inhibitor to be active. The second class of products considered (**9** and **35**) did not possess a steroidal skeleton, but has a size and a hydrophobicity similar to that of steroids. Ro 4-4445 (**35**) is a compound very similar to triparanol, a hypocholesterolemic drug previously shown to inhibit C-24-methyltransferase activity in a pea seedling homogenate (36). 2-Aza-2,3-dihydrosqualene (**9**) has been shown to be a strong inhibitor of the 2,3-oxidosqualene-sterol cyclases (24). Neither **35** nor **9** were inhibitory at the highest concentration (100 μM) tested (Table 5).

In conclusion, this study of chemical structure-inhibitory activity relationships has led to the following results: (i) the presence of a positive charge at position 25 was the major cause of the inhibition as electrostatically neutral isosteric compounds possessing a carbon in place of the nitrogen atom are not inhibitory. The positive charge leading to inhibition may be conferred by a protonated amine or a quaternary ammonium group, as well as by a sulfonium or an arsonium group; it should be located at position 25; (ii) a steroid-like structure of the inhibitor is also important, and (iii) the presence of a free 3β-hydroxy group and the typical conformation of cycloartenol, essential molecular features of the substrate for the methylation reaction, are no longer required to observe inhibition. This last result is important, as it suggests that the conformation of the Adomet-cycloartenol-C-24-methyltransferase which recognizes 25-azacycloartanol is different from the conformation of the enzyme in the initial cycloartenol-AdoMet complex, reflecting the fact that the 25-azasteroids have been designed to mimic a carbocationic HEI such as **14** occurring later in the reaction pathway. This agrees with the literature (10,37,38) suggesting that the molecular features which determine the affinity of TS (or HEI) analogues should be different from those affecting the recognition of the substrate(s) in the ground state. Therefore, the data obtained strongly support the idea that C-25 heteroatoms (N, S, and As) substituted triterpenoid derivatives possessing a positive charge at position 25, are analogues of a carbocationic HEI involved during the reaction catalyzed by the AdoMet-cycloartenol-C-24-methyltransferase. However, such a hypothesis remains to be tested by an appropriate experimentation. This could be done since the binding of true TS or HEI analogues displays features allowing them to be distinguished easily from ground state analogues (11,37). In particular, while ground state analogue inhibitors are bound essentially instanteously, slow binding would be characteristic of TS or HEI analogue inhibitors and might be a reliable indicator of this class of inhibitors (37).

Inhibition of the S-Adenosyl-L-methionine-sterol-C-28-methyltransferase. The sterol-C-28-methyltransferase catalyzes the insertion of a methyl group into a $\Delta^{24(28)}$ acceptor sterol. It has been demonstrated that one good $\Delta^{24(28)}$ sterol substrate for the C-28-methyltransferase from suspension cultures of bramble cells was 24-methylene lophenol (**36**) (39) whereas 24-methylene cholesterol (**37**) was the best substrate in *Uromyces phaseoli,* a phycomycete which synthesizes fucosterol (**38**) as major sterol (40). **36,** when incubated in the presence of [methyl-^{14}C]AdoMet and microsomes from suspension cultures of bramble

cells, is converted into a compound cochromatographing with cold 24-ethylidene lophenol (**39**) (39). Before studying the inhibition of the AdoMet-24-methylene lophenol (**36**)-C-28-methyltransferase (AdoMet LMT), it appeared important to get some data on the relationships between the chemical structure of the substrate and the enzymatic activity.

(a) Substrate requirements of the AdoMet-24-methylene lophenol-C-28-methyltransferase. The compounds listed in Table 1 have been used as potential substrates. As expected following previous studies (39), **36** appeared to be the best substrate of the series; however, 4α-methyl fecosterol (**40**) was almost as efficient as **36.** Episterol (**41**), which lacks the 4α-methyl group, gave only 50% of the maximal activity, fecosterol (**42**) was still less efficient and 24-methylene cholesterol (**37**) gave very little activity. 24-Methylene cycloartenol (**13**), cycloeucalenol (**6**) and obtusifoliol (**7**) were not substrates for this enzyme. Therefore the molecular features involved in the ability of $\Delta^{24(28)}$-sterols to be substrates of the AdoMet LMT are the following: (i) the 4α-methyl group and the Δ^7 (or Δ^8) double bond appear to be important, and (ii) the presence of a methyl group at C-14 (and possibly also of the cyclopropane ring) abolishes the substrate capacity; therefore, while $\Delta^{24(25)}$-9β,19-cyclopropyl sterols (e.g., **3**) are the best substrates for the AdoMet CMT, $\Delta^{24(28)}$-9β,19-cyclopropyl sterols (e.g., **6**) are not substrates for the AdoMet LMT. Thus the 9β,19-cyclopropane ring must be opened and the 14α-methyl group removed before that sterol would be methylated by the AdoMet LMT. These results and those reported above concerning substrate requirements of the AdoMet CMT are in accordance with a biosynthetic scheme outlined previously (1,41). In this scheme, the first sterol containing a carbon atom unit at C-24 is indeed **13**, and the first sterol containing a two-carbon unit at C-24 is **39**, whose formation evidently results from C-28 transmethylation of **36** (Fig. 1).

A possible mechanism for the AdoMet LMT reaction is summarized in Figure 4. The C-28 methylation reaction can be considered in its initial step as a nucleophilic attack of the $\Delta^{24(28)}$ double bond of various sterols on the sulfonium methyl of AdoMet (1). The reaction leads to the formation of an HEI (**43**) possessing a carbocation at C-24. Then a stereospecific elimination of a proton at C-28 occurs, giving a 24-ethylidene sterol with generally the Z-24(28) configuration in higher plant systems. As suggested above in the case of the AdoMet CMT, one possibility would be the involvement of a transient interaction between the C-24 carbocationic HEI and the active site of the enzyme. With these considerations in mind, we thought it possible to mimic **43** by the azasteroids already used in the present study and by 25-azacycloartenol (**15**) in particular. Microsomes obtained from maize embryos were incubated in the presence of AdoMet and various concentrations of **15.** Then the amount of **39** formed from **36** present endogenously in microsomes was quantified. The results obtained showed that the AdoMet LMT activity was strongly reduced ($I_{50} \pm 100$ nM) (42) by **15,** giving evidence that **15** also is a potent inhibitor of the C-28-methyltransferase. However, the I_{50} found is higher than that (30 nM) found in the inhibition of the AdoMet CMT by **15.** Such an observation agrees with the fact that the position of the positive charge present in **15** is not identical with that of the positive charge present in the HEI (**43**). In addition, the structure of the tetracyclic moiety in **15** differs from that present in **43.** Nevertheless, the difference between the inhibitory activity of **15** on the AdoMet CMT and the AdoMet LMT is not very important, and this observation is consistent with the rather delocalized character of the positive charge in both carbocationic HEI (**14** and **43**) and in the ammonium derivative of **15** as discussed previously (16,43,44). To obtain more potent and specific inhibitors of the AdoMet LMT, azasteroids mimicking more closely the HEI (**43**) should be synthesized.

(b) In vivo experiments. When the culture medium of suspension cultures of bramble cells was supplemented with **15,** the growth was severely inhibited at concentrations of **15** close to 1 μM. Concentrations of **15** higher than 2 μM stopped cell growth (41). The sterol profile of cells treated with **15** (1 μM) has been reported previously (41). The major result was a strong decrease of the content in 24-ethyl sterols and particularly in sitosterol (**1**) and a striking accumulation of sterols nonalkylated at C-24, such as cycloartenol (**3**), cholesterol (**44**) and desmosterol (**19**). This result shows that the two C-24 and C-28 methyltransferases catalyzing the insertion of the two extra carbon atoms present at position C-24 in **1** are inhibited in vivo in agreement with the in vitro experiments (41). Furthermore, the strong accumulation of **3** and **36** (41) is consistent with the biosynthetic pathway of Figure 1 where the central positions occupied by **3** and **36** in the two C-methylation reactions have been thrown into relief.

Finally, C-25 heteroatom (N, As and S) substituted steroids derivatives are potent molecular tools to manipulate sterol alkylation in higher plant and yeast (35) membranes. One major question raised by the above results is whether there are relationships between C-24-methyltransferase inhibition and the severe growth reduction observed upon treatment of bramble cells with **15.**

Inhibition of $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and of COI. COI catalyzes the cleavage of the 9β,19-cyclopropane ring of **6** to give **7** (Fig. 5). The first step of this reaction consists of the C-19 protonation of cyclopropane leading to the HEI (**45**) bearing a carbocation at C-9. Then there is a *cis* regiospecific elimination of the H-8β to give the Δ^8-double bond (17). To explain these stereochemical features, it has been suggested that the HEI (**45**) could be stabilized by a suitable subsite (anionic or electron-rich subsite) of the enzyme (1,17). The $\Delta^8 \rightarrow \Delta^7$-sterol isomerase catalyzes the $\Delta^8 \rightarrow \Delta^7$ isomerization of the Δ^8-sterol such as **46** to give the Δ^7-sterol (**39**) (Fig. 5). According to Wilton et al. (45), the reaction involves first an α-protonation of the Δ^8-double bond giving an HEI (**47**) possessing a carbocation at C-8 (a localized form of a three center H^+ bridging a C-8–C-9 bond). Then there is elimination of a C-7 proton to give the Δ^7-double-bond. Loss of the 7β-hydrogen atom occurs in rat liver homogenates (46), whereas in yeast the enzyme action proceeds with loss of the 7α-hydrogen atom (47,48). In higher plants, in vivo experiments suggest that the H-7β is eliminated, as in rat liver (1,49). To our knowledge no experiments using cell-free extracts to study the isomerase in higher plants have been reported. With

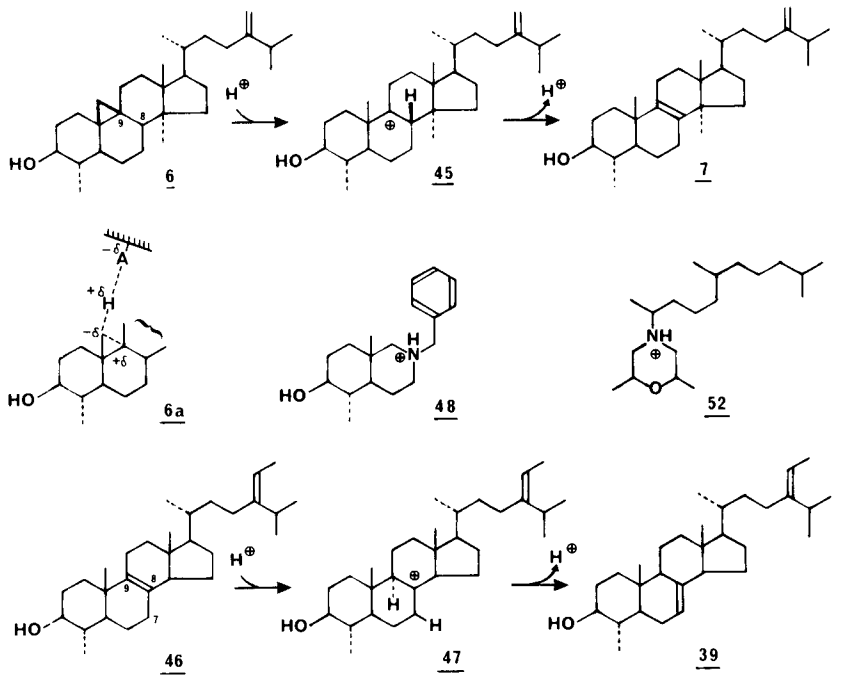

FIG. 5. Hypothetical reaction pathways for cycloeucalenol-obtusifoliol isomerase (6→45→7) and $\Delta^8\rightarrow\Delta^7$-sterol isomerase (46→47→39).

these considerations in mind, we have designed model molecules, such as N-benzyl-8-aza-4α,10-dimethyl-*trans*-decal-3β-ol (**48**), which can be considered low MW analogues of HEI (**45** and **47**) occurring in the reactions catalyzed by the COI and the $\Delta^8\rightarrow\Delta^7$-sterol isomerase. Indeed, these tertiary amines present structural and electronic similarities with the HEI (**45** and **47**) involved during the reactions catalyzed by the two enzymes (Fig. 5). In particular, the location of the positive charge conferred by the ammonium group in **48** is identical to that of the C-8 carbocation of **45**. Alternatively, the location of the positive charge in **48** is spatially very close to that occupied by the carbocation at C-9 in 45. Accordingly, we hoped that **48** and its derivatives could be inhibitors of the COI and of the $\Delta^8\rightarrow\Delta^7$-sterol isomerase. The results show that **48** is an extremely potent inhibitor of these enzymes both in vivo and in vitro (21,50).

The inhibition of COI and $\Delta^8\rightarrow\Delta^7$-sterol isomerase activities by **48** was measured in vitro in an enzymatic system prepared from maize seedlings as described earlier (17,21,51). In the case of the COI, the microsomes were incubated in the presence of **6** at 30 C for 45 min. The product (**7**) of the reaction was identified and quantified using gas chromatography-mass spectroscopy (GC-MS) (17). A preliminary study of the $\Delta^8\rightarrow\Delta^7$-sterol isomerase (Taton, M., unpublished results) as well as biosynthetic considerations arising from Figure 1 have given evidence that **46** was a good substrate of the enzyme. Therefore, the microsomes were incubated in the presence of **46** at 30 C for 2 hr. The product of the reaction, **39**, was identified by MS and ^{1}H NMR and quantified by capillary GC. The putative inhibitor (**48**) was shown to inhibit strongly both the COI and the $\Delta^8\rightarrow\Delta^7$-sterol isomerase activities. From the inhibition curves it was possible to calculate I_{50} values

TABLE 6

Inhibition of Enzymes of Sterol Biosynthesis by 8-Azadecalins (48 and 49), by 4α,10α-Dimethyl-*trans*-Decal-3β-ol (50) and by the N-Substituted Morpholines (51 and 52)

Inhibitor	COI	$\Delta^8\rightarrow\Delta^7$-sterol isomerase ($I_{50}$ [μM])	AdoMet CMT
48	0.10±0.05	0.13±0.05	50
49	17.5	10	200
50	_[a]	_[a]	_[a]
51	0.4±0.1	0.6	_[b]
52	0.4±0.1	_[c]	_[c]
53	0.4	_[c]	_[c]

In all the enzymatic assays, the microsomes from maize seedlings (0.5 ml) were incubated in the presence of substrate (100 μM), various concentrations of inhibitors (10 nM-100 μM) and Tween-80 (final concentration 0.1% [w/v]) at 30C for 45 min.

[a]No inhibition at the highest concentration (100 μM) tested.

[b]20% Inhibition at 100 μM of inhibitor.

[c]Not determined.

of 0.1 and 0.13 μM for the COI and the $\Delta^8\rightarrow\Delta^7$-sterol isomerase, respectively (Table 6). Under the assay conditions used in this study, where the concentrations of the substrates were close to their Km values (100 μM for both **6** and **46**), I_{50} is in the order of the inhibition constants (52) and the values obtained for **48** indicate the COI and the $\Delta^8\rightarrow\Delta^7$-sterol isomerase had a much higher affinity (three orders of magnitude) for the HEI analogues than for their best substrates (**6** and **46**, respectively). To gain more information about the molecular features involved in

the inhibition, **48** was compared to 8-aza-4α-10-dimethyl-*trans*-decal-3β-ol (**49**) and to 4α,10-dimethyl-*trans*-decal-3β-ol (**50**). **49** differs from **48** in lacking a benzyl substituent on the nitrogen atom. **50** is a neutral isosteric analogue of **49**. In addition, **48** has been compared to fenpropimorph (**51**) and tridemorph (**52**). **51** and **52** are systemic fungicides used in agriculture which have been shown to inhibit strongly sterol biosynthesis in plants in vivo (15,22) at the level of the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase (see below). Therefore they appear to be good candidates also to inhibit these enzymes in vitro. A rationale for their inhibitory properties similar to that proposed for **48** also could be suggested for **51** and **52** as these morpholines, having a pKa in the order of 7.5, would be present in both neutral and protonated forms at physiological pH. Therefore, as the morpholinium cations present some resemblances with the HEI **45** and **47**, they would be expected to interact with the active sites of both the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase (Fig. 5). The data of Table 6 show that this is indeed the case as **51** and **52** powerfully inhibit both enzymes although they appear to be less potent than **48**. Compound **53**, the N-oxide derivative of **51**, was shown to be as active as **51**. When tested on the AdoMet-cycloartenol-C-24-methyltransferase, none of these products significantly inhibited this enzyme, at least for the concentrations which lead to strong inhibition of the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase.

In vivo inhibition of the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase. In recent years it became evident that several chemicals having fungicidal activities were also inhibitors of sterol biosynthesis (53). One important group of these fungicides is derived from morpholines such as **51** and **52** (54–56). Evidence has been given that **52** inhibits Δ^8- to Δ^7-sterol isomerization in *Botrytis cinerea* (57,58) in vivo, leading to accumulation of Δ^8-sterols such as fecosterol (**42**). Inhibition of ergosterol biosynthesis by **51** also was noticed in *Ustilago maydis* culture. In this case, ergosta-8,14-dien-3β-ol (**54**) has been reported to accumulate as a major sterol (59). It therefore was suggested that the fungicide inhibited the Δ^{14}-reductase in this material. We have performed similar studies in suspension cultures of bramble cells (22), in maize (15,60) and in wheat (Costet, M.F., unpublished results) seedlings. We were able to show that treatment of maize seedlings with **51** and **52** resulted in a spectacular accumulation of cyclopropyl sterols (Fig. 1). Among them, 24-methyl pollinastanol (**55**) (40% of total sterols), cycloeucalenol (**6**) and 24-dihydrocycloeucalenol (**56**) predominate. In addition to the 9β,19-cyclopropane ring, more than 95% of these sterols possess a methyl group at C-24 in place of the ethyl group normally found in higher plant sterols. This agrees with the inability of 24-methylene cyclopropylsterols to be substrates of the AdoMet LMT. A striking feature of these sterols concerns their configuration at C-24. For instance, high field ^{1}H-NMR spectroscopy has shown that **55** was a mixture of two isomers containing 85% of the 24-S epimer and 15% of the 24-R one. This contrasts strongly with control cells where the major sterol, sitosterol (**1**) is a 100% 24-R sterol. When the plant cells were treated with low concentrations (1 mg/l) of **51** or **52**, the following Δ^8-sterols were shown to accumulate in addition to cyclopropyl sterols: (24-ξ)-24-ethyl-5α-cholest-8-en-3β-ol (**57**); (24-ξ)-24-ethyl-5α-cholesta-8,22-dien-3β-ol (**58**), and 5α-stigmasta-8,Z-24(28)-dien-3β-ol (**59**) (15). These sterols are essentially 24-ethyl Δ^8-sterols. Very low amounts of 24-methyl Δ^8-sterols were found, suggesting that the C-28 methyltransferase is facilitated on Δ^8-sterols. Therefore our results (15,22,60), recent data from other authors (61) and the data reported above with cell free systems (Table 6) give a large array of evidence that one major target of **51** and **52** in plant cells is the COI. Not only did the chemicals lead to an accumulation of sterol intermediates (mainly 9β,19-cyclopropyl sterols), but they also led to changes in the stereochemistry of some enzymatic reactions. A recent study performed in our group (Bladocha, M., unpublished results) has given evidence that **52** affects indirectly the stereochemistry of the hydrogenation involved in the last step of the C-24 alkylation process. A secondary target of **51** and **52** is probably the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase.

RESULTS AND DISCUSSION

Results obtained in the present work show that molecules bearing a tertiary amine function in a suitable position behave as strong SBIs. For instance, molecules such as 2-aza-2-dihydrosqualene (**9**), 25-azacycloartenol (**15**) and N-benzyl-8-aza-4α,10-dimethyl-5α-decal-3β-ol (**48**) inhibit powerfully (Ki/Km in the order of 10^{-3}) the following enzymes involved in sterol biosynthesis: ESA(C)C, AdoMet CMT and both COI and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, respectively. As shown in Figure 2, the reactions catalyzed by these enzymes are supposed to involve positively charged HEIs; therefore it is tempting to suggest that the protonated amines are the active species because they mimic the carbocationic HEI. This assumption has received strong support because in all three cases, quaternary ammonium derivatives of the tertiary amines, which possess a permanent positive charge, are as active as the parent amines (16,18). Moreover, in the case of the methyltransferase of the COI and of the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, electrostatically neutral isosteric analogues of the protonated tertiary amines or of their quaternary ammonium derivatives were practically devoid of activity (16,21). This work suggests therefore that positively charged intermediates, involved in the biosynthesis of a sterol, can be mimicked by ammonium (sulfonium, arsonium) group-containing molecules, resulting in a strong (e.g., charge-charge) interaction between the ammonium function and the active site of the enzyme. Such a conclusion is strengthened by a recent report showing that the farnesyl-pyrophosphate-squalene synthetase can be inhibited by a nitrogen-containing analogue of a carbocationic HEI involved in the reaction pathway (62). As shown in Figure 2, carbocationic species were frequently involved in the enzymology of sterol biosynthesis; the results here have important implications both for understanding the molecular mechanisms involved during the action of enzymes on sterol biosynthesis and for the search for new inhibitors.

For ESA(C)C, AdoMet CMT and COI, the N-oxide derivative of tertiary amines was shown to be at least as active as the parent amine. In the case of the two first enzymes, the N-oxide derivative (**10**) and (**23**) were shown

to be three and two times as active, respectively, as the parent amines. Such a result was unexpected at first because N-oxides can be considered globally neutral. However, these molecules have a strong permanent dipolar moment (4-5 dipolar moment units) (28). This is supported by theoretical calculations (Wipf, G., unpublished results) which indicate that amine N-oxides have some similarities with the ammonium cations (charge distribution, binding affinity for H_2O, etc.). The potency of **10, 23** and **53** also could result from the fact that in N-oxides at least two coordination modes are possible; it is well-established that tertiary amine N-oxides are known ligands of both cations and anions (63). Therefore one can suggest that positively charged intermediates involved in sterol biosynthesis also can be mimicked by tertiary amine N-oxide group-containing molecules, resulting in a strong (charge-charge or dipole-dipole) interaction between the N-oxide function and the active site of the enzyme. In addition, the polarized character of the $-N^+-O^-$ bond is well-suited to mimic TS (such as **4a** and **6a** of Figures 3 and 5, respectively) involved in the first step (general acid-catalyzed oxiran or cyclopropane ring opening) of the enzymatic reaction catalyzed by the cyclase and the COI, respectively. The assumption that N-oxide function could mimic TS or HEI involved in sterol biosynthesis is a new concept which may be of more general value. It has to be pointed out that pyridine N-oxide already has been designed as a TS analogue inhibitor of the protocatechuate dioxygenase (63).

When SBI was given to suspension cultures of bramble cells or to maize seedlings, the substrate of the inhibited enzyme was shown to accumulate strongly, resulting in profound qualitative and quantitative changes in the sterol profile of the treated cells and confirming the results obtained in vitro with the isolated enzymes: **9** inhibited the ESA(C)C leading to an accumulation of **4; 15** inhibited the AdoMet CMT and the AdoMet LMT resulting in a strong accumulation of **3** and **44,** and **48** and **51** interfered with both the COI and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase resulting in a spectacular accumulation of cyclopropyl- (mostly **55**) and Δ^8-sterols. The effects on cell growth were quite different depending on the inhibitor. In the case of a treatment of the cells with **15,** complete growth inhibition occurred at a concentration of **15** higher than 1 to 2 μM (23). Also, severe effects on growth were observed in the case of cells treated with **9,** and no growth occurred when **9** was present at concentrations in the order of 10 μM in the culture medium. By contrast, **51** led to little effect on growth of bramble cells at concentrations lower than 30 μM. However, complete growth inhibition could be obtained at ca. 150 μM. The link between the phytotoxicity and the nature of the enzymatic target is far from being evident except perhaps in the case of cultures treated with **9,** where the phytotoxicity could be explained by the inability of **4** to fulfill the role devoted to sterols in membranes. In the case of cells treated with **51,** the almost total replacement of Δ^5-sterols by 9β,19-cyclopropyl sterols seems to be well-tolerated, whereas the accumulation of **3, 19** and **44** in cells treated with **15** is accompanied by a strong growth inhibition. The difficulty of linking biological effects with the aimed enzymatic target in the present work possibly reflects the existence of other, still unknown, cellular targets. Some recent results dealing with the action of SBI in fungi support the idea that inhibition of ergosterol biosynthesis is not the lone mode of toxicity of these inhibitors (64). These considerations suggest that future research in the field of SBI include direct and indirect cellular effects of the modification of the sterol pattern. As discussed above, the membranes (essentially the plasma membrane) particularly are concerned and different types of effects of SBI would be expected at this level: effects of changes in the sterol profile on polar lipid composition, membrane fluidity, and the activity of intrinsic enzymes such as the PM (KCl dependent) ATPase (65) or the cellulose synthetase systems.

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Biosynthesis of 24-Methylcholest-5-en-3β-ol and 24-Ethylcholest-5-en-3β-ol in *Zea mays*

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The relative rates of synthesis of 24-methylcholest-5-en-3β-ol and 24-ethylcholest-5-en-3β-ol in *Zea mays* shoots were determined using [2-^{14}C]mevalonic acid and [methyl-^{14}C]methionine as substrates. The 24-ethylsterol had a higher specific activity and it apparently was synthesized at about 3–4 times the rate of the 24-methylsterol. ^{1}H NMR spectroscopy showed that the 24-ethylsterol was predominantly the 24α-epimer but the 24-methylsterol was a mixture of the 24α-epimer (30–40%) and the 24β-epimer (60–70%). The results are discussed in relation to the involvement of $\Delta^{24(28)}$-, Δ^{23}, Δ^{25}- and $\Delta^{24(25)}$-sterol intermediates in 24-methyl- and 24-ethylsterol production.
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Much is now understood about the overall biosynthetic routes to sterols in a range of algae and higher plants (1,2). Also, detailed information is accumulating on aspects such as the formation and subsequent metabolism of cycloartenol (3,4) and the mechanisms of C-24 and C-28 alkylation (5-9) required for the elaboration of the 24-methyl- and 24-ethylsterols which are typical of plants. However, some details, such as the precise roles of the Δ^{23}-sterol, cyclosadol (10), and the Δ^{25}-sterol, cyclolaudenol (11-13), in 24β-methylsterol synthesis and of 24-alkyl-$\Delta^{25(25)}$-sterols in the production of 24α-methyl- and 24α-ethylsterols in vascular plants, remain to be clarified (5,10-17).

Attention now is turning to the problems of regulation of plant sterol synthesis (18) and studies have been reported on, for example, plant HMG-CoA reductase (19,21), which has been recognized for some years as a key regulatory enzyme in sterol biosynthesis in animal tissues (22). In the past other steps have been suggested as rate limiting in plants, and some studies have been conducted to investigate the effects of environmental conditions and various plant hormones on phytosterol synthesis (review ref. 18).

In a previous publication (13) on the biosynthesis of *Zea mays* sterols we indicated that the 24-ethylcholest-5-en-3β-ol (sitosterol) was apparently synthesized at a faster rate than 24-methylcholest-5-en-3β-ol (campesterol). The biosynthesis of these and other sterols in *Sorghum bicolor* also has been the subject of a study by Heupel and Nes (23). We now report our further investigations on the determination of the relative rates of biosynthesis of 24-methyl- and 24-ethylcholest-5-en-3β-ol in *Zea mays* and discuss the results in relation to the possible involvement of Δ^{23}-, Δ^{25}-, $\Delta^{24(25)}$- and $\Delta^{24(28)}$-sterol intermediates.

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MATERIALS AND METHODS

DL-[2-^{14}C]Mevalonic acid lactone (50 mCi/mmol) and L-[methyl^{14}C]methionine (50 mCi/mmol) were purchased from Amersham International (Amersham, United Kingdom). Gas liquid chromatography (GLC) was on a 0.25 mm × 25 m BP-1 capillary column (SGE), argon carrier gas [2 ml/min, initial temp 240 C then 4 C/min to 280 C. Gas chromatography/mass spectroscopy (GC-MS) was on a VG-70-70F instrument. ^{1}H NMR (400 MHz) spectra were run in $CDCl_3$ with TMS as the internal standard. Radioactivity was assayed by liquid scintillation counting using 0.7% butyl-PBD in toluene as the counting cocktail. Sterols were acetylated in the standard manner using acetic anhydride/pyridine (1:1, v/v).

Zea mays (cv Caldera 535) seeds were washed and soaked in water for 24 hr prior to germination in trays of moist cotton wool overlaid with paper. The seedlings were grown in the dark for up to 12 days when the coleoptiles reached 6-10 cm. Shoots were excised from the seeds, and typically 20 shoots placed in a vial containing [2-^{14}C]MVA or [methyl-^{14}C]methionine and water (4 ml). Usually 60-100 shoots were incubated per experiment. The shoots were placed in a gentle draft to enhance transpiration and uptake of the solution and harvested 8-48 hr later. The shoots were homogenized in acetone and filtered and the plant residue was re-extracted three times with acetone. The combined acetone extracts were reduced in volume by rotary evaporation to leave an aqueous residue containing the total lipids. This was saponified by reflux (1-2 hr) with 100 ml of 8% KOH in 85% aqueous EtOH. The nonsaponifiable lipid was extracted into petroleum ether in the usual manner. Thin layer chromatography (TLC) (silica gel; $CHCl_3$/EtOH, 98:2, v/v) and radioscanning showed that the major radioactive components of the nonsaponifiable lipids were 4-demethylsterols and 4,4-dimethylsterols but with low incorporation also into squalene. The labeled 4-demethylsterols (containing 24-methylcholest-5-en-3β-ol, 24-ethylcholest-5-en-3β-ol and 24-ethylcholesta-5,22-dien-3β-ol) were obtained by preparative TLC (silica gel; $CHCl_3$-EtOH, 98:2, v/v), acetylated and separated by further preparative TLC on 10% $AgNO_3$-silica gel (EtOH free $CHCl_3/Et_2O$, 98:2, v/v). This gave two bands of material; the band at Rf 0.52 contained a mixture of 24-methylcholest-5-en-3β-yl acetate and 24-ethylcholest-5-en-3β-yl acetate and the band at Rf 0.42 contained only 24-ethylcholesta-5,22-dien-3β-yl acetate. The 24-methylcholest-5-en-3β-yl acetate and 24-ethylcholest-5-en-3β-yl acetate were separated by high performance liquid chromatography (HPLC) on a 250 mm × 4.6 mm column of Ultrasphere 5ODS eluted with MeOH/H_2O (97:3, v/v) at 1.5 ml/min. The eluant was monitored with a UV detector at 206 nm and the eluant fractions containing the 24-methylcholest-5-en-3β-yl acetate (RT 62 min)

TABLE 1

Incorporation of [2-^{14}C] Mevalonic Acid or [Methyl-^{14}14C] Methionine into the Nonsaponifiable Lipids of *Zea mays* Shoots and the Specific Activities of the Isolated Steryl Acetates

	Nonsaponifiable lipid (dpm)	Sp act (dpm/μmole)			Ratio of sp act
		C_{28}[a]	C_{29}	$C_{29}\Delta^{5,22}$	C_{28}:C_{29}
Experiment 1[b]	9.55×10^5	49060	86640	--	1:1.77
Experiment 2[c]	3.11×10^4	760	1180	31	1:1.55
Experiment 3[d]	2.28×10^5	9000	15940	1610	1:1.77
Experiment 5[e]	8.07×10^5	4180	11670	2260	1:1.39

[a] C_{28} is 24-methylcholest-5-en-3β-yl acetate; C_{29} is 24-ethylcholest-5-en-3β-yl acetate; $C_{29}\Delta^{5,22}$ is 24-ethylcholesta-5,22-dien-3β-yl acetate.

[b] Sixty shoots (7-day-old) incubated for 30 hr with 6.0 μCi [2-^{14}C]MVA.

[c] Eighty shoots (5-day-old) incubated for 24 hr with 8.4 μCi [2-^{14}C]MVA.

[d] Eighty shoots (7-day-old) incubated for 24 hr with 5.0 μCi [2-^{14}C]MVA.

[e] Three hundred fifty shoots (8-day-old) incubated for 24 hr with 155 μCi [methyl-^{14}C]methionine.

and 24-ethylcholest-5-en-3β-yl acetate (RT 70 min) were collected. The quantity of each steryl acetate recovered was estimated from the UV detector absorption values using standard curves prepared by injections of a range of known amounts of the two steryl acetates.

To characterize the sterols, a large-scale isolation of the 24-methylcholest-5-en-3β-yl acetate and 24-ethylcholest-5-en-3β-yl acetate was performed starting with 271 g (fresh weight) of coleoptiles from 7-day-old *Zea mays* seedlings. The 4-demethylsterols (60 mg) were isolated from the nonsaponifiable lipids (450 mg) by column chromatography on alumina, Brockman grade 3, eluting with petroleum ether/Et_2O mixtures. After acetylation the 4-demethylsteryl acetates (59 mg) were fractionated on a column of 15% $AgNO_3$-alumina eluted with hexane-Et_2O mixtures. GLC monitoring identified a fraction which contained a mixture of only 24-methylcholest-5-en-3β-yl acetate (27%) and 24-ethylcholest-5-en-3β-yl acetate. (73%). These compounds then were separated by HPLC as described above to give essentially pure GLC samples which were analyzed by GC-MS and ^{1}H NMR spectroscopy. 24-Methylcholest-5-en-3β-yl acetate, MS m/z (rel. int.): 382 (100), 367 (24), 340 (4), 274 (19), 262 (18), 255 (20), 228 (5), 213 (17). ^{1}H NMR (400 MHz, $CDCl_3$) 24α/24β: δ 0.677/0.688 (s, H-18), 1.020/1.020 (s, H-19), 0.772/0.776 (d, J, = 6.8 Hz), 0.804/0.784 (d, J = 6.8 Hz, H-27), 0.851/0.857 (d, J = 6.8 Hz, H-26), 0.912/0.919 (d, J = 6.5 Hz, H-21). 24-Ethylcholest-5-en-3β-yl acetate, MS m/z (rel. int.): 396 (100), 381 (25), 354 (4), 288 (19), 275 (19), 255 (24), 228 (5), 213 (20). ^{1}H NMR (400 MHz, $CDCl_3$):0.678 (s, H-18), 1.020 (s, H-19), 0.817 (d, J = 6.5 Hz, H-27), 0.834 (d, J = Hz, H-26), 0.845 (t, J = 7.2 Hz, H-29).

RESULTS AND DISCUSSION

Zea mays shoots readily incorporated [2-^{14}C]mevalonic acid into the nonsaponifiable lipid fraction from which the labeled 4-demethylsterols were isolated by preparative TLC on silica gel. GLC analysis of the 4-demethylsterols from the various batches of shoots used showed them to consist of a mixture in which the main components were cholesterol (traces-1.5%), 24-methylcholest-5-en-3β-ol (18-24%), 24-ethylcholest-5-en-3β-ol (41-48%) and 24-ethylcholesta-5,22-dien-3β-ol (30-39%). This is in agreement with previous analyses of *Zea mays* shoots (10,13,24). Acetylation of the 4-demethylsterols followed by preparative TLC on $AgNO_3$-silica gel gave a less polar fraction containing the acetates of 24-methylcholest-5-en-3β-ol (30%), 24-ethylcholest-5-en-3β-ol (70%) and cholesterol (traces). A more polar band containing only the acetate of 24-ethylcholesta-5,22-dien-3β-ol also was eluted from the plate. The specific activities (dpm/μmol) of these compounds were found to vary widely from one experiment to another. The specific activity values obtained for three experiments with [2-^{14}C]mevalonic acid and one experiment with [methyl-^{14}C]methionine are given in Table 1. The wide ranging values for the incorporation of labeled precursor into the nonsaponifiable lipid and specific activities of the isolated sterols are a result of the different experimental conditions employed and they also presumably have been affected by the physiological status of the shoots used. A major factor in determining the extent of incorporation was the efficiency of uptake of the solution of labeled substrate by the shoots. In some cases all the solution was taken up but in others a significant amount of substrate solution remained unabsorbed at the termination of the experiment. However, in each incubation the 24-ethylcholest-5-en-3β-ol had a higher specific activity than the 24-methylcholest-5-en-3β-ol. Moreover, the ratios of the specific activities of these two sterols biosynthesized from [2-^{14}C]mevalonate in the different experiments were in the same range (Table 1). A similar ratio for the specific activities of these sterols was obtained when [methyl-^{14}C]methionine was used as the precursor. In this case a correction was applied to account for the fact that two labeled carbons are introduced into the C_{29}-sterol during elaboration of the 24-ethyl group compared to one labeled carbon incorporated as the 24-methyl group of the C_{28}-sterol. These results agree with our previous observations (13); one interpretation is that the C_{29}-sterol is biosynthesized at a significantly faster rate than the C_{28}-sterol. In a

TABLE 2

Relative Rates of Synthesis of 24-Methylcholest-5-en-3β-ol and 24-Ethylcholest-5-en-3β-ol by *Zea mays* Shoots

	Relative rate of synthesis (pmol/hr/100 shoots)		Ratio of relative rates of synthesis, C_{28}:C_{29}
	C_{28}[a]	C_{29}	
Experiment 1[b]	1.07	4.20	1:3.94
Experiment 2	0.026	0.122	1:4.67
Experiment 3	0.20	0.81	1:4.05
Experiment 4	0.91	2.95	1:3.24
Experiment 5	0.36	1.18	1:3:27

[a] C_{28} is 24-methylcholest-5-en-3β-ol; C_{29} is 24-ethylcholest-5-en-3β-ol.

[b] For experiments 1-4 the substrate was [2-^{14}C]MVA; for experiment 5 the substrate was [methyl-^{14}C]methionine. In experiments 1-3 and 5 the conditions were as described in Table 1. In experiment 4, [2-^{14}C]MVA (10 μCi) was incubated for 24 hr with 100 shoots (12-day-old).

study on *Sorghum bicolor* (23), when leaves were exposed to [2-^{14}-C]acetate the C_{29}-sterol had a specific activity only a little higher than that of the C_{28}-sterol but somewhat less than that of the cholesterol also isolated from the leaves.

The conclusion that the 24-ethylsterol probably is biosynthesized at a faster rate than the 24-methylsterol by *Zea mays* shoots was strengthened when the relative rates of synthesis of these two sterols were calculated (Table 2). Although the relative rates of 24-methyl- and 24-ethylsterol synthesis varied widely between the different experiments, the 24-ethylsterol apparently was always synthesized at a rate from 3.3 to 4.7 times faster than that of the 24-methylsterol.

Relative rather than absolute rates of synthesis were calculated in these experiments for the following reasons. Much effort has been devoted to developing methods for the measurement of absolute rates of cholesterol synthesis in animal tissues (25). The prime requirement is a knowledge of the specific activity of the labeled precursor after it has entered the cell. The presence of large pools of endogenous substrate or intermediates will cause dilution of the added labeled precursor with the lowering of its specific activity and a consequent underestimation of the absolute rate of synthesis of sterol if this dilution is not taken into account. This situation can be complicated further by the existence of two or more pools of the endogenous material in different cellular compartments, which are not in free equilibrium and are not used equally for sterol synthesis. Also, to measure absolute rates of synthesis it is important that the labeled substrate enters the pathway prior to the regulatory step(s) and that uptake of the precursor into the cell and its passage to the site of synthesis are not rate limiting factors. Additionally, for the measurement of the absolute rate of synthesis the recovery of labeled product must be corrected for losses occurring during the isolation and purification procedure. This usually can be achieved by addition of an internal standard. Given these and other factors, the most satisfactory precursor for measurements of sterol synthesis in animal tissue is tritiated water (25), which permits introduction of tritium into the sterol at the several reduction steps in the biosynthetic pathway. As most studies on phytosterol production have been concerned with identification of intermediates and elucidation of reaction mechanisms, these points have yet to receive detailed consideration in plant studies. We have conducted some preliminary investigations on this problem using *Acer pseudoplatinus* tissue cultures (8, 26 and Ryder, N.S. and Goad, L.J., unpublished results) and found that labeled mevalonic acid gives somewhat higher rates of sterol synthesis than labeled acetate, methionine or tritiated water. These results may indicate that in this plant tissue culture HMG-CoA reductase is a rate limiting enzyme as suggested in other plants (19-21). Similar studies designed to optimize methods for determining absolute rates of sterol synthesis in whole plant tissues have not been reported.

In the case of *Zea mays* shoots we do not know if the labeled substrate is being diluted significantly by endogenous pools in the plant cell or if HMG-CoA reductase is the principal regulatory enzyme. Moreover, the unavailability of suitable tritium-labeled sterols for use as internal standards precluded correction for losses of biosynthesized [^{14}C]-labeled 24-methyl- and 24-ethylcholest-5-en-3β-ol during their purification. However, the 24-methyl- and 24-ethylcholest-5-en-3β-ol would behave identically through the isolation procedure up to the stage of HPLC separation of their acetates. The collection of these compounds in the appropriate HPLC eluant fractions should be achieved with reasonably equal recoveries of each compound. Thus, although losses of both compounds would occur in the work up procedure, the percentage recovery of the two purified steryl acetates should be very similar. Also, the production of these two sterols would follow a common route from precursor to cycloartenol and therefore be subjected to the same constraints of precursor uptake and dilution by endogenous pools and regulation of early enzymic steps. It is only at the C-24 alkylation step that their biosynthetic pathways would diverge and be subject to independent regulatory processes. Therefore a calculation of their relative rates of synthesis based upon the radioactivity associated with the two compounds and the specific activity of the [^{14}C]-labeled precursor administered to the plant shoot will give results useful for comparison and will permit some conclusions regarding the differential regulation of synthesis of these two sterols. The relative rates measured, however, will underestimate the true absolute rates of synthesis.

Mevalonic acid may enter the pathway after the regulatory step, and it is possible that addition of excess of this compound may perturb the normal flow of precursors to the C_{28}- and C_{29}-sterols. As only tracer amounts (12-27 μg/60-100 shoots) of [2-^{14}C]MVA were used in these particular experiments this may not have presented a problem. This was examined by measuring the relative rates of C_{28}- and C_{29}-sterol synthesis using [methyl-^{14}C]methionine as the precursor. The incorporation of this precursor depends upon its conversion to S-adenosylmethionine (SAM) which then is used in the C-24 transmethylation reactions to yield the 24-methyl- and 24-ethylsterols. Its rate of entry into the pathway is determined by the flux of precursors arising from acetyl-CoA and providing the substrates for the C-24 and C-28 alkylation reactions at a rate which corresponds to the true physiological rate of sterol synthesis. The added [methyl-^{14}C]methionine again

TABLE 3

Specific Activities and Relative Rates of Synthesis of 24-Methylcholest-5-en-3β-ol and 24-Ethylcholest-5-en-3β-ol Synthesized from [2-^{14}C]Mevalonic Acid by *Zea mays* Shoots during 8, 24 and 48 Hr Incubations

Time (hr)	Sp act (dpm/μmol)			Ratio sp act	Relative rate of synthesis (pmol/hr/100 shoots)		Ratio of relative rates of synthesis
	C_{28}	C_{29}	$C_{29}\Delta^{5,22}$	C_{28}:C_{29}	C_{28}	C_{29}	C_{28}:C_{29}
8[a]	740	1150	4	1:1.55	0.069	0.275	1:3.99
24	9000	15940	1615	1:1.77	0.200	0.812	1:4.06
48	20360	28970	3217	1:1.34	0.225	0.725	1:3.22

[a]For each incubation 80 shoots (7-day-old) were incubated with 5.0 μCi [2-^{14}C]MVA.

is subject to dilution by endogenous pools so that only relative rather than absolute rates of synthesis can be determined.

Using [methyl-^{14}C]methionine gave values for the ratios of C_{28}:C_{29} sterol specific activities (Table 1) and relative rates of synthesis (Table 2) comparable to those obtained with [2-^{14}C]mevalonic acid. It therefore can be concluded that the C_{29}-sterol is synthesized at a faster rate than the C_{28}-sterol and that the results obtained with [2-^{14}C]mevalonic acid are not a consequence of disturbing the equilibrium of normal physiological sterol metabolism.

The experiments summarized in Tables 1 and 2 all employed incubation periods of 24-30 hr. To investigate whether the relative rates of sterol synthesis changed markedly with time after excision of the shoots, three incubations were conducted for eight, 24 and 48 hr (Table 3). The results obtained for the ratios of C_{28}:C_{29} specific activities and relative rates of synthesis were essentially the same as in other experiments. The 48-hr incubation did produce somewhat lower ratios than the eight- and 24 hr incubations but whether this is significant will require further experimentation. However, it may be relevant that in the experiments with *Sorghum bicolor* that produced 24-methyl- and 24-ethylcholest-5-en-3β-ol with rather close specific activities (ratio 1:1.07) the leaves were exposed to [2-^{14}C]acetate and incubated for 7 days (23).

Included in Tables 1 and 3 are specific activities for the 24-ethylcholesta-5,22-dien-3β-ol ($C_{29}\Delta^{5,22}$, stigmasterol) isolated from the *Z. mays* shoots. In all cases the specific activity of this sterol was much lower than the 24-ethylcholest-5-en-3β-ol (C_{29}, sitosterol) isolated in the same experiment. A similar observation was made in the experiments with *Sorghum bicolor* (23). This could be interpreted to mean that stigmasterol is elaborated slowly compared to the other sterols, which would be the conclusion if the rate of synthesis were calculated on the basis of total radioactivity incorporated and the specific activity of the precursor [2-^{14}C]mevalonic acid. For example, from the data for the experiment presented in Table 3, the relative rates of synthesis of stigmasterol calculated for the eight-, 24- and 48-hr incubations would be 0.008, 0.073 and 0.100 pmol/hr/80 shoots, respectively. However, there is evidence that stigmasterol is produced from sitosterol (23,27). Accordingly, if the relative rate of stigmasterol biosynthesis is calculated using the specific activity of the 24-ethylcholest-5-en-3β-ol isolated from the same experiment the values for the eight-, 24- and 48-hr incorporations are 3.7, 2.5 and 1.9 nmol/hr/80 shoots, respectively. These values are an order of magnitude greater than the corresponding relative rates of 24-ethylcholest-5-en-3β-ol synthesis and must be an overestimation. The results also highlight the difficulty of determining absolute rates of plant sterol synthesis. In this case a possible interpretation would be that there are two pools of 24-ethylcholest-5-en-3β-ol in the *Z. mays* cell. One could be a large pool with a low turnover rate, possibly fulfilling a structural role in membranes. The other pool could be smaller and consist of newly synthesized material which is metabolically more active and used as the precursor pool for synthesis of stigmasterol or other derivatives. In short-term incubations with [2-^{14}C]mevalonic acid, the smaller pool would become more rapidly labeled and have a higher specific activity than the larger structural pool which only would become appreciably labeled in long-term incubations as the two pools equilibrate. During extraction of the sterols from the leaves the two pools would be mixed and the resulting specific activity of the total sterol would be lower than that of the smaller metabolically active pool. Consequently, use of this "average" low specific activity would result in an overestimation of the amount of stigmasterol synthesized, as appears to be the case above.

Previous investigations on *Z. mays* sterols have shown that while the 24-ethylcholest-5-en-3β-ol has the 24α-configuration (24R-), the 24-methylcholest-5-en-3β-ol is a mixture of the 24α- and 24β-epimers (10,11). As the rates of production of the 24-epimeric sterols may be different, knowledge of the proportions of these compounds in the *Z. mays* shoots used for these experiments might help in understanding the processes underlying the differential rates of 24-methyl and 24-ethylsterol synthesis. The proportions of the 24α- and 24β-epimeric sterols can be deduced from the ^{1}H NMR spectra of the sterol mixtures (28,29). The ^{1}H NMR spectrum of the 24-ethylcholest-5-en-3β-yl acetate isolated from *Z. mays* shoots (Fig. 1) showed that it was predominantly the 24α-epimer (sitosterol) with the 24β-epimer (clionasterol) comprising no more than a few percent of the mixture. In contrast, the ^{1}H NMR spectrum of the 24-methylcholest-5-en-3β-ol (Fig. 2) revealed that it was a mixture of the 24α- and 24β-methyl epimers. The doublets for H-21, H-26 and H-27 show different chemical shifts for the 24α- and 24β-epimers.

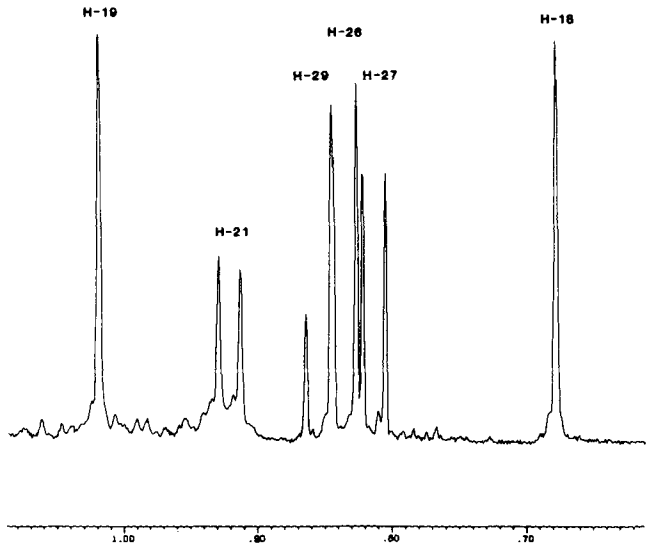
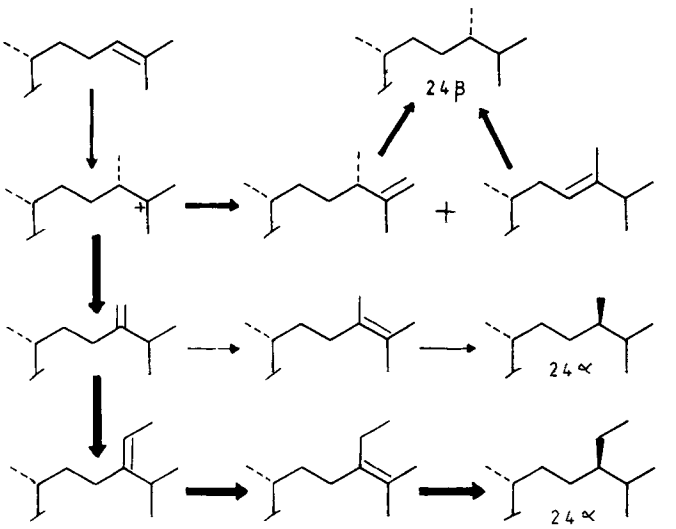


FIG. 1. ^{1}H NMR spectrum (400 MHz) of the 24-ethylcholest-5-en-3β-yl acetate isolated from *Zea mays* shoots.



SCHEME 1. Biosynthetic routes leading to the major 24-methyl and 24-ethylsterols in *Zea mays* shoots.

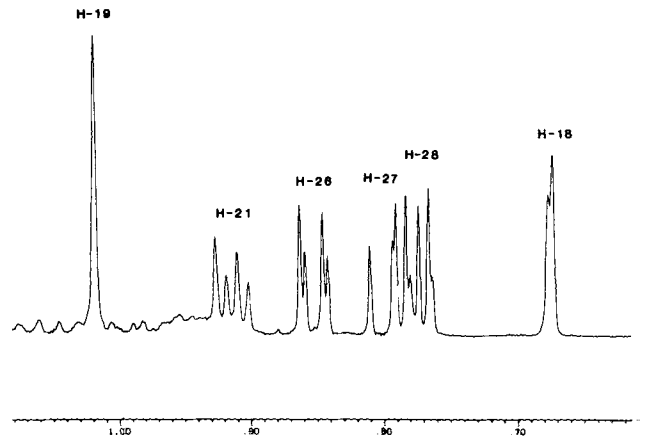


FIG. 2. ^{1}H NMR spectrum of the 24-methylcholest-5-en-3β-yl acetate isolated from *Zea mays* shoots.

From the intensities of the H-21 and H-26 doublets it was estimated that the mixture comprised 30-40% of the 24α-methyl epimer and 60-70% of the 24β-epimer. These are similar to the proportions reported previously (11).

An explanation of the differential rates of 24-methyl- and 24-ethylsterol synthesis may be found by considering the differing routes to the 24α- and 24β-epimers (Scheme 1). It has been suggested that at the first transmethylation step from SAM a cation is produced which can stabilize by expulsion of a proton in three ways. Loss of a proton from C-23 or C-26 will give either cyclosadol (Δ^{23}) or cyclolaudenol (Δ^{25}), respectively (10-13). The 24β-methylcholest-5-en-3β-ol is postulated to arise from these compounds by side chain double bond reduction and appropriate modifications of the ring system (10-14). Loss of a proton from C-28 will produce 24-methylenecycloartanol which then is suggested to act as a precursor to the 24 α-methylsterol, possibly via an isomerization to an intermediary $\Delta^{25(25)}$-sterol (11-15). Alternatively, the 24-methylenecycloartanol is converted by ring modification to a further intermediate, probably 24-methylenelophenol, which then is a substrate for the second SAM transmethylation to produce a 24-ethylidene sterol. This is ultimately converted into 24α-ethylcholest-5-en-3β-ol, probably again via a $\Delta^{24(25)}$-sterol intermediate (5,11-17).

On the basis of these biosynthetic relationships, the amount of 24β-methylsterol generated can be determined by the proportions of cyclosadol or cyclolaudenol produced at the first transmethylation step. In incubations of *Z. mays* shoots with radioactive precursors, cylosadol, cyclolaudenol and 24-methylenecycloartanol were identified as labeled products (11,13). Using a microsomal preparation from *Z. mays* coleoptiles, we found that the products of cycloartenol methylation were cyclosadol, which constituted about 3-4% of the products, cyclolaudenol (ca. 1%) and 24-methylenecycloartanol (88%) (12). However, Scheid et al. (10) found with a similar cell-free preparation from *Z. mays* that 24-methylenecycloartanol again was the major product but cyclosadol accounted for 25% of the products and cyclolaudenol was not detected. These differences may be due either to different varieties of *Z. mays* or to differences in preparation methods of microsomes and incubation conditions. It is not known if only one enzyme is involved in the elaboration of the three C-24 methylated products or if different enzymes are required.

In some algae, such as *Trebouxia* species, the 24-methylenesterol produced at the first transmethylation step is utilized solely for the second transmethylation reaction required for 24-ethylsterol production (5); the 24-methylenesterol is not reduced to yield a 24-methylsterol (5). In higher plants, dual labeling studies suggest that 24-ethylidenesterols are isomerized to a $\Delta^{24(25)}$-sterol prior to reduction to the 24 α-ethylsterol (11,15,16). It can be postulated that the $\Delta^{24(28)}$-sterol $\rightarrow$ $\Delta^{24(25)}$-sterol isomerase may not be specific to a 24-ethylidene sterol but also will promote a similar isomerization of a 24-methylenesterol to a 24-methyl-$\Delta^{24(25)}$-sterol. Consequently this would di-

vert a portion of the flow of precursors away from C_{29}-sterol production, and subsequent reduction of the 24-methyl-$\Delta^{24(25)}$-sterol could give the 24α-methylsterol epimer (Scheme 1). Thus competition between the C-28 transmethylation enzyme and the $\Delta^{24(28)} \rightarrow \Delta^{24(25)}$-sterol isomerase for the 24-methylenesterol intermediate may be a factor in determining the rates of synthesis of the 24α-methylsterol and the 24-ethylsterols. The balance of the rates of these various reactions thus will lead to the proportions of C_{28} and C_{29} sterols characteristic of higher plants. These considerations highlight the need for further studies (a) to determine if one or more transmethylase enzymes is involved at the cycloartenol alkylation step; (b) to establish unequivocally that 24-alkyl-$\Delta^{24(25)}$-sterols are obligatory intermediates in 24α-methyl- and 24α-ethylsterol synthesis in higher plants, and (c) to examine the possibility that a 24-methylene sterol also can be reduced directly in higher plants to yield either a 24 α-methyl or 24 β-methyl sterol.

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Sterol Composition and Biosynthesis in Sorghum: Importance to Developmental Regulation

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Sterol composition and biosynthesis have been examined in seeds, germinating seeds and blades from fully matured leaves of *Sorghum bicolor* in various stages of development—from seedlings (seven-day plants) to flowering (66-day) plants. The profile of the dominant free sterols of seeds was similar to that of leaf blades; both contained cholesterol, 24α-methylcholesterol (campesterol), 24β-methylcholesterol (dihydrobrassicasterol), 24α-ethylcholesterol (sitosterol) and 24α-ethylcholesta-5,22-dienol (stigmasterol). Sufficient sterol intermediates were identified in the plant to indicate separate post-cycloartenol pathways to sterolic end products. The total free sterol content of the seed (μg/seed) increased somewhat during the 20 hr germination period. However, as the plant developed (seven to 48 days), there was a logarithmic increase in the leaf blade sterol content (μg/leaf blade) which plateaued at the onset of floral differentiation (ca. day 41). Over the next 18 days (48 to 66 days—period of inflorescense development), the sterol content rapidly decreased. In the early stages of plant development, the leaf blade pentacyclic triterpenoid (PT) content was negligible. With the onset of floral differentiation, PT content increased logarithmically, reaching a plateau level that surpassed the sterol content as flowering progressed. These results imply that a critical mass of sterol is associated with sorghum for floral induction. Sterol loss from the leaves of the flowering plants presumably was compensated for by the diversion of 2,3-oxidosqualene (SO) from sterol synthesis to PT production. Additional feeding and trapping experiments with [2-^{14}C]mevalonic acid, [2-^{3}H]cycloartenol, [24-^{3}H]lanosterol [4-^{14}C]sitosterol and [4-^{14}C]cholesterol fed to germinating seeds and leaves from flowering plants demonstrated that sorghum possessed a cycloartenol-based pathway; germinating seeds synthesized 24-alkylsterols but not cholesterol, although cholesterol was identified in both dry and germinating seeds by gas chromatography-mass spectroscopy (GC-MS); and mature leaves synthesized cholesterol and 24α-alkylsterols but not 24β-methylcholesterol.

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In our studies on the occurrence and biosynthesis of sterols and PT in C-3 (1) and C-4 (2-5) plants (e.g., wheat and sorghum, respectively), we have assumed that the genes involved in 24-desalkyl- and 24-(α- and β-) alkylsterol and PT (amyroid and migrated hopanoid series) production are distributed generally throughout the Graminae. This uniformity in the gene pool presumably has led to similarity in the structure of functional end products, notably the sterols, at the familial level. Because gene expression may be temporally and spacially affected, the kinds and amounts of these polycyclic compounds (sterols and PT) may change as they are biosynthesized during the plant life cycle. To shed additional light on developmentally induced changes in sterol composition and biosynthesis, we studied sorghum as a model gramineous crop from seed to flowering plant. There is some leading information obtained through studies with grasses and legumes that the biosynthesis of PT also may fluctuate during germination and seedling development (6-9) and in grain maturation (10). Since the potential of removing SO for sterol production and committing it for PT synthesis may, in sorghum, be under developmental control, we also have monitored PT levels of the mature leaves at various stages of development. The results reported here show that regulation of the sterol and PT biosynthetic pathways exists during development. The impact of this regulation on sorghum physiology is discussed.

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MATERIALS AND METHODS

Seeds of *Sorghum bicolor* (L.) Moench, cv. G 499 GBR were obtained from Funk Seeds International (Bloomington, Illinois). Plants were grown in the greenhouse as described previously (3-5) except in the developmental study, for which greater detail is given in the Results section. In general, following acetone extraction of the fresh material, free sterols were separated from free PT and glycosylated and esterified sterols by adsorption thin layer chromatography (TLC). Free sterol and PT were quantified by gas liquid chromatography (GLC) using three packed columns with cholesterol as the internal standard as described (3,4). Glycosylated and esterified material was hydrolyzed and liberated compounds were quantified by GLC (5). The sterol and PT content presented on a dry weight basis was an estimation derived from an equal amount of compound in oven-dried (48 hr, 80 C) material of similar maturation and size. Spectral methodology (EI-MS and ^{1}H-NMR) was as described (2-5).

Radioactive compounds were purchased from New England Nuclear (Boston, Massachusetts) and Amersham (Arlington Heights, Illinois) or synthesized in this laboratory according to established methods for the introduction of ^{3}H into steroids (11,12). Specific activities for the labeled compounds used in this study were as follows: [2-^{14}C]mevalonic acid (MVA), 51.5 μCi/μmol; [2-^{3}H]cycloartenol, 0.9 μCi/μmol; [24-^{3}H]lanosterol, 1,077.0 μCi/μmol; [4-^{14}C]sitosterol, 58.0 μCi/μmol; [4-^{14}C]cholesterol, 58.4 μCi/μmol. The labeled steroids were purified by high performance liquid chromatography (HPLC) prior to use. [2-^{14}C]MVA was supplied

to germinating seeds as follows: 50 seeds were placed in a petri dish with ca. 2.4 ml of an aqueous solution containing the labeled compound and 10 μl of Tween 80. The seeds then were imbibed for 20 hr under continuous light at 28 C. Each of the labeled steroids was applied uniformly to an 8 cm section, beginning 4 cm from the tip of the blades of the second leaf from the flag leaf of plants with inflorescences just emerging. The compounds were applied as a solution of 0.1% silicon oil and 0.1% D,L-α-tocopherol in 95% aq. EtOH (3). The plants were allowed to incubate for 72 hr with the labeled substrates.

RESULTS AND DISCUSSION

Structure determinations. Many of the sterols (Tables 1 and 2) endogenous to the dry seed of G 499 GBR previously were characterized by Palmer and Bowden (10) in their study of sorghum grain cv. Pers DC 36. Our ^{1}H-NMR data were consistent with the literature (13 and refs. cited therein). The chemical shifts confirm most stereochemical implications of the structures proposed by the earlier investigators except for the stereochemical purity of campesterol. 24β-Methylcholesterol, which cochromatographed with campesterol,

TABLE 1

Chromatographic and Spectral Characteristics of Sterols Isolated from Sorghum Seeds[a,b]

Sterol	GLC RRT_c	RP-HPLC α_c	TLC R_f	MS: Some diagnostic ions	^{1}H-NMR: Some diagnostic chemical shifts
Cholesterol[c]	1.0	1.00	0.48	M^+ (386, 100%), M^+-CH_3(371, 33%), M^+-H_2O (368, 43%), M^+-SC (273, 28%), M^+-85 (301, 40%) and M^+-111 (275, 75%) characterize Δ^5.	N.E.[d]
Campesterol	1.28	1.15	0.48	M^+ (400, 100%), M^+-CH_3 (385, 31%), M^+-H_2O (382, 58%), M^+-SC (273, 19%), M^+-85 (315, 25%) and M^+-111 (289, 36%) characterize Δ^5.	H-21 (3H, d, J = 6.1 Hz) at 0.910 ppm, H-26 (3H, d, J = 6.0 Hz) at 0.800 ppm, H-27 (3H, d, J = 6.0 Hz) at 0.850 ppm and H-6 (1H, br s) at 5.359 ppm characterize Δ^5, 24α CH_3.
Dihydrobrassicasterol	1.28	1.15	0.48	M^+ (400, 100%), M^+-CH_3 (385, 31%), M^+-H_2O (382, 58%), M^+-SC (273, 19%), M^+-85 (315, 25%) and M^+-111 (289, 36%) characterize Δ^5.	H-21 (3H, d, J = 6.1 Hz) at 0.921 ppm, H-26 (3H, d, J = 6.0 Hz) at 0.784 ppm, H-27 (3H, d, J = 6.0 Hz) at 0.862 ppm and H-6 (1H, br s) at 5.359 ppm characterize Δ^5,24α CH_3.
Sitosterol	1.62	1.29	0.48	M^+ (414, 100%), M^+-CH_3 (399, 23%), M^+-H_2O (396, 58%), M^+-SC (273, 17%), M^+-85 (329, 27%) and M^+-111 (303, 29%) characterize Δ^5.	H-21 (3H, d, J = 6.5 Hz) at 0.921 ppm, H-29 (3H, t, J = 7.3 Hz) at 0.845 ppm and H-6 (1h, br s) at 5.359 ppm characterize Δ^5, 24α C_2H_5.
Stigmasterol	1.42	1.10	0.48	M^+ (412, 67%), M^+-CH_3 (397, 6%), M^+-H_2O (394, 19%), M^+-SC (273, 12%), M^+-43 (369, 13%) and ratio of ion intensities of M^+ vs M^+-SC and m/z 314 (7%) characterize $\Delta^{5,22}$.	H-29 (3H, t, J = 7.1 Hz) at 0.804 ppm, H-6 (1H, br s) at 5.359 ppm, H-22 (1H, dd, J = 7.5 Hz) at 5.015 ppm and H-23 (1H, dd, J = 7.5 Hz) at 5.159 ppm characterize $\Delta^{5,22}$, 24α C_2H_5.
Isofucosterol[c]	1.65	1.00	0.48	M^+ (412, 7%), M^+-CH_3 (397, 2%), M^+-H_2O (394, 3%), M^+-SC-2H (271, 7%), M^+-43 (369, 0%), and M^+-98 (314, 76%) due to allylic cleavage thru 22(23) characterize $\Delta^{5,24(28)}$.	H-25 (1H, m) at 2.819 ppm, H-28 (1H, m) at 5.115 ppm, H-6 (1H, br s) at 5.359 ppm characterize $\Delta^{5,24(28)}$, with the configuration of the ethylidene bond as the Z-isomer.
Avenasterol	1.85	1.00	0.43	M^+ (412, 3%), M^+-CH_3 (397, 3%), M^+-H_2O (374, 1%), M^+-SC-2H (271, 60%), M^+-43 (369, 0%), M^+-85 (327, 0%), M^+-98 (314, 40%) and ratio of ion intensities of M^+ vs M^+-SC characterize $\Delta^{7,24(28)}$.	H-29 (3H, d, J = 6.6 Hz) at 1.591 ppm, H-25 (1H, m) at 2.830 ppm, H-7 (1H, br s)) at 5.153 ppm characterize $\Delta^{7,24(28)}$, with the configuration of the ethylidene bond as the Z-isomer.
24-Methylenelophenol	1.63	1.00	0.65	M^+ (412, 11%), M^+-CH_3 (397, 9%), M^+-H_2O (394, 2%), M^+-SC (285, 42%), M^+-43 (369, 0%), M^+-85 (328, 20%) due to allylic cleavage thru 22(23) and ratio of ion intensities of M^+ vs M^+-SC characterize $\Delta^{7,24(28)}$.	H-18 (3H, s) at 0.538 ppm, H-19 (3H, s) at 0.828 ppm, H-30 (3H, d, J = 6.5 Hz) at 0.969 ppm and H-28 (1H, br d, J = 12.0 Hz) at 4.680 ppm characterize $\Delta^{7,24(28)}$, 4α CH_3 methyl groupings.

TABLE 1 *(continued)*

Citrastadienol	2.19	1.20	0.65	M^+ (426, 6%), M^+-CH_3 (411, 6%), M^+-H_2O (408, 2%), M^+-SC (285, 100%), M^+-43 (383, 0%), M^+-98 (328, 20%) and ratio of ion intensities of M^+ vs M^+-SC characterize $\Delta^{7,24(28)}$.	H-18 (3H, s) at 0.538 ppm, H-19 (3H, s) at 0.828 ppm, H-30 (3H, d, J = 6.5 Hz) at 0.969 ppm and H-25 (1H, m) at 2.835 ppm characterize $\Delta^{7,24(28)}$, 4α methyl groupings and the ethylidene having the Z configuration.
Graminasterol	2.10	1.03	0.71	M^+ (440, 20%), M^+-CH_3 (425, 35%), M^+-H_2O (422, 0%), M^+-SC-2H (313, 45%), m/z 300 (0%) (characteristic of cyclopropane group cleavage), M^+-85 (355, 10%) due to allylic cleavage thru 22(23) characterize $\Delta^{24(28)}$ sterols; lack of M^+-H_2O and ratio of ion intensities of M^+ vs M^+-SC and M^+ vs M^+-CH_3 characterize Δ^7, 14α methyl grouping.	N.E.
Obtusifoliol	1.49	0.82	0.63	M^+ (426, 44%), M^+-CH_3 (411, 83%), M^+-H_2O (408, 3%), M^+-SC (301, 1%), M^+-99 (327, 15%) due to allylic cleavage thru 22(23) with hydrogen transfer and ratio of ion intensities of M^+ vs M^+-CH_3 characterize 14α methyl substituent.	N.E.

[a]Conditions. GLC: Varian 1440, flame ionization detector (310 C), 3% SE-30 packed column on Gas Chrom Q 100/120 mesh, 1.8 m × 2 mm i.d; oven temp. 240 C; flow rate of He (carrier) 30 $ml.^{-1}$; RRT_c = relative retention time to cholesterol. RP-HPLC: Altex column packed with Ultrasphere ODS (5 μm p.s.), 4.6 mm i.d. × 250 mm; 100% MeOH, flow rate 1.0 m/min^{-1}; detector at 205 mm; compounds chromatographed as acetates; α_c = retention volumes relative to cholesterol. TLC: silica gel G (250 μm × 20 cm × 20 cm) developed twice (as the free sterol) with $C_6H_6/(C_2H_5)_2O$ (9:1, v/v). MS: EI-MS, 70 eV, VG micromass (70/70) mass spectrometer; ion source temp. 150 C for direct probe or GLC-MS (2-5). ^{1}H-NMR: Nicolet 200 mHz spectrometer operating at RT in the Fourier transform mode; solvent $CDCl_3$; δ values relative to Me_4Si.

[b]Quantities of the major seed sterols are given in Table 2. Amounts of minor sterols varied from 3–25 μg/g dry seed.

[c]Cholesterol and isofucosterol were resolved as acetates by $AgNO_3$-TLC. The mixture was spotted onto $AgNO_3$ impregnated silica gel G-TLC plates and developed twice in C_6H_{14}/C_6H_6 (40:60, v/v). The typical R_f was for cholesteryl acetate 0.56 and isofucosteryl acetate 0.43.

[d]N.E. = Not examined.

and graminasterol (not to be confused with gramisterol) (Fig. 1) are two previously unidentified sterols synthesized by sorghum (Table 1). The seed PT composition, unlike the sterol profile, was different from that of the variety studied by Palmer and Bowden (10). For instance, we could not detect significant levels of fernenol or isoarborinol. In G 499 GBR, two major PT were apparent with M^+ 426 and 428, respectively. Both compounds produced mass spectra with diagnostic ions at m/z 274, characteristic of migrated hopanoids with a simiarenol skeleton.

In our earlier report (3) on the leaf sterols of 66-day flowering sorghum, we failed to discriminate the epimeric purity of 24-methylcholesterol and 24-ethylcholesta-5,22-dienol; the former is an epimeric mixture of campesterol and dihydrobrassicasterol, as we show by ^{1}H-NMR (Table 1), and the latter is pure stigmasterol. The ^{1}H-NMR spectrum obtained earlier on the 24-ethylcholesterol sample was consistent with that of an authentic standard of sitosterol. Additional confirmation for the structure of sitosterol was obtained through an X-ray crystallographic study of the free alcohol. The partial three-dimensional structure is shown in Figure 2. (Full details of the computer analysis will be presented elsewhere by Wong and Nes.) It is apparent from the X-ray structure that sitosterol in the crystalline state, analogous to its presumed conformation in the membrane, possesses a planar nucleus and a right-handed side chain (cf. the X-ray analysis of euphol and tirucallol [14]). This view of the molecule usually is not depicted in illustrations of the sterol side chain. The X-ray also depicts the C-22(23) bond directed toward the observer or in a skew conformation (cf. Fig. 2) in which the C-20 H is skew to the alkyl chain bearing C-22. Of the rotameric possibilities (some examples in Fig. 2) for the side chain as the sterol complexes with fatty acids in the lipid bilayer, the staggered conformation is most favored on energetic grounds such as in Figure 2C. This suggestion is supported by physiochemical studies (15).

Sterol composition during development. There are

TABLE 2

Occurrence of Major Free Sterols in *Sorghum bicolor* During Development[a]

Dominant sterol	Dry seeds (caryopses)	Germinating seeds	Leaf blade			
			Day 7	Day 34	Day 48	Day 66
Cholesterol	0.08 (1)[b]	0.04 (tr)[c]	0.10 (tr)	4.20 (2)	13.00 (1)	5.70 (1)
Campesterol[d]	0.75 (9)	{2.60	{0.50	{33.70	{169.00	38.60 (6)
Dihydrobrassicasterol[d]	1.13 (13)	(23)	(14)	(14)	(15)	57.60 (10)
Stigmasterol	1.94 (23)	2.50 (23)	2.10 (59)	110.20 (47)	492.00 (43)	237.90 (40)
Sitosterol	4.50 (54)	6.10 (54)	1.00 (27)	88.20 (37)	465.00 (41)	254.80 (43)
Total in μg/seed or leaf blade	8.40	11.24	3.70	236.30	1139.00	594.60
Total as percent dry wt	0.032	0.042	0.256	0.037	0.13	0.105

[a]Values are expressed in μg/seed or leaf blade. Plant material used throughout was *S. bicolor* (L.) Moench, cv G 499 GBR. Seeds were extracted and the extracts chromatographed without saponification before (dry) and after (germinating) 20 hr imbibition with water (continuous light). Leaves were derived from 7-day plants sown in vermiculite, watered and grown in an incubator (28 C) with discontinuous light (14 hr light, 10 hr dark). In the later stages, plants were grown in the green house in soil under normal light conditions. On the 7-day seedling, the primary leaf blade was 1.5 cm in length and the second leaf was just emerging from the primary sheath. On the 34-day plant, eight leaves were visible and the blade of the 4th leaf from the flag leaf (length 39.3 cm) was excised. On the 48-day plant, nine leaves were visible and the blade of the fifth leaf from the flag leaf was excised (length 55.5 cm). Floral differentiation, depending on the growing time, appeared between 41 and 48 days from the point of sowing. On the 66-day plant, 13 leaves were visible and the blade of the second leaf from the flag leaf was excised (length 52.5 cm).

[b]Values in parentheses are percentages of individual sterols in the mixture.

[c]Tr: trace levels ca. 1 to 5 ng.

[d]^{1}H-NMR determinations were conducted only on seeds and mature leaves. Braces refer to unresolved α/β mixture in the germinating seeds and leaf blades from 7-, 34- and 48-day plants.

four major stages of sorghum development: seed germination, seedling development, inflorescence development or flowering and senescence. Vegetative growth is continuous in stages 2 and 3; however, by ca. 60 days (stage 3) when the inflorescence (a panicle in sorghum) emerges from the boot (shoot whorl), the shoot height has been established. Floral induction was determined by dissecting out the apical dome of plants 34 to 48 days from sowing. The appearance of reproductive meristems (immature inflorescence) varied depending on the growing conditions (e.g., day length). Most of our experiments were carried out in the winter/spring months when floral induction was detected between 41 and 48 days. Panicles were noticeable 12 to 25 days following floral induction. Grain maturation occurs in stage 3 after anthesis (pollination), usually 25 to 55 days after blooming depending on the variety (16).

As shown in Table 2, the major free sterol content of the seeds increased somewhat during the first 20 hr of germination. Because much is happening to the grain in the succeeding two to five days independent development of hypocotyl, roots and shoots, each of which is assumed to have specialized capabilities for sterol and PT synthesis (8,9)—we elected to continue our study using mature leaf blades which could be defined uniformly throughout stages 2 and 3. Free sterol content (μg/leaf blade) of the mature leaf blades increased from day seven to day 48, then decreased (Table 2). The amount of sterol esters and glycosides was relatively low until flowering. The ratio of free to ester and glycoside in the leaves increased with development but never exceeded 70:30 in the leaves at anytime during stages 2 and 3. The increase in the derivatized sterols could not account for the total decrease in free sterol. This indicates that a critical mass of free sterol may be required for reproduction to be induced. It remains to be determined whether the increase in derivatization has additional physiological importance or its role is to effectively control the free

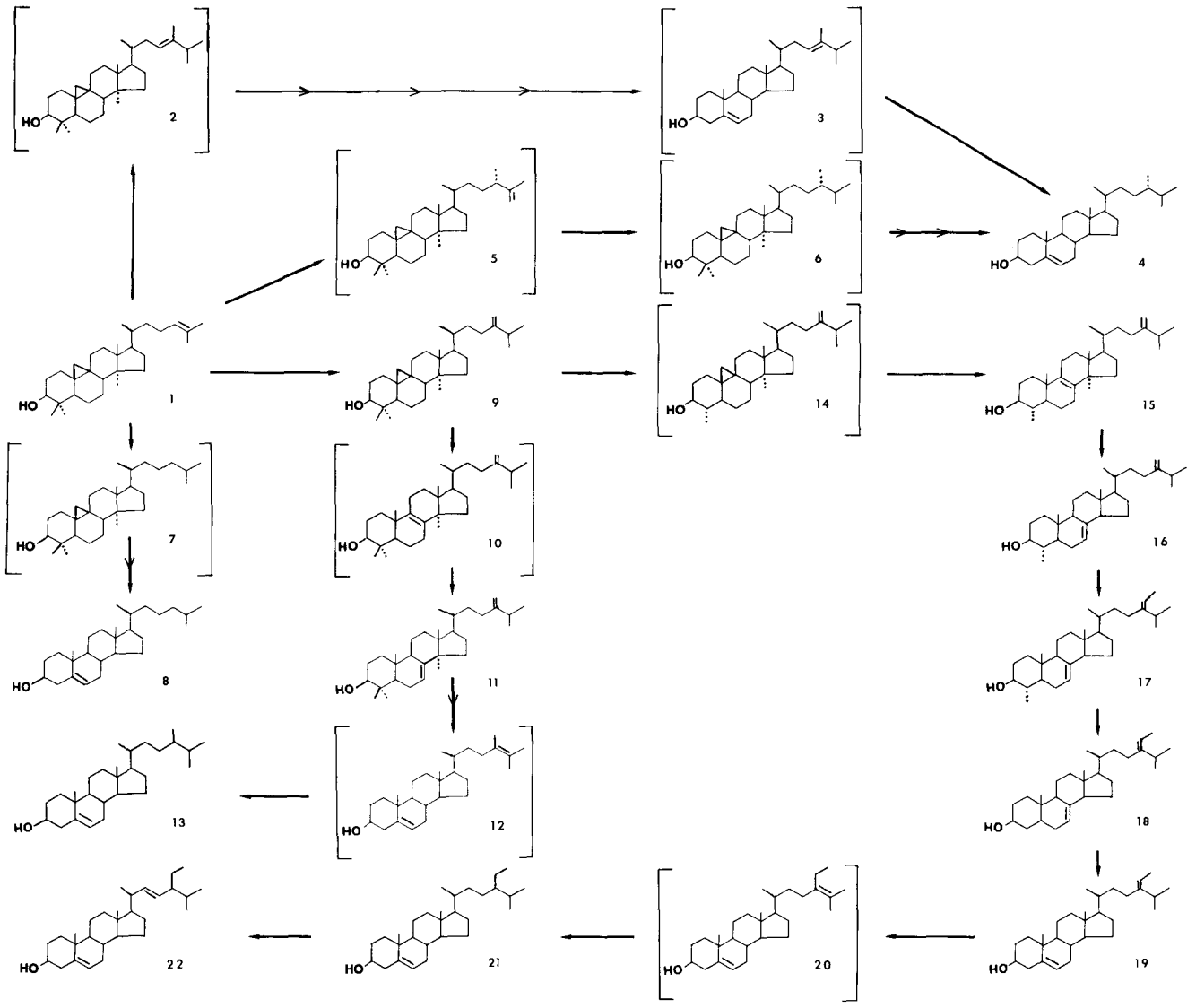

FIG. 1. Proposed separate pathways to multiple sterol end products of sorghum. Bracketed compounds are proposed intermediates; nonbracketed compounds have been identified endogenous to seeds or leaves. 1, Cycloartenol; 2, cyclosadol; 3, ergosta-5,23-dienol; 4, dihydrobrassicasterol; 5, cyclolaudenol; 6, 24-methylcycloartanol; 7, cycloartanol; 8, cholesterol; 9, 24-methylenecycloartanol; 10, 24-methylenedihydrolanosterol; 11, graminasterol; 12, ergosta-5,24-dienol; 13, campesterol; 14, cycloeucalenol; 15, obtusifoliol; 16, 24-methylenelophenol; 17, citrostadienol; 18, avenasterol; 19, isofucosterol; 20, stigmasta-5,24-dienol; 21, sitosterol; 22, stigmasterol.

sterol levels. The suggestion that a critical mass of free sterol plays a physiological role in the initiation of flowering in sorghum differs from the general view that if sterols regulate flowering they must be metabolized to a hormone (17). As we have suggested, sterols in crops may have multiple nonmetabolic roles such as bulk and regulatory (3). In sorghum one or both roles appear to minimally influence floral induction.

While monitoring the sterol content of the mature leaf blades, we also examined the PT content. GLC analysis of the leaf blade PT in the four-week period from seven to 35 days showed that the profiles were similar and the contents were negligible (both as free alcohols and esters). The PT content suddenly increased at floral induction from a few μg/leaf blade to an amount somewhat greater than that of the leaf blade sterol in 66-day plants. The ratio of free to esterified PT was 3:1 in the mature leaves (cf. ref. 5 for details of sterol to PT ratios in mature leaves of three varieties of flowering sorghum) but one to 20 in the seven-day leaves. A direct probe MS of the hydrolyzed seven-day PT esters showed mass fragmentation which corresponded to a mixture of PT (m/z: 426, 411, 279 and 218). Whether the change with age in the free to ester ratio was influenced by incoming translocated PT from other plant parts such as roots or due to interconversion or perhaps a cessation of esterification in the older plants requires further study. Nevertheless, that free PT appears as free sterol and disappears

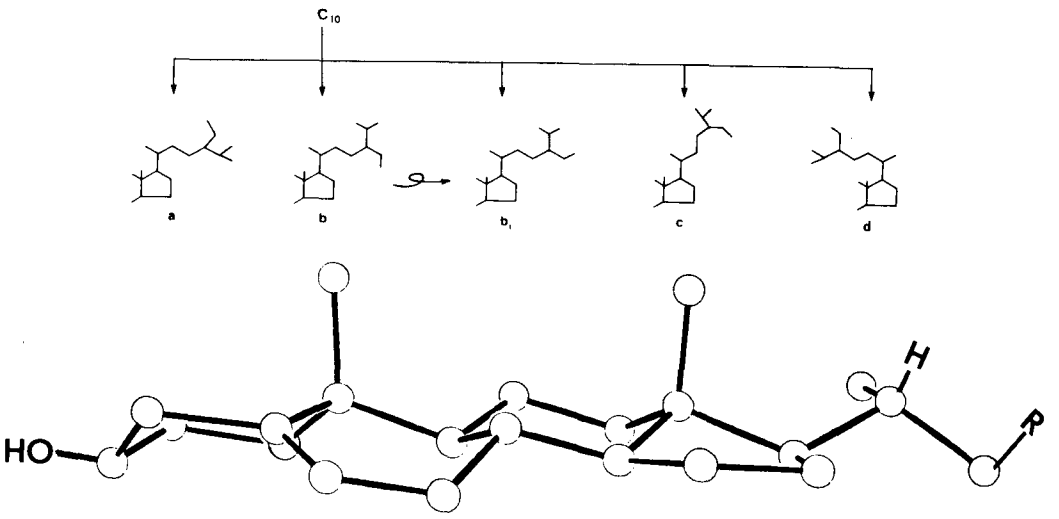

FIG. 2. Partial X-ray crystal structure of sitosterol. A through D represent some of the different rotameric conditions that potentially exist for a C_{10}-aliphatic side chain (R represents C_{10}).

indicates that the SO, normally cyclized to cycloartenol, is shunted into the PT pathway, thereby effectively removing precursor sterol material. Newly formed PT may replace the sterol in the membranes (18) so that the preformed sterol is removed from its primary function of an architectural component, translocated and/or metabolized for other physiological functions.

Differences in sterol biosynthesis during development. In a preliminary experiment in which [2-^{14}C]MVA was imbibed into seeds (25 μCi), the percentage incorporation of the available isomer into sterols was low (ca. 0.26%), as expected (7). After sequential chromatographies developed to radiochemically purify each of the 4-desmethylsterols—by adsorption TLC, $AgNO_3$-Anacil B column chromatography, RP HPLC and $AgNO_3$-TLC followed by, in selected cases, recrystallization—only the 24-alkylsterols were found to possess radioactivity; no radioactivity was associated with cholesterol. Thus, the cholesterol in the seed may have been derived from maternal tissue, synthesized by the embryo or translocated from other plant parts (leaves) during grain maturation.

Further differences in sterol biosynthesis were apparent in sorghum's developmental stage 3. From a [2-^{3}H]cycloartenol feed to a leaf blade from a plant with its inflorescence just emerging (9.20×10^5 dpm/200 μg/blade) we isolated four radioactive sterols; only cholesterol eluted from the HPLC column with significant radioactivity to continue with its radiochemical purification. The sterol fraction corresponding to cholesterol was diluted with carrier material and recrystallized to constant specific activity: initial, 932 dpm/21.9 mg; first crystals, 15.7 dpm/ mg from Me_2CO (100%); second crystals, 15.4 dpm/mg from Me_2CO/H_2O (1:1); third crystals, 15.6 dpm/mg from EtOAc/C_6H_{14}/H_2O (1:1:tr, v/v/v). The percent conversion of [2-^{3}H]cycloartenol into cholesterol was very low (0.09%). From a [2-^{3}H]lanosterol (3.11×10^8 dpm/55 μg/blade) incubation to a similar leaf blade, only radiolabeled cholesterol was isolated by HPLC, analogous to the [2-^{3}H]cycloartenol incubation. Its conversion to cholesterol (0.002%) was less than that of the cycloartenol conversion to cholesterol. While radioactivity was associated with the 24-alkylsterols by HPLC of the desmethylsterol fraction from [2-^{3}H]cycloartenol-treated leaves, no 24-alkylsterols were labeled from the [24-^{3}H]lanosterol treatment. Thus, the label in the 24-methylsterol fraction from the [2-^{3}H]cycloartenol feed must be associated entirely with campesterol. This follows, assuming that sorghum possess a similar mechanism of alkylation to other grasses (19-23) and that in the formation of C-24α-alkylsterols the ^{3}H from [24-^{3}H]lanosterol is transferred to C-25 and lost following the isomerization of $\Delta^{24(28)}$ to $\Delta^{24(25)}$. Alternatively, in C-24β-alkylsterol formation the ^{3}H should be retained at C-25 (19-23).

When [4-^{14}C]sitosterol (1.84×10^7 dpm/56 μg/blade) was applied to a blade from a plant with its inflorescence just emerging, it was oxidized to unidentified polar compounds (30% conversion), of which only a few percent represented sterylglycosides and acylated sterylglycosides, or desaturated to stigmasterol (0.11% conversion). Unequivocal proof for sitosterol to stigmasterol conversion was obtained by diluting the HPLC-purified stigmasterol with carrier material and recrystallizing to constant specific activity (initial, 29,622 dpm/20.4 mg; sp act [dpm/mg] from Me_2CO [100%], Me_2CO/H_2O [1:1, v/v], EtoAc/C_6H_{14}/H_2O, [1:1:tr, v/v/v] and MeOH/Me_2CO/CH_2Cl_2 [1:1:0.25, v/v/v] were 1547, 645, 684 and 668, respectively). When a similar experiment was performed with [4-^{14}C]cholesterol (2.78×10^7 dpm/93 μg/blade), no evidence was obtained for the metabolism of cholesterol to 24-alkysterols.

The identification and composition studies indicate that sorghum may synthesize the alkylated sterols by similar mechanisms to other grass plants (19-23). Some similarities in sequencing to the multiple end

products in the post-cycloartenol pathways also are apparent (Fig. 1). The specific occurrence of cycloartenol in mature leaves (5) coupled with biochemical data obtained with seedlings (19-23) indicates that cycloartenol rather than lanosterol is the tetracyclic product of SO cyclization throughout the plant's life cycle.

In summary, the functional expression of the post-cycloartenol pathways may be developmentally regulated, resulting in differences in sterol biosynthesis and composition. A relationship seems to exist between the biosynthetic "switching on and off" of specific polycyclic isopentenoids and the transition from one developmental stage to another (e.g., vegetative to reproductive). Physiological phenomena appear regulated by the availability of certain combinations of sterols and/or PT or their metabolites during development. For floral induction a high content of free sterol is required by sorghum. Inhibition of sterol synthesis through inhibitors such as SKF 7997 can inhibit growth and flowering in tobacco (24, 25) and may affect sorghum physiology similarly. The decrease in sterol levels during the flowering stage may indicate sterol translocation to other target sites, such as maturing seeds, or sterol metabolism to hormones. The resultant loss of free sterol then may be compensated for by the synthesis of replacement membrane architectural components—PT. Patterson and coworkers have shown recently in whole plant studies that similar changes occur in sterol composition through the life cycle of squash and soybean (26).

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The Fate of Radiolabeled Steroids in Ovaries and Eggs of the Tobacco Hornworm, *Manduca sexta*

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To determine the precursors and the fate of 26-hydroxyecdysone in eggs, the fate of labeled putative ecdysteroid precursors was examined in the tobacco hornworm, *Manduca sexta*. Following injection of [^{14}C]cholesterol, 22,25[^{14}C]dideoxyecdysone or [^{3}H]ecdysone into female pupae (day 16), only [^{14}C]cholesterol was incorporated and metabolized. It was converted to labeled nonecdysteroid and ecdysteroid conjugates, of which the latter in ovaries and 48- to 64-hr-old eggs is mainly 26-hydroxyecdysone 26-phosphate (>85% in ovaries). Quantitation of the ecdysteroid conjugate by reversed-phase high performance liquid chromatography (RP-HPLC) showed that the levels of 26-hydroxyecdysone 26-phosphate were 31 μg/g of ovaries from 4-day-old females and 25 μg/g and 17 μg/g of 48- to 64-hr-old and 72- to 88-hr-old eggs, respectively. The RP-HPLC of the conjugate fraction of 48- to 64-hr-old eggs showed an additional peak of radioactive material eluting about three min before the 26-hydroxyecdysone 26-phosphate. The quantity of this material increased in the 72- to 88-hr-old eggs, though it was not detected in the analyses of the conjugate fraction from ovaries. Additional peaks of radioactive material eluting before the 26-hydroxyecdysone 26-phosphate peak were observed in the chromatogram of the conjugates of 72- to 88-hr-old eggs. These radioactive materials need to be identified to determine the ultimate fate of ecdysteroids in the developing embryos of the tobacco hornworm. No radioactive free ecdysteroids were detected in either egg age group.
Lipids 21, 76–81 (1986).

The ovaries and newly laid eggs of various insect species contain comparatively large quantities of ecdysteroid conjugates and only minute amounts of free ecdysteroids (1–3). In ovaries and newly laid eggs (0- to 1-hr-old) of the tobacco hornworm, *Manduca sexta*, conjugates account for more than 95% of the total ecdysteroids present (4). These conjugates, unlike conjugates in eggs of other insect species, are comprised primarily of 26-hydroxyecdysone (21 μg/g of eggs) and ecdysone (0.73 μg/g). 26-Hydroxyecdysone 26-phosphate (Fig. 1) has been identified as the major conjugate in 48- to 64-hr-old eggs of *M. sexta* (5). In batch-collected, 1- to 18-hr-old eggs stored at −20 C until analyzed, more than 63% of the ecdysteroids are in the free form with 26-hydroxyecdysone accounting for 97% and ecdysone accounting for 3% (4). Interestingly, 26-hydroxyecdysone first isolated from 48- to 64-hr-old tobacco hornworm eggs (6) is devoid of molting hormone activity in the house fly bioassay (7) and has been reported to occur at such a high level only in eggs of the tobacco hornworm. Although low levels of other ecdysteroids such as 20-hydroxyecdysone, 20,26-dihydroxyecdysone, 3-epi-26-hydroxyecdysone and 3-epi-20,26-dihydroxyecdysone (Fig. 1) as well as six other unidentified ecdysteroids have been isolated from kg quantities of tobacco hornworm eggs of different age groups (7), nothing is known of the precursors or the fate of 26-hydroxyecdysone in these eggs. We now have examined putative ecdysteroid precursors in *Manduca sexta*. [4-^{14}C]Cholesterol, 22,25-[4-^{14}C]dideoxyecdysone or [23,23-^{3}H]ecdysone was injected into female tobacco hornworm pupae on day 16 after pupation. Steroid analyses of ovaries from 4-day-old adult females (ca. five days after injection) showed that only the [^{14}C]cholesterol was incorporated and appreciably metabolized. In this paper we will discuss the metabolism of [^{14}C]cholesterol in *M. sexta* and its incorporation into ecdysteroid and nonecdysteroid conjugates in ovaries and eggs. We also will discuss recent developments concerning the fate and/or state of labeled ecdysteroid conjugates of different age groups of tobacco hornworm embryonated eggs.

MATERIALS AND METHODS

Instrumentation. RP-HPLC analyses were performed with a Spectra-physics SP8700 solvent delivery system equipped with an SP8500 dynamic mixer. Absorbance of the effluent was monitored at 254 nm with a Waters model 441 absorbance detector and automatically recorded by a Shimadzu model C-R1B. Samples were counted in a Packard TriCarb 460CD Liquid Scintillation System or Beckman LS 5801. Samples were analyzed by high performance thin layer chromatography (HP-TLC) (precoated plates for Nano TLC, Silica Gel 60F 254, E. Merck, Darmstadt, West Germany).

Steroids. [23,24-^{3}H]Ecdysone was a gift of Zoecon Corp. (Palo Alto, California). After dilution with unlabeled ecdysone, it was purified to >95% radiopurity by RP-HPLC on a radial compression C_{18} column (Waters Assoc., Midland, Massachusetts). The [^{3}H]ecdysone was eluted isocratically with a mixture of methanol (Burdick and Jackson, American Scientific Products, Columbia, Maryland) and glass-distilled water (38:62, v/v) at 2 ml/min. Fifty μg of ecdysone, 2.49×10^5 dpm, were injected per pupa. 22,25-[4-^{14}C]Dideoxyecdysone was prepared from [4-^{14}C]cholesterol according to procedures used for the synthesis of unlabeled compound (8), and 9.5×10^4 dpm (50 μg) were injected into each pupa. [4-^{14}C]Cholesterol was purchased from Amersham Corp. (Arlington Heights, Illinois). After purification by column chromatography the radiochemical purity was >99%. One μCi was injected into each pupa.

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Nomenclature—Ecdysone: 2β, 3β, 14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one; 20-hydroxyecdysone: 2β,3β,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one; 26-hydroxyecdysone: 2β,3β,14α,22R,25,26-hexahydroxy-5β-cholest-7-en-6-one; 20,26-dihydroxyecdysone: 2β,3β,14α,20R,22R,25,26-heptahydroxy-5β-cholest-7-en-6-one; 3-epi-20,26-dihydroxyecdysone: 2β,3α,14α,20R,22R,25,26-heptahydroxy-5β-cholest-7-en-6-one; 3-epi-26-hydroxyecdysone: 2β,3α,14α,22R,25,26-hdexahydroxy-5β-cholest-7-en-6-one; 22,25-dideoxyecdysone: 2β,3β,14α-trihydroxy-5β-cholest-7-en-6-one, and 26-hydroxyecdysone 26-phosphate: 2β,3β,14α,22R,25,26-hexahydroxy-5β-cholest-7-en-6-one 26-phosphate.

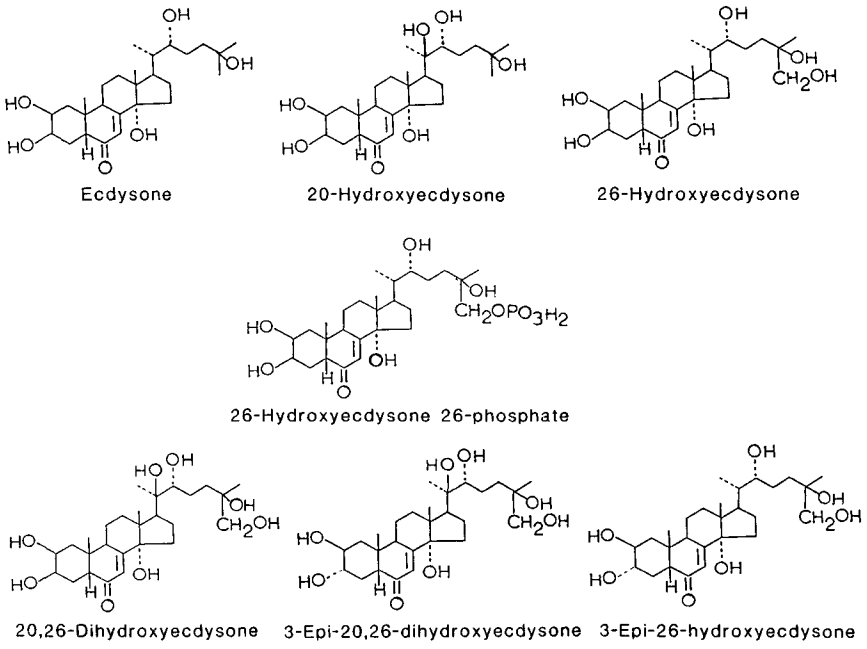

FIG. 1. Ecdysteroids isolated from *Manduca* eggs.

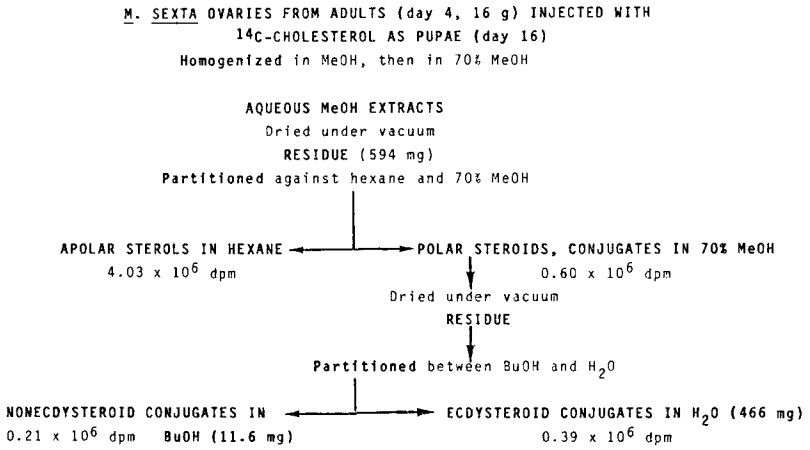

FIG. 2. Procedure used for the separation from ovaries and eggs of apolar and polar steroids which were separated further into nonecdysteroid and ecdysteroid conjugate fractions.

Biological material. Tobacco hornworms were reared as described previously (9). The labeled steroid, in 25 μl of a saline solution (10) containing 3% Tween 80, was injected with a microsyringe into each female pupa (day 16) through the ventral intersegmental membrane between the fifth and sixth abdominal segment (11). As the moths emerged they were used for dissection of ovaries or allowed to oviposit on tobacco plants in cages with equal numbers of adult males. Ovaries were excised from adult females 93 hr after eclosion and samples were held in methanol at −20 C until at least 10 g were accumulated for extraction. Females ordinarily would begin laying eggs the night following the 93-hr time period. Eggs for the 48- to 64-hr and 72- to 88-hr samples were removed from the tobacco plant at ca. 18 hr and kept in Petri dishes for the remainder of the time interval (6). The eggs were weighed, transferred into screw cap glass bottles and stored in methanol at −20 C until 10 g or more were available for workup.

Extraction and separation of nonecdysteroid and ecdysteroid conjugates. Tobacco hornworm ovaries (28 pairs) and eggs were extracted and processed as described previously (5) and according to the schemes shown in Figures 2 and 3 for isolation and purification of radioactive materials from ovaries.

Purification of ecdysteroid conjugates. The aqueous phase from the butanol-water partition (Fig. 2) was reduced to dryness under vacuum. The residue was redissolved in 4 ml of water and the solution was adjusted to pH 4 with 2N acetic acid, then placed on a 1.5 × 13 cm column (bed volume 25 ml) of Amberlite XAD-2 beads (Rohm and Haas, Philadelphia, Pennsylvania). The column (Fig. 3) was eluted with 150 ml of water followed by 150 ml of ethanol. The ethanol, which contained the con-

jugates, was removed under vacuum and the residue was further fractionated on a C_{18} Sep-Pak cartridge (Millipore, Waters Chromatography Division, Milford, Massachusetts) as shown in Figure 3.

Radioassay and quantification of ecdysteroid conjugates by HPLC. The ecdysteroid conjugates (Sep-Pak fractions 3 and 4, Fig. 3) were separated by ion suppression RP-HPLC on an IBM C_8 column (4.6 mm × 15 cm; Danbury, Connecticut) by isocratic elution with 30% methanol in 0.03 M aqueous NaH_2PO_4 solution (pH 5) at a flow rate of 1.0 ml/min. 26-Hydroxyecdysone 26-phosphate in the Sep-Pak fractions was quantified by comparison of the peak areas with calibrated areas obtained with known amounts of authentic 26-hydroxyecdysone 26-phosphate. When fractions (0.5 ml) were collected for monitoring radioactivity, scintillation fluid (10 ml Hydrofluor, National Diagnostics, Somerville, New Jersey) was added directly to the solvent.

RESULTS

Steroid analyses of ovaries (28 pairs) from 4-day-old females showed that of the three steroids injected into female hornworm pupae on day 16, only the [^{14}C]cholesterol (Table 1) was incorporated and appreciably metabolized. The schemes in Figures 2 and 3 were used effectively for the partial purification and subsequent analyses of polar steroids from both ovaries and eggs. The general procedure, however, is given only for steroid analyses of ovaries. The results show that effective use of the scheme (Fig. 2) separates the apolar and polar steroids. Further, the butanol-water partition as previously employed (4) efficiently separates polar steroids into two fractions. The butanol phase contains the free ecdysteroids and nonecdysteroid conjugates of hornworm ovaries and eggs whereas the aqueous phase contains the ecdysteroid conjugates (Table 2).

Ecdysteroid conjugates. After applying the aqueous solution (4 ml) adjusted to pH 4 with 2N acetic acid to

TABLE 1

Incorporation of Radioactive Material into Ovaries and Eggs of *M. sexta* Injected with ^{14}C-Cholesterol as Day-16 Pupae

Tissue	Weight (g)	Total radioactivity (dpm × 10^6)	dpm × 10^5/g of tissue
Ovaries[a]	16.34	4.63	2.83
Eggs[b]	12.27	9.60	7.83
Eggs[c]	14.85	8.21	5.53

[a]From 4-day-old females
[b]48- to 64-hr-old
[c]72- to 88-hr-old

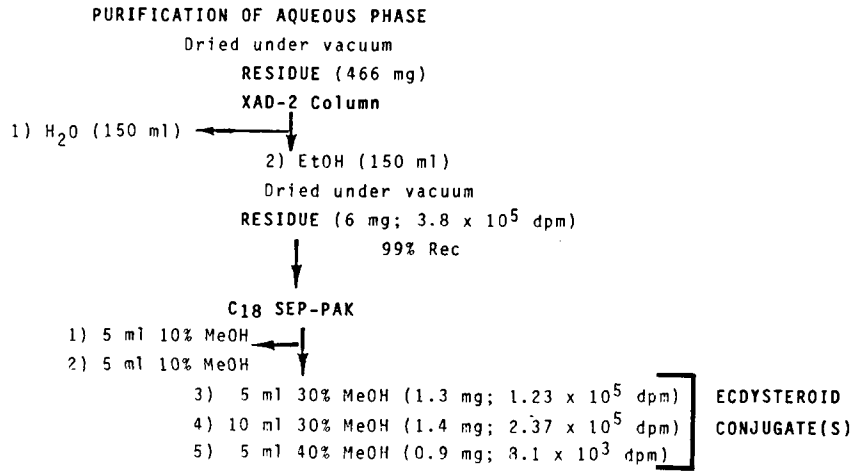

FIG. 3. Additional procedure used for isolation and purification of ecdysteroid conjugates.

TABLE 2

Conversion of ^{14}C-Cholesterol Injected into Day-16 *M. sexta* Pupae to Nonecdysteroid and Ecdysteroid Conjugates

		Nonecdysteroid conjugate	Ecdysteroid conjugate
Tissue	Weight (g)	Radioactivity (dpm × 10^5/g of tissue)	Radioactivity (dpm × 10^5/g of tissue)
Ovaries[a]	16.34	0.126	0.238
Eggs[b]	12.27	0.96	0.34
Eggs[c]	14.85	1.60	0.42

[a]From 4-day-old females.
[b]48- to 64-hr-old.
[c]72- to 88-hr-old.

an XAD-2 column (Fig. 3), 98% of the impurities were removed from the column with water and the partially purified conjugates were eluted with ethanol. The Sep-Pak fractionation removed additional impurities. The ecdysteroid conjugates (from ovaries of 4-day-old females) in fractions 3 and 4 accounted for 94% of the total activity added to the XAD-2 column. Analyses of 1.0 and 0.6% of fractions 3 and 4, respectively, by ion suppression RP-HPLC and radioassay gave the chromatograms shown in Figure 4. The material eluting at 7.62 min is the major component of both fractions. This retention time and R_f 0.17 in the solvent system of chloroform/methanol/10N NH_4OH (15:35:3.5, v/v/v) are identical to those of authentic 26-hydroxyecdysone 26-phosphate (5). The structure of the conjugate eluting at 6.45 min remains to be determined. The radioactivity was associated only with these two peaks.

Analyses of 0.66% of each of fraction 3 and 4 (from processing 48- to 64-hr-old eggs through Sep-Pak separation) by ion suppression RP-HPLC and radioassay gave the chromatograms shown in Figure 5. Interestingly, a polar radioactive material eluting at 4.90 min appears in the chromatogram of fraction 3 (Fig. 5A). The 26-hydroxyecdysone 26-phosphate and the unidentified conjugate eluted at 7.74 and 6.54 min, respectively, in the chromatograms of fractions 3 and 4. Most of the 26-hydroxyecdysone 26-phosphate and radioactivity was eluted in Sep-Pak fraction 4 (Fig. 5B).

Anticipating that the polar radioactive material eluting at 4.90 min in the chromatogram of Sep-Pak fraction 3 (from processing 48- to 64-hour-old eggs through Sep-Pak separation) would increase in 72- to 88-hr-old eggs, we altered the elution scheme (Table 3) to obtain a purer fraction. Although we were expecting the more polar or radioactive metabolite eluting at 4.90 min (fraction 3, Fig. 5A) to elute before 26-hydroxyecdysone 26-phos-

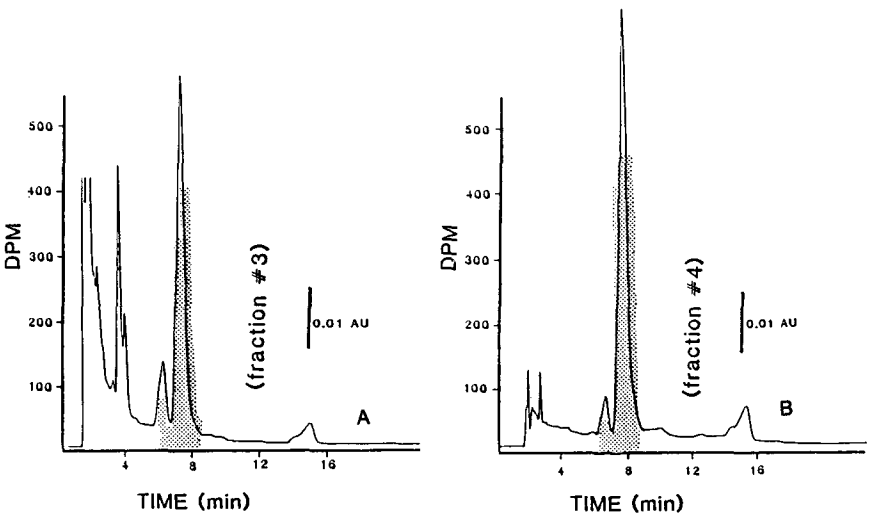

FIG. 4. Ion suppression RP-HPLC trace and radioassay analysis of partially purified ecdysteroid conjugates from ovaries of day-4 female *Manduca*, Sep-Pak (Fig. 3) fraction 3 (A), fraction 4 (B). On IBM C_8 column (4.6 mm × 15 cm) by isocratic elution with 30% methanol in 0.03 M aqueous NaH_2PO_4 solution (pH 5) at flow rate of 1.0 ml/min. Shaded area indicates radioactivity.

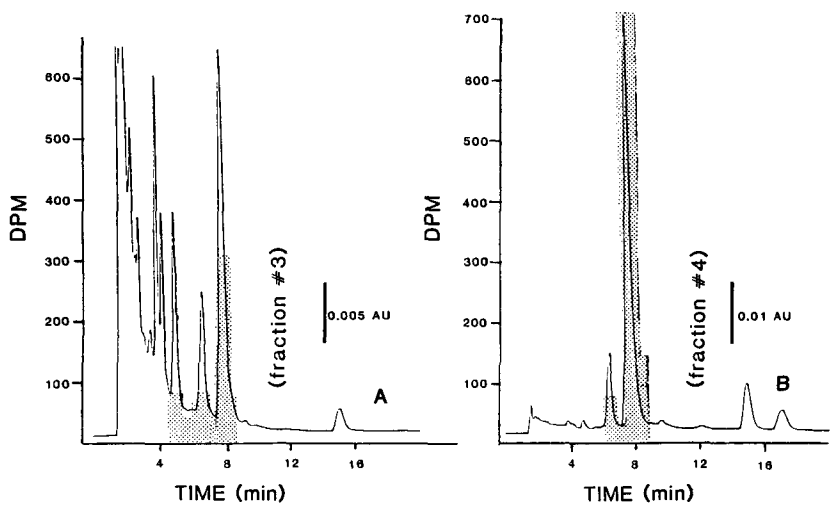

FIG. 5. Ion suppression RP-HPLC trace and radioassay analysis of partially purified ecdysteroid conjugates from 48- to 64-hr-old *Manduca* eggs, Sep-Pak fraction 3 (A), fraction 4 (B). Column conditions are identical to Fig. 4. Shaded area indicates radioactivity.

phate in the Sep-Pak separations (Table 3), analyses by ion suppression RP-HPLC and radioassay of 0.5% of Sep-Pak fraction 4 (from processing 72- to 88-hr-old eggs) gave the chromatogram in Figure 6A. This fraction contained 73% of the total amount of 26-hydroxyecdysone 26-phosphate (elution time 7.74 min) present in this age group of eggs plus the unidentified conjugate (elution time 6.54 min). RP-HPLC and radioassay of Sep-Pak fraction 3 (chromatogram not shown) also showed a small quantity of 26-hydroxyecdysone 26-phosphate and radioactive material eluting between 3 and 4 min.

Ion suppression RP-HPLC and radioassay analyses of 0.5% of fraction 5, however, showed an increase in the more polar radioactive metabolite eluting at 4.90 min (Fig. 6B). The total quantity of this metabolite is ca. 62 μg (based on comparison with peak areas of known amounts of 26-hydroxyecdysone 26-phosphate). This chromatogram also exhibited a shoulder on the back side of the peak in which the 26-hydroxyecdysone 26-phosphate elutes (7.74 min). Moreover, the total radioactivity (Table 3 and Fig. 6B) far exceeds the relative UV absorbance accounted for by 26-hydroxyecdysone 26-phosphate, suggesting that the radioactive material absorbs weakly or not at all at 254 nm.

Our results show that the ecdysteroids of ovaries from 4-day-old females and the two different age groups of eggs are present mainly as 26-hydroxyecdysone 26-phosphate. The levels of 26-hydroxyecdysone 26-phosphate were 31 μg/g for ovaries, 25 μg/g for 48- to 64-hr-old eggs and 17 μg/g for 72- to 88-hr-old eggs.

Nonecdysteroid conjugates. The butanol phase (Fig. 2) which should contain free ecdysteroids contained a large quantity of radioactivity (Table 2). This was unexpected, as our previous study had shown that no free ecdysteroids could be detected in this age group of ovaries (4). Nevertheless, the residue was applied in 10% aqueous methanol to a C_{18} Sep-Pak cartridge and eluted as shown in Figure 3 followed by two additional fractions, 5 ml each of 60% and 100% methanol. The 60% aqueous methanol fraction which should elute the free ecdysteroids (12,13) contained 76% (0.13×10^6 dpm) of the total radioactivity of the butanol phase but no ecdysteroids. All radioactivity remained at the origin after HP-TLC in the solvent system of chloroform/methanol (65:35, v/v) (14). In the solvent system of chloroform/methanol/10N NH_4OH (15:35:3.5, v/v/v), the radioactive material (>95%) from both ovaries and eggs migrated with an R_f of 0.22 (R_f of 26-hydroxyecdysone 26-phosphate = 0.17).

No radioactive free ecdysteroids were detected in the 60% aqueous methanol fraction from either ovaries or eggs, and the radioactivity appeared associated with a nonecdysteroid conjugate. This nonecdysteroid conjugate has been partially characterized as a steroid glycoside and its identification will be reported elswhere.

TABLE 3

Sep-Pak Fractionation of Material from Processing of 72- to 88-Hr-Old *M. sexta* Eggs Through XAD-2 Purification

Fraction	Eluent (% MeOH in H_2O)	Volume (ml)	Radioactivity (dpm × 10^5)
1	10	5	—
2	10	5	—
3	15	5	0.17
4	20	10	1.58
5	30	10	4.56
6	40	5	0.37
7	60	5	0.29
8	100	5	0.05

DISCUSSION

The experiments indicate that, following injection into *M. sexta* female pupae (day 16), [^{14}C]cholesterol is converted to labeled nonecdysteroid and ecdysteroid conjugates, of which the latter is mainly 26-hydroxyecdysone 26-phosphate. The ovaries and eggs contain relatively large amounts of both conjugates; however, there is a greater abundance of the nonecdysteroid conjugates also obtained from ovaries and eggs of similar ages from untreated insects. Previously, our qualitative and quantitative analyses of the ecdysteroid conjugates of tobacco hornworm ovaries and eggs were conducted on the released ecdysteroids following enzymatic hydrolysis of the conjugates (4). Our ability to analyze directly for the ecdysteroid conjugates by ion suppression RP-HPLC (5) and

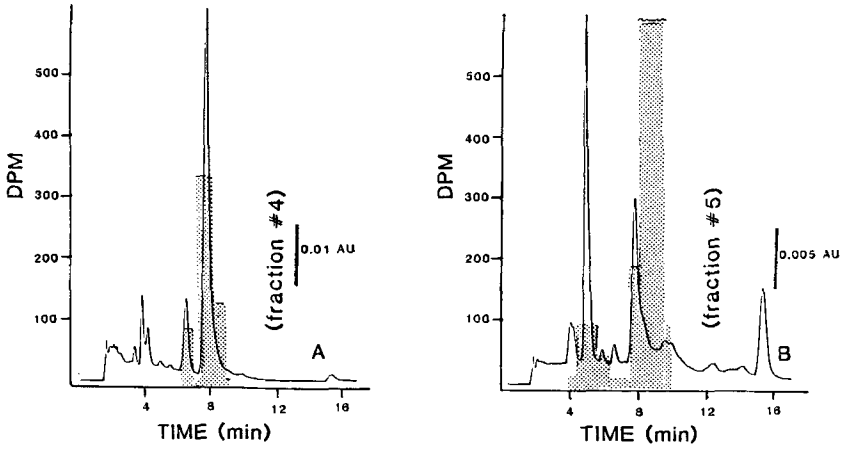

FIG. 6. Ion suppression RP-HPLC trace and radioassay analysis of partially purified ecdysteroid conjugates from 72- to 88-hr-old *Manduca* eggs, Sep-Pak (Table 3) fraction 4 (A), fraction 5 (B). Column conditions are identical to Fig. 4. Shaded area indicates radioactivity.

radioassay permits us to follow the fate of these molecules directly in developing embryos.

The RP-HPLC and radioassay analyses of the ecdysteroid conjugates of ovaries (Fig. 4) confirm our earlier finding that 26-hydroxyecdysone 26-phosphate is the major ecdysteroid conjugate of ovaries and that ecdysteroid conjugates at this developmental stage occur only as phosphates. The minor radioactive material eluting at 6.45 min is probably a phosphate conjugate of ecdysone or 26-hydroxyecdysone at a position other than C-26 or a mixture of both.

In this study we have not detected any free ecdysteroids in 48- to 64-hr or 72- to 88-hr-old eggs. There is, however, a reduction in the content of 26-hydroxyecdysone 26-phosphate from 25 μg/g in 48- to 64-hr-old eggs to 17 μg/g in 72- to 88-hr-old eggs. This reduction in the older eggs is accompanied by the appearance of a radioactive peak eluting at 4.9 min, several minutes earlier than the conjugate of 26-hydroxyecdysone. This material is not present in ovaries and is first noticeable in 48- to 64-hr-old eggs (Fig. 5A). It is not a free ecdysteroid because in the ion suppression RP-HPLC, 26-hydroxyecdysone elutes far later (ca. 16 min).

In the RP-HPLC of 72- to 88-hr-old eggs (Fig. 6B) a shoulder also occurs on the back side of the peak of 26-hydroxyecdysone 26-phosphate. This area is very highly radioactive (1200 dpm), which suggests that the material responsible for the high radioactivity is not derived from 26-hydroxyecdysone 26-phosphate nor is it an intermediate in the conversion of [^{14}C]cholesterol to ecdysteroid conjugates. Whether the shoulder described is associated with the very high radioactivity remains to be determined.

The absence of free ecdysteroids in 48- to 64-hr-old eggs was unexpected and completely different from results of our earlier studies (4,6,7). In fact we showed that in 1- to 18-hr-old eggs, more than 63% of the ecdysteroids existed in the free form. The proportion was similar in 48- to 64-hr-old eggs, though the sum of the free and conjugated ecdysteroids was far less in 48- to 64-hr-old eggs. In our search for an explanation for the different results of this and earlier studies (4,6,7), we noted that in this study we had immediately placed and stored all biological material in methanol at −20 C. Previously, this was done only with ovaries and 0- to 1-hr-old eggs (4). Older eggs routinely were placed in glass bottles and kept at −20 C until workup. Perhaps certain phosphatases of these eggs were activated by lowering the temperature and subsequent hydrolysis of the ecdysteroid conjugates caused an accumulation of free ecdysteroids. There also could have been a sudden burst in enzyme activity at a critical temperature during cooling of the eggs to −20 C. Undoubtedly, the low temperature destroyed the normal developmental processes of eggs, but did not eliminate the hydrolytic activity. Efforts are in progress to determine the exact conditions that cause this hydrolysis.

Our results of analyses of ovary ecdysteroids in this study and an earlier one (4) agree with respect to no detection of free ecdysteroids, although improvements in the method of analyses of ecdysteroid conjugates could account for the greater amount of 26-hydroxyecdysone conjugate (31 μg/g of ovaries) being found in this study as compared to 20 μg/g previously (4). We caution others to make every effort to destroy or eliminate any potential for initiation or continuation of enzyme action before storing any biological material. This is very important if analytical results or final conclusions derived from such material are to be considered valid. On the other hand, if eggs initially had been stored in methanol at −20 C, the discovery of 26-hydroxyecdysone in *M. sexta* eggs (6) would have been at least delayed.

It has been surmised that formation of conjugates allows for storage of large quantities of ecdysteroids in insect eggs, to be released during a developmental stage (embryogenesis) incapable of ecdysteroid biosynthesis (2,3,5,15,16). This study suggests that the controlled release of free ecdysteroids in *M. sexta* eggs is more limited than previously thought. Certainly no significant amounts of free ecdysteroids could be detected. The data at this stage of development are complex and not clearly understood. If free ecdysteroids are being released, they are bound immediately or are converted to other metabolites. Perhaps the ecdysteroid conjugates are being converted to more complex molecules. Presently, the fate of 26-hydroxyecdysone 26-phosphate will have to await the identification of the unknown material eluting at 4.9 min in the chromatogram of Figure 6B as well as the radioactive compounds of Sep-Pak fraction 3 (from 72- to 88-hr-old eggs) eluting between 3 and 4 min. Having [^{14}C]cholesterol converted to labeled ecdysteroid conjugates in the tobacco hornworm should make it easier to determine the fate of ecdysteroids in developing embryos of the tobacco hornworm.

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Hydroxymethylglutaryl-CoA Reductase, a Key Enzyme in Phytosterol Synthesis?

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Hydroxymethylglutaryl-CoA reductase (HMGR) regulates the synthesis of mevalonic acid (MVA), the precursor of the myriad of isoprenoid compounds functional in plant cells, with phytosterols representing one class of major importance. Recently, it has shown possible to solubilize and purify the membrane-bound enzyme from a heavy membrane fraction (P 16,000 x g) isolated from a cell-free homogenate of etiolated radish seedlings. What is presently known about the molecular and kinetic properties of radish HMGR is reported.

***Mevinolin*, a highly specific competitive inhibitor of HMGR, has been valuable as a research tool in studying the regulatory role of HMGR activity for the growth and development of intact seedlings and cell cultures. The results obtained indicate a primary effect of mevinolin on phytosterol accumulation, whereas other endproducts of the multibranched isoprenoid pathway, such as ubiquinone in the mitochondria or chlorophylls and carotenoids in the plastids, are less or not at all affected. This and other data can be interpreted to mean that the organelles are autonomous in their capacity to synthesize MVA. Since the mevinolin-induced drop in free sterol accumulation is paralleled by significant plant growth retardation, a rate-limiting role of HMGR activity for phytosterol synthesis and normal development of plants is suggested.**
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Hydroxymethylglutaryl - CoA reductase (HMGR, EC 1.1.1.34) regulates the synthesis of MVA, the specific precursor of the vast array of diverse isoprenoid compounds functional in plant biochemical processes (Fig. 1). It is widely accepted that in animal cells this enzyme catalyzes the major rate-limiting step in cholesterol synthesis. Because of the close relation between increased serum cholesterol levels and atherosclerosis in man and the possible involvement of HMGR in the regulation of the cell cycle, this enzyme has generated a great deal of interest (1–6).

First, the presence of HMGR activity in plant tissue was demonstrated indirectly through incorporation studies using $^{14}CO_2$, [2-^{14}C]acetate (7,8), [3-^{14}C]HMG (9) and [3-^{14}C]HMG-CoA (10). An early attempt to determine HMGR activity in latex of *Hevea brasiliensis* parallel to other enzymes involved in rubber biosynthesis (11) failed because the enzyme did not survive freezing (10).

Since then, the in vitro assay of the conversion of HMG-CoA to MVA using membrane preparations has been achieved in an array of different higher plants. These comprise pea seedlings (12–16), sweet potato roots (17–20), radish (21–26) and barley seedlings (23), latex of *H. brasiliensis* (27–29), spinach (30), catnip leaf tissue (31), carrot cell cultures (32), soybean tissue and cell cultures (33) and pepper chromoplasts (34); this list certainly will be expanded in the near future. The experimental results of most of these publications deal with membrane fractions of more or less defined purity. From some of these studies, it appears that plant HMGR is an enzyme regulated by phytochrome (14,16,23,25), herbicides (21), phytohormones (14,15,25), feedback mechanisms (15) and endogenous protein factors (28).

To investigate the regulation of plant HMGR in greater detail and to learn more about molecular and kinetic properties of the enzyme, solubilization of the enzyme from the membrane and its purification is required, a problem recently solved by using radish seedlings as an enzyme source (24,26).

Although the synthesis of sterols or of other isoprenoids (see Fig. 1) strictly correlated to HMGR activity is frequently postulated, hard data yielding clear evidence are scarce in the literature. Mevinolin, an antibiotic isolated from *Aspergillus terreus* (35) and reported to be a highly specific inhibitor of mammalian (35) and plant (36,37) HMGR, was used as a molecular probe to study the role

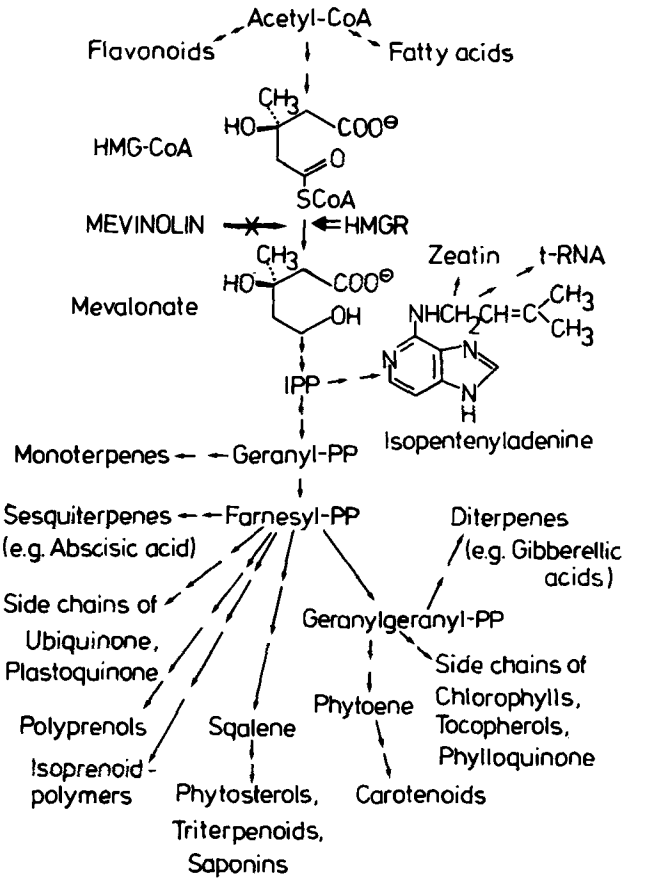

FIG. 1. Role of HMGR-directed synthesis of MVA and its flow into various classes of isoprenoid compounds in plant cells. Inhibition of HMGR by mevinolin is indicated.

of this enzyme in the regulation of plant growth and development. Mevinolin and its analogue, compactin (38), have been helpful tools in numerous investigations to determine the features HMGR regulation and isoprenoid synthesis in mammals (4–6,39,40); some of these features might be useful in explaining the physiological control of HMGR activity in plants. For example, compactin was a prerequisite for the development of a line of Chinese hamster ovary (CHO) cells, designated as UT-1 cells, in which the HMGR gene was amplified 15-fold. The 100-fold increase of HMGR mRNA in UT-1 cells allowed the cloning and sequencing of several overlapping cDNAs spanning the entire hamster HMGR gene (ref. [41] and literature cited therein).

It was revealed that mevinolin applied to radish and wheat seedlings did significantly inhibit root growth already at concentrations between 2.5 x 10^{-8} and 2.5 x 10^{-7}M (36). In the root and hypocotyl tissue of radish seedlings, mevinolin treatment appeared primarily to affect the accumulation of sterols (43,44); some central data will be discussed in this contribution. In addition, with the help of mevinolin, it is possible to study the compartmentation of isoprenoid synthesis in intact plants (42–44), thereby yielding further support for the presence of HMGR in different organelles of the plant cell (cf. [13,16–18,21, 22,25,31]) The occurrence of HMGR in compartments other than the endoplasmatic reticulum is a matter of current debate (30,45,46).

MATERIALS AND METHODS

The commercial sources of chemicals have been listed elsewhere (23,47). Mevinolin was provided by A.W. Alberts, Merck Sharp & Dohme Res. Labs. (Rahway, New Jersey) and was converted to its water-soluble sodium salt as described (48).

Four-day-old etiolated radish seedlings were used for the solubilization and purification of HMGR. The protocol developed for the isolation of membrane pellets (25,37), the detergent solubilization of radish HMGR from a heavy membrane fraction (P 16,000 x g) and its subsequent purification by the aid of column chromatography on DEAE-Sephadex, Blue Dextran-agarose and HMG-CoA-hexane-agarose are described elsewhere (47). For the measurement of kinetic parameters, purified enzyme was used, which was eluted from the HMG-CoA-hexane-agarose column at 500 to 600 mM KCl in the presence of ca. 2% detergent (Brij W-1) and stabilized by addition of glycerol and dithioerythritol (47).

The composition of the radioactive assay system for radish HMGR activity resembled that described elsewhere (25,37), but the assay volume was reduced to 60 μl. Where possible, an NADPH regenerating system was used. Unreacted substrate was separated from doubly labeled mevalonolactone by TLC on silica gel (5) and the samples were assayed for radioactivity. Kinetic data were plotted as described previously (25).

Protein was determined as described (47).

Conditions for the cultivation of radish seedlings in the light to study mevinolin-induced effects on growth and prenyllipid accumulation have been described in detail (43,44).

To study the incorporation of ^{14}C-acetate and ^{3}H-MVA into digitonine-precipitable sterols, radish seedlings were grown for six days on water (control) and on water supplemented with bentazone, DCMU, amitrol (10^{-4} M each) and on SAN 6706 (10^{-5} M) in white light (Fluora 65 W, 2000 lux, 8 W m^{-2}). Two hundred seedlings per condition were cut and the remainder, consisting of hypocotyls and cotyledons, were incubated in the light for another 24 hr with 1 mCi [2-^{14}C]acetate (s.a. 40-60 mCi/mmol) and 1 mCi [2-^{3}H]MVA (s.a. 100-500 mCi/mmol). Lipids were extracted with acetone and transferred into petrolether. Free sterols from 50% of the various extracts were separated from other lipids and pigments by TLC on silica gel, with $CHCl_3$ as the solvent system. Sterol bands were eluted with 5 ml acetone, and the solutions were supplied with 0.2 ml of 1% cholesterol in 95% EtOH as a carrier, followed by addition of 2 ml of 0.5% digitonine in 50% EtOH. After two hr at 6 C the fluffy precipitates were sedimented for 10 min at 5,000 x g and washed four times with 2 ml EtOH/acetone (1:1, v/v) followed by 2 ml pure acetone. The precipitates were dried and assayed for radioactivity by liquid scintillation counting.

RESULTS AND DISCUSSION

Solubilization and purification of radish HMGR. Several detergents were evaluated for their abilities to release the enzyme from the membrane without interference with the assay system for HMGR activity (47). Brij W-1 substantially activated HMGR activity of a membrane preparation sedimented at 16,000 x g, while sodium deoxycholate strongly inhibited the enzyme (47). A combination of the freeze-thawing procedure commonly used to solubilize HMGR from rat liver microsomes (49–51) and a detergent extraction with Brij W-1 was the most effective.

Usually, the incubation at 37 C of membranes in the presence of Brij W-1, glycerol and dithioerythritol resulted in an increase of measurable HMGR activity (26,47). Because the release of HMGR from rat liver microsomes by the usual freeze-thawing procedure requires the activity of lysosomal membrane-bound proteases (52), the role of proteolysis in the freeze-thaw steps of the solubilization process was checked. There was essentially no difference in solubilization behavior in the absence or presence of protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) and leupeptin (Table 1). Thus it seems reasonable to assume that the solubilized preparation obtained in this way is identical with the native membrane-bound enzyme. The solubilization process usually released 90% of the total activity (26).

The concentration and partial purification of HMGR then was achieved by ammonium sulphate precipitation. Increasingly purified HMGR was inhibited to about 96% at 10^{-6} M mevinolin, which can be taken as proof that HMGR was measured exclusively throughout the experiments (25). Both the solubilized HMGR preparation and the redissolved ammonium sulphate precipitate were stable when stored at −20 C in the presence of high concentrations of dithioerythritol, and even repeated thawing and refreezing did not substantially decrease the apparent enzyme activity.

The bulk quantity of protein and contaminating caro-

TABLE 1

Effectivity of Radish HMGR Solubilization in the Presence of Protease Inhibitors

Treatment/fraction	Specific activity (pmoles/min/mg)	Recovery of initial activity
P 16,000[a]	331.2	100%
P 16,000 (control)		
+Glycerol	314.8	95%
Solubilized fraction	491.9	148.5
P 16,000[a]	384.7	100%
P 16,000 (PMSF)[b]		
+Glycerol	379.7	98.7%
Solubilized fraction	588.3	152.9
P 16,000[a]	277.5	100%
P 16,000 (leupeptin)[c]		
+Glycerol	351.6	126.7%
Solubilized fraction	644.9	232.4

[a]In the presence of 2% Brij W-1.

[b]5 mM.

[c]10 μM.

tenoids was removed by chromatography on DEAE-Sephadex (24,26,47). The time required to load and elute the enzyme from the column was experimentally variable, the effect of such a high amount of detergent. Prolonged time to elute the enzyme could result in substantial inactivation, possibly due to conformational changes under those chromatographic conditions. Further purification of radish HMGR was achieved through chromatography on Blue Dextran-agarose and HMG-CoA-hexane-agarose. The salt concentrations needed to elute the enzyme activity from these affinity materials were similar to those reported for the rat liver HMGR (51).

The results obtained with sucrose density centrifugation in the presence of Triton X-100 suggest an apparent molecular weight of 180 kDa for purified radish HMGR with subunits of 45 kDa as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (47). The presence of at least trace amounts of detergent was mandatory to maintain detergent-solubilized and purified active HMGR, especially under high pressure conditions such as sucrose density centrifugation (47). Since reference proteins were incubated in salt-detergent solution and centrifuged under identical conditions, it was assumed that these proteins were complexed comparably by detergent and that the calculated value for radish HMGR truly reflects its real molecular weight (47), not excluding that the enzyme is forming a dimer under such conditions. Therefore, considering the early controversies surrounding the molecular weight of the rat liver enzyme (5), final assessment of the plant enzyme awaits application of molecular biology techniques (41).

In addition to high concentrations of glycerol, an increased salt content was beneficial for the activity as well as the stability of purified radish HMGR, presumably through the enhancement of hydrophobic interaction. Under such conditions, the enzyme activity remained stable over 30 min at 67.5 C (47). The increase of apparent enzyme activity throughout the first solubilization steps possibly was caused by a combination of detergent effects and the reduction of essential thiol groups in the presence of dithioerythritol (47). Such a thiol dependency of HMGR activity seems common to all eukaryotic HMGRs (5). The possible involvement of free thiol groups in the regulation of HMGR has been suggested for yeast (53), plant (22) and mammalian enzymes (54–57). Recently the occurrence of enzymic systems in radish, such as thioredoxin f being involved in the regulation of enzymes through reduction of functional thiol groups, has been reported (58).

Determination of kinetic properties. When HMG-CoA concentration was varied and that of NADPH was kept constant and vice versa, intersecting patterns were obtained in double-reciprocal plots (26). Concentrations above 150 μM NADPH caused substrate inhibition at low HMG-CoA concentrations. At higher fixed concentrations of NADPH with the concentration of HMG-CoA being varied, a change to irregular intersecting patterns was observed in the double-reciprocal plot (47).

Additional insight into the reaction mechanism of radish HMGR was obtained by study of the inhibition by-products NADP, HS-CoA and MVA. NADP was revealed to act as a linear competitive inhibitor with respect to NADPH (Fig. 2a), having a K_i of 65.6 μM, and uncompetitive with respect to HMG-CoA (Fig. 2b). At concentrations higher than 1.2 mM, the inhibition by NADP against HMG-CoA changed to a noncompetitive pattern. The K_{ii}-value as determined from the linear part of a secondary plot (intercepts against inhibitor concentration) was 1.21 mM. When NADPH was varied at constant initial concentrations of HMG-CoA (Fig. 2C), HS-CoA acted as a noncompetitive inhibitor with K_i and K_{ii}-values of 921 and 899 μM, respectively. Secondary plots of intercepts or slopes against inhibitor concentration were linear, although there seemed to be a slight upward curve in the latter case, indicating a possible parabolic dependency. However, HS-CoA was linearly competitive with respect to HMG-CoA (Fig. 2d). Although MVA was converted into the free acid, high concentrations were necessary to inhibit the enzyme. The inhibition was linearly competitive with respect to HMG-CoA (Fig. 2f), with a K_i of 990 μM, and noncompetitive with respect to NADPH (Fig. 2e). In the latter case, while a replot of intercepts against MVA concentration was linear, yielding a K_{ii}-value of 4.1 mM, a secondary plot of slopes against MVA concentration demonstrated the calculated points lay on a parabolic curve. The K_i value was calculated to be 2.69 mM. The data of the product inhibition study are summarized in Table 2.

The K_m value of 1.5 μM with respect to (S)-HMG-CoA (26) is in the range of that reported for purified mammalian and yeast HMGRs (5). Quite different values exist in the literature for HMGRs from different plant sources (Table 3). These values were determined by the use of crude membrane fractions and not in purified enzyme where no side reactions occur. Interesting is the difference of the K_m values for the cytosolic and the plastidic HMGR assayed in preparations of pea seedlings, which led to the assumption that isoenzymic forms of HMGR are present in plant cells (16). A break in the double reciprocal plot when a particulate preparation from sweet potato containing mitochondria and microsomes was used (17) may have been caused by the presence of isoenzymes having

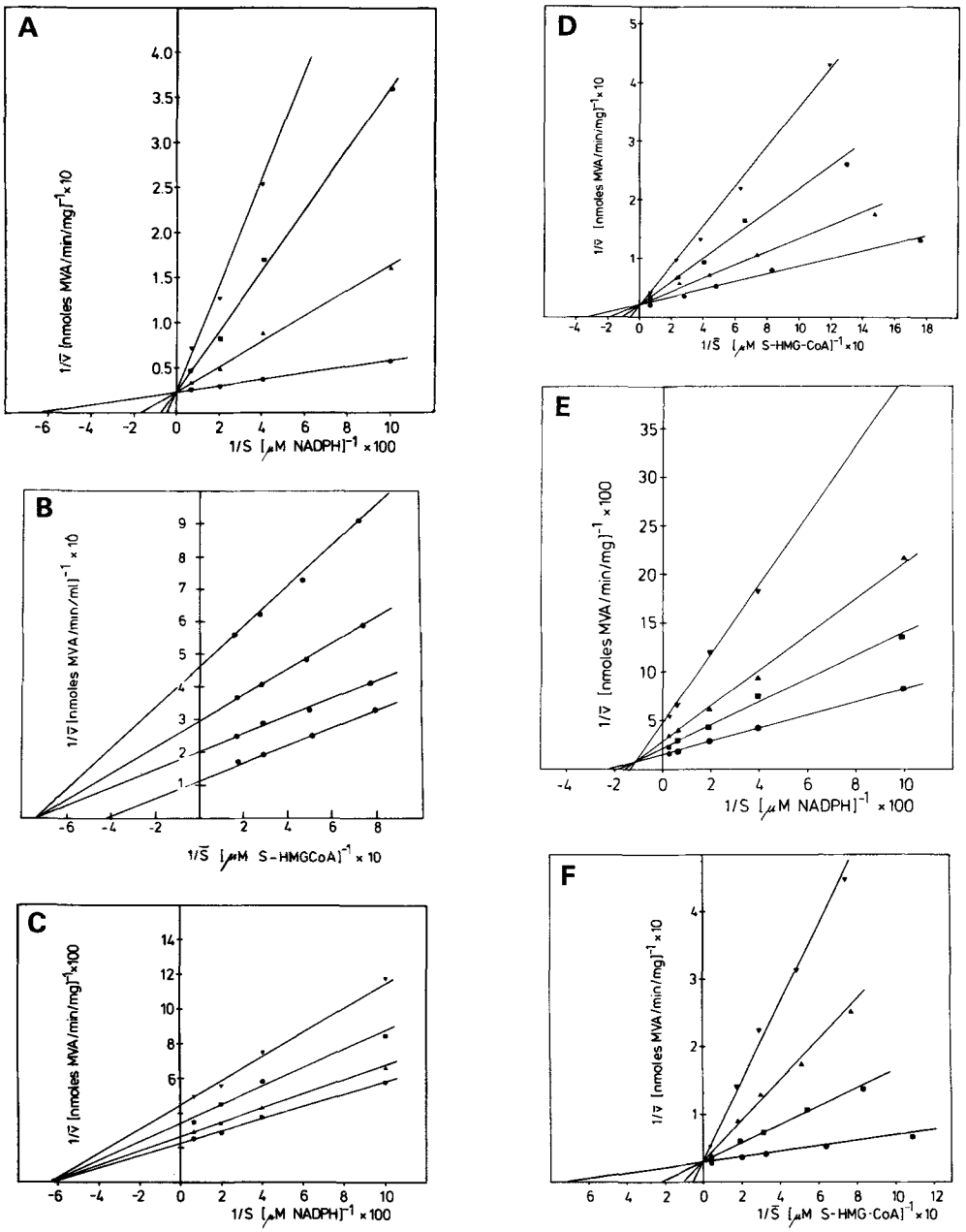

FIG. 2. Product inhibition pattern of purified radish HMGR. (A) NADP with respect to NADPH. Initial concentrations of (S)-HMG-CoA: 15 μM (= 10 × K_m); inhibitor concentrations: 0, 0.2, 0.5, 1.0 mM. (B) NADP with respect to HMG-CoA. Initial concentrations of NADPH: 300 μM; inhibitor concentrations: 0, 1, 2, 5 mM. (C) HS-CoA with respect to NADPH. Initial concentrations of (S)-HMG-CoA: 15 μM; inhibitor concentrations: 0, 0.2, 0.4, 0.8 mM. (D) HS-CoA with respect to HMG-CoA. Initial concentration of NADPH: 300 μM; inhibitor concentrations: 0,0.2, 0.4, 0.8 mM. (E) MVA with respect to NADPH. Initial concentration of (S)-HMG-CoA: 18.9 μM; inhibitor concentrations: 0, 2, 4, 8 mM. (F) MVA with respect to HMG-CoA. Initial concentration of NADPH: 300 μM; inhibitor concentrations: 0, 2, 4, 8 mM.

different substrate affinities.

The value of 27 μM for the K_m against NADPH as evaluated with purified radish HMGR (26) is slightly lower, but in the same order of magnitude as reported for purified mammalian and yeast HMGRs (5). The converging patterns in double-reciprocal plots (26) when both substrates were varied indicated a sequential mechanism of binding of the substrates to the enzyme (59). Substrate inhibition by NADPH, and the complete release of this effect when HMG-CoA concentrations were increased, was explained by postulating HMG-CoA as the first molecule to bind to the enzyme, followed by NADPH (26,47). Since NADPH binds twice to the enzyme through the course of HMG-CoA reduction, straight patterns when the first substrate HMG-CoA is kept constant while the concentration of the second substrate NADPH is varied indicate that an irreversible step occurs between the points of addition (60). This implies that NADP leaves before

TABLE 2

Kinetic Constants of the HMGR Reaction Obtained from Product Inhibition Studies

Inhibitor	Variable substrate	Inhibition type	K_i (μM)	K_{ii} (μM)
NADP	HMG-CoA[a]	Uncompetitive[d]	—	1210
	NADPH[b]	Competitive	65.5	—
HS-CoA	HMG-CoA[a]	Competitive	204	—
	NADPH[b]	Noncompetitive	921	899
MVA	HMG-CoA[a]	Competitive	990	—
	NADPH[c]	Noncompetitive[e]	2690	4100

[a]Fixed concentrations of NADPH: 300 μM.

[b]S_o of [S]-HMG-CoA: 15 μM.

[c]S_o of [S]-HMG-CoA: 18.9 μM.

[d]At concentrations higher than 1.2 mM NADP tends to act as a noncompetitive inhibitor with respect to HMG-CoA.

[e]Parabolic dependency of slopes against MVA concentration in the secondary plot.

TABLE 3

K_m-Values of Membrane-Bound Plant HMGRs from Various Sources with Respect to [S]-HMG-CoA

Plant source	Membrane fraction	K_m-value [μM]	Reference
Pea seedlings	Microsomes	80	16
Pea seedlings	Plastids	0.385	16
Sweet potato	Mitochondria pelleted at 105,000 x g	6.5[a]	17
	Microsomes pelleted at 105,000 x g	21.0[b]	17
Radish seedlings	Microsomes (P 105,000 x g)	2.0-2.9[c]	25
Radish seedlings	Heavy membranes (P 16,000 x g)	2.2-7.3[c]	25
Hevea brasiliensis	Latex, 103,000 x g Pellet	28	29

[a]Below 50 μM [S]-HMG-CoA.

[b]Above 50 μM [S]-HMG-CoA.

[c]Varies with treatment of plants.

TABLE 4

Effect of Some Herbicides on Apparent Microsome-Bound Radish HMGR and on [2-^{14}C]Acetate[a] and [2-^{3}H]Mevalonate[a] Incorporation into Digitonin-Precipitable Sterols

Treatment[a]	(Rel) HMGR activity (nmol/h/mg)	^{3}H dpm x 10^{-6}	^{14}C dpm x 10^{-5}	Ratio ^{3}H:^{14}C
6 d Fluora (H_2O control)	0.74 (100%)	2.936	3.798	7.73
10^{-5} M SAN 6706	0.73 (98.6%)	1.812	1.384	13.09
10^{-4} M Amitrol	0.17 (23.0%)	2.538	3.167	8.01
10^{-4} M Bentazon	0.20 (27.0%)	1.982	1.259	15.74
10^{-4} M DCMU	0.13 (17.6%)	2.809	1.186	23.69

[a]1 mCi each; 24 hr incubation under Fluora white light; excised seedlings (200 per condition).

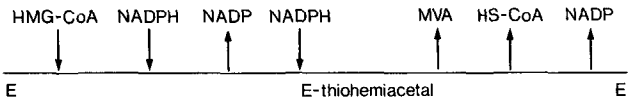

FIG. 3. Proposed reaction pathway of radish HMGR.

the next NADPH can bind. From the product inhibition pattern and the estimates of the inhibition constants, it seems plausible to assume a reaction sequence of product release with MVA leaving the enzyme before HS-CoA and NADP probably the last product dissociating. The reaction pathway therefore could be designated as bi-uni-uni-ter-ping-pong (Fig. 3).

Correlation between microsomal HMGR activity and incorporation of acetate and MVA into sterols. It was shown earlier that treatment of light-grown radish seedlings with some selected herbicides, affecting either the photosynthetic electron transport at the site of photosystem II (bentazon, DCMU) or the synthesis of photosynthetic pigments (amitrol, SAN 6706), results in changes of apparent HMGR activity of the heavy membrane and the microsomal fractions (21). The strongly increased mol-specific incorporation of acetate into plastidic pigments of SAN 6706-treated seedlings coincided with the increase in the apparent HMGR activity connected with the so-called organelle fraction (21). None of these compounds up to millimolar concentrations had any effect on the in vitro activity of HMGR. Some correlation between apparent microsomal HMGR activity and the incorporation of [^{14}C]acetate and [^{3}H]MVA is shown in Table 4. An increase in the ^{3}Y:^{14}C ratio indicates discrimination of acetate with respect to MVA, thereby pointing to a regulated step between acetyl-CoA and MVA. Decrease of MVA incorporation indicates inhibition of substrate flux at a site positioned later in the pathway. Though MVA incorporation into digitonine-precipitable sterols appears to be affected by the herbicides, the effects on acetate incorporation are more obvious. However, only in the case of treatment with the photosystem II herbicides does the relative discrimination of acetate correlate with the drastically decreased HMGR activity.

In vivo inhibition studies using mevinolin. Inhibition of an enzyme at least close to coarse control of substrate flux into essential endproducts should result in, besides changes in the biochemistry, clear morphological and developmental responses. Mevinolin, the highly specific inhibitor of HMGR, proved extremely helpful in defining the role of MVA synthesis and the flow of this compound to the major isoprenoid entities in the plant cell (cf. Fig. 1). The existence of mevinolin and of related antibiotics directed solely against HMGR emphasizes the central position of this enzyme in the cellular metabolism. From the occurrence and natural localization of the various strains of ascomycetes producing these compounds in the upper rhizosphere, it can be suspected that the biological function of these inhibitors, if any, is to be directed against plants (roots) and microbes competing for the same substrates and minerals in soil. In view of this hypothesis it was not surprising that mevinolin was revealed to cause plant growth retardation (36,42,43). However, even at very high concentrations mevinolin did not prevent germination of radish seeds, thereby indicating that during this

early part of the life cycle, the MVA-derived storage products in the seeds permit the development of the embryo as well as the initial formation of roots and hypocotyls (42,43). The predominant effect of mevinolin on sterol accumulation in intact seedlings (43,44) and cell suspension cultures (61) points to the rate of MVA synthesis being limiting for these isoprenoids as demonstrated for animal cells and yeast (1–6). This seems to be true also for ubiquinone, but apparently not for other compounds investigated so far (44,61). Even though there is conclusive evidence to date for a MVA-synthesizing mechanism in plant mitochondria (13,18,22), mevinolin exerts some, though a limited, effect on ubiquinone synthesis (44,61). This can be explained if mevinolin can penetrate to some extent the mitochondrial envelope. The mevinolin-induced shift in the ubiquinone pattern toward monologues containing shorter isoprenoid side-chains (44,61) might reflect the ability of mitochondria to adjust the usage of isopentenyl pyrophosphate (IPP) units to the available substrate inside or outside of the organelle. This might include the possibility that mitochondrial MVA utilization—and thereby ubiquinone synthesis—might be linked to cytoplasmic MVA production, depending on the need of the organelle for additional IPP units (cf. [46]). On the other hand, the inability of mevinolin at physiologically reasonable concentrations to prevent the synthesis and accumulation of plastidic chlorophylls, carotenoids (42,43) and plastidic prenylquinones such as plastoquinone, phylloquinone and α-tocopherol (44), can be explained easily by a nearly complete impermeability of the plastidic envelope toward the inhibitor. The recent hypothesis (30) that IPP is synthesized exclusively from acetyl-CoA by cytoplasmic enzymes and then is transferred into the organelles to serve as the basic building block of the various prenyllipids would require that different competing prenyltransferases and translocators utilize the IPP units very differently. In particular, if it is possible to virtually knock out de novo sterol synthesis through inhibition of microsomal HMGR activity, these sequential enzymic systems must have substrate affinities that differ by orders of magnitude in order to maintain an unchanged substrate flux into one particular endproduct. Although there are little conclusive data on the substrate affinities and specificities of those enzymes or translocators, the even enhanced accumulation of plastidic prenyllipids in radish seedlings treated with low concentrations of mevinolin (44) can hardly be explained in this way. Thus, it seems more reasonable to assume plastids possess their own HMGR; independent data from several groups support this view (13,16,31,34). HMGR activity usually is high in rapidly developing tissue such as young seedlings (12,22), where a high synthetic or turnover rate for isoprenoids can be observed. However, for the preparation of "pure" chloroplasts more mature leaves frequently are used. If the construction of the photosynthetic apparatus is completed, only small amounts of newly synthesized MVA may be needed to maintain, for example, a basic turnover rate. Thus, only a very low HMGR activity can be expected in such organelles, possibly even too low to be measurable.

The apparent effect of mevinolin on the accumulation of plastidic prenyllipids in cell cultures of *Silybum marianum* (61), even though the inhibition was much less dramatic than that of sterol synthesis, was due not only to an easy penetration of the envelope but also a general inhibition of cell growth and division as indicated by the decreased biomass production (protein content, dry weight, etc.) (61).

More secondary responses to mevinolin treatment point to induced shifts in the developmental program of young plants (43,44) which might be due to changes in the phytohormone balance as discussed elsewhere (43,62).

The experimental work discussed here is evidence for the importance of a functioning biosynthetic pathway that directs HMG-CoA to MVA to IPP and to all various isoprenoid compounds depending upon the needs of the plant cell for these materials. The regulation at this particular step, especially concerning sterol accumulation, might be thought of as a coarse control, not ruling out that further enzymes of the pathway might be tuned for fine control. The availability of a purified enzyme preparation opens new avenues to studying the regulation of plant HMGR at the molecular level.

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An Essential Fungal Growth Factor Derived from Ergosterol: A New End Product of Sterol Biosynthesis in Fungi?

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Concentrations of ergosterol as low as 1.2 mM are effective in satisfying the essential high specificity microrequirement for C5,6 unsaturated sterol in yeast. In a sterol auxotroph supplemented with saturated sterol (cholestanol), aqueous extracts of yeast provide a growth factor that eliminates the ergosterol microrequirement. The factor is easily differentiated from ergosterol by solubility, thermostability, and thin layer and liquid chromatography and is functional at levels equivalent to those of vitamins, hormones and pheromones.
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Yeast sterol auxotrophs are unable to grow on cholestanol (saturated cholesterol) (12 μM) unless a minute quantity (25 mM) of a C-5,6 unsaturated sterol such as ergosterol is available (1,2). We have designated this phenomenon the "sparking" of growth, in which cholestanol fulfills an overall membrane sterol requirement and ergosterol satisfies a high specificity microrequirement. Under sparking conditions, ergosterol appears to function as a signal molecule for the initiation of growth. However, ergosterol concentrations previously observed as necessary for sparking may have been higher than the absolute amount required to satisfy the sparking function(s) for a number of reasons: (i) the Km of ergosterol uptake is 25 μM so uptake efficiency is low at 25 nM; (ii) sterol solubility properties and the higher level of cholestanol (500×) in the medium may diminish competitively the availability of ergosterol, and (iii) because of its hydrophobic nature, much of the ergosterol may be sequestered in cell membranes during transport.

It is equally possible that ergosterol per se may not be the sparking component but is metabolized to another form or elicits the synthesis of another compound which is the active sparking substance. We present here data indicating that the concentration of ergosterol required for sparking is substantially less than previously reported and that ergosterol can be replaced by a compound with distinctly different properties from ergosterol.

MATERIALS AND METHODS

Growth of strains for bioassay. For sparking analyses, the yeast auxotroph RD5-R (a, *heml, erg3, erg7*) was depleted of C-5,6 unsaturated sterol by growing the organism on cholestanol as described (1). In the bioassay of sparking concentrations of ergosterol or sparking ergosterol replacement factor (SERF), the sterol-depleted cells were preincubated with varying concentrations of ergosterol or SERF prior to cholestanol addition.

To investigate the possibility that low levels of ergosterol were localized or compartmentalized at specific site(s) in the cell, RD5-R was preincubated with 1.2 nM [^{3}H] ergosterol for 30 hr prior to addition of cholestanol (12 μM). These cells were harvested when they reached stationary phase, spheroplasts were prepared and the cellular components (floating lipid layer, plasma membrane, mitochondria and cell pellet) were fractionated on sucrose gradients (3).

SERF isolation, extraction and characterization. A sterol wildtype, X2180-1A, was cultured in minimal medium, harvested in late log phase, washed with distilled water and lyophilized. The dried cells (9 g) were powdered and extracted three times with stirring at room temperature with 100 ml methanol/water (9:1, v/v). The methanol/water extract was dried by rotoevaporation at 40 C and the evaporation vessel was washed sequentially with ethanol (I), methanol (II) and water (III). The extracts (I-III) were clarified through C-18 reverse phase Sep-Pak cartridges (Waters, Milford, Massachusetts) and tested for the ability to replace ergosterol under sparking conditions. The aqueous solution (III) alone contained material that could fulfill that function. To ensure that the aqueous extract (III) was not contaminated with ergosterol, it was chromatographed by high performance liquid chromatography (HPLC) using a solvent system that separates free sterols (4). Eluent from this system was monitored for UV absorption at 210 nm. Fractions were collected at five min intervals, resuspended in 1 ml distilled water, filter-sterilized and assayed for their sparking ability. Additional HPLC solvent systems were utilized to purify SERF from all material absorbing ultraviolet light at 210 nm (Figs. 2B and 2C).

Since large batch cultures of X2180-1A were required for SERF extraction, we used a readily available supply of active dry yeast. SERF was extracted from 48 g of commercial dry bakers' yeast (Fleischmann's) using the procedure described above. These extractions yielded a pale yellow liquid which became extremely viscous upon rotoevaporation of the aqueous phase. Prior to purification by HPLC, the extract was spotted on preparative thin layer chromatography (TLC) plates and developed using benzene ethyl acetate (5:1, v/v) (5). This solvent system is known to separate sterols from yeast polysaccharides which solubilize sterols (5,6).

Tests for contaminating sterols in the aqueous extract (III) included methanolic pyrogallol saponification (5) and treatment with dimethyl sulfoxide (Me_2SO) (7). Both saponified and Me_2SO SERF samples were extracted into n-hexane and evaporated to dryness with N_2 and gentle heating. Gas liquid chromatography (GLC) (SE-30, 235 C, with a nitrogen carrier gas flow rate of 20 ml min^{-1}) and HPLC analyses, using the solvent system as described (Fig. 2A), were performed using an analytical 5 μ ODS

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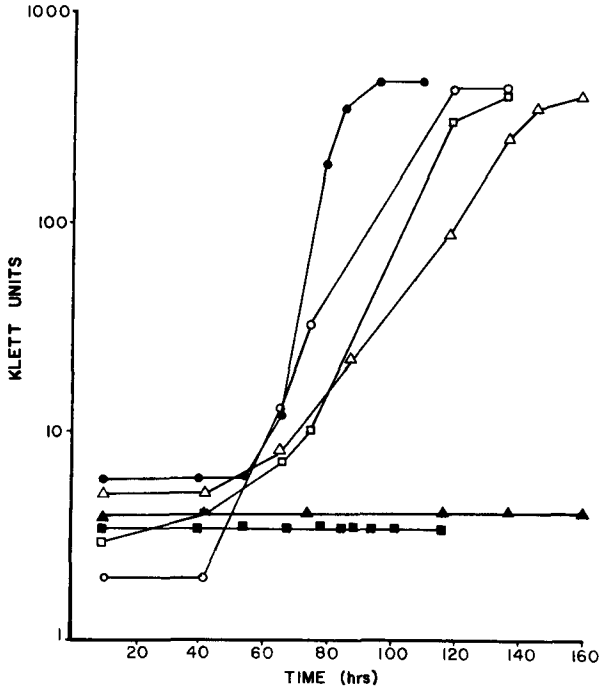

FIG. 1. Cholestanol-cycled RD5-R cells were inoculated into medium containing 25 nM (○), 2.5 nM (□), 1.2 nM (△) and 0.25 nM (▲) ergosterol and incubated for 30 hr. Then 25 μM cholestanol was added to each culture and growth was monitored turbidimetrically with a Klett-Summerson photoelectric colorimeter (one Klett unit is ca. 1.7×10^5 cells/ml^{-1}). All cultures were incubated at 28 C. Also shown is growth of cholestanol-cycled RD5-R cells inoculated into medium containing cholestanol and SERF (●). A control culture of cholestanol-cycled cells inoculated into medium containing cholestanol alone also is presented (■).

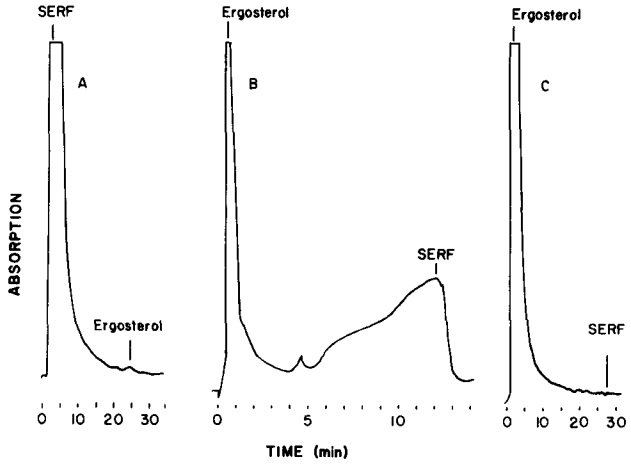

FIG. 2. HPLC scans of each system used with arrows indicating the elution time for SERF and ergosterol. (A) 250 μl of the aqueous solution was chromatographed on a semipreparative 5μ ultrasphere column (10.0 mm i.d. × 150 mm, Altex) under isocratic conditions with a mobile phase of methanol/100% ethanol/water (85:10:5, v/v/v) at a flow rate of 6 ml/min^{-1}. (B) The first five min fraction from system A was resuspended in 1 ml distilled water and 250 μl was passed through an analytical 5μ ultrasphere column (4.0 mm i.d. × 250 mm, Altex) using a mobile phase of 100% ethanol for 20 min at a flow rate of 1.5 ml/min^{-1}, then a gradient of methanol from 5–25% over 20 min at a flow rate of 1.0 ml/min^{-1}. (C) The fraction which eluted from system B in 20–25% methanol was resuspended in 250 μl distilled water and passed through the same column under isocratic conditions using a mobile phase of methanol/100% ethanol (15:85, v/v) at a flow rate of 1.0 ml/min^{-1}.

ultrasphere (4 mm × 250 mm, Altex, Berkeley, California) column (methanol/ethanol/water, 85:10:5, v/v/v at a flow rate of 1.5 ml/min^{-1}).

Analyses for phosphate (8), protein (BioRad) (9) using bovine serum albumin as a standard and total carbohydrate (10) were conducted.

RESULTS AND DISCUSSION

Growth of the sterol auxotroph RD5-R on cholestanol appeared to have a threshold concentration of 1.2 nM ergosterol (Fig. 1), a 20-fold reduction over previously observed amounts. This does not preclude the possibility that ergosterol concentrations less than 1.2 nM may be effective, but growth could be prevented by the conditions previously described. To investigate the possibility that ergosterol is localized or compartmentalized at specific site(s) in the cell, RD5-R was preincubated with 1.2 nM [^{3}H]ergosterol (1.44×10^8 dpm/μmol^{-1}). Our results indicated no enrichment of radioactive ergosterol in any of the cellular fractions when compared to gradients of cellular fractions from a culture of RD5-R grown on [^{14}C]-cholestanol (data not shown). From these results, it is clear that yeast has a specific growth requirement for a C-5,6 unsaturated sterol.

We tested aqueous extracts (I-III) from X2180-1A to determine if ergosterol was metabolized to another form or if it caused the synthesis of another compound which was the active molecule involved in fulfilling the sparking requirement. During this portion of the study, it was found that only fraction III (SERF) could fulfill the role played by sparking ergosterol. Further purification of fraction III by HPLC revealed that SERF (Fig. 2A) eluted in the solvent front while ergosterol eluted much later. In all cases, SERF was assayed by demonstrating the ability to replace ergosterol for sparking growth of RD5-R on cholestanol (Fig. 1). Additional HPLC systems were utilized to separated SERF from all material that absorbed ultraviolet light at 210 nm (Figs. 2B and 2C). Purified SERF remained insoluble in 95% ethanol and methanol but was very soluble in water.

In order to obtain larger quantities of SERF, the same procedures for extracting X2180-1A were used to extract commercial dry yeast. The aqueous fraction, after passage through the C-18 Sep-Pak column and the HPLC solvent system described in Fig. 2A, was analyzed for phosphate, protein and total carbohydrate. SERF from X2180-1A was analyzed in parallel. The results were negative for phosphate and protein and positive for total carbohydrate in the extracts for both sources.

Bioassays of SERF from commercial yeast and X2180-1A (Fig. 3) indicated SERF extracted from commercial yeast was as effective as SERF extracted from X2180-1A in satisfying the sparking ergosterol microrequirement. There did not appear to be a significant difference in growth rate or yield between heat-treated (autoclaved 20 min) and non-heat-treated SERF (Fig. 3).

As it is possible to isolate a water-soluble form of ergosterol (5), it was necessary to ensure that SERF extracts from yeast did not contain sterol enveloped in polysaccharide. Analysis of SERF extracts from dry yeast (after TLC in benzene/ethyl acetate and HPLC purifica-

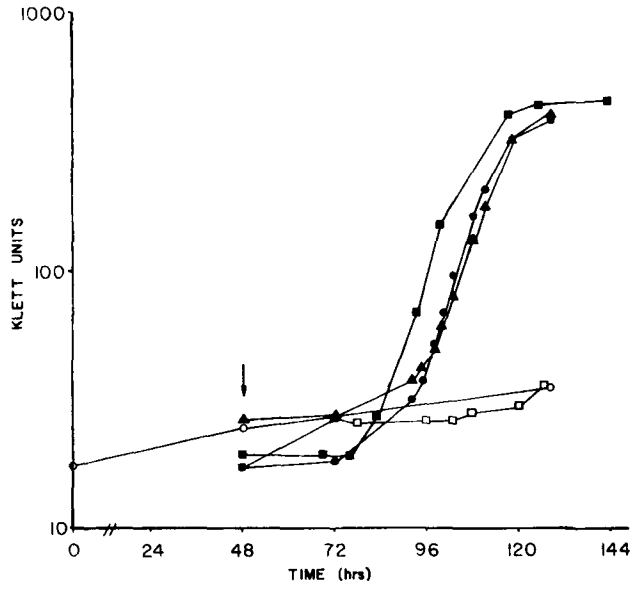

FIG. 3. Comparison of SERF from X2180-1A and commercial dry bakers' yeast. Cholestanol-cycled cells were inoculated into minimal medium containing 5 $\mu g/ml^{-1}$ cholestanol and 100 μl of the extract added. A cholestanol control culture (no SERF added) (□) and the original inoculum (no SERF added) (○) are shown for comparison. SERF from X2180-1A (●), SERF from Fleischmann's yeast (△) and autoclaved SERF from Fleischmann's yeast (■) were added to cycled RD5-R cells. The arrow indicates the time that the original inoculum of RD5-R cells was subinoculated into fresh medium containing the above components.

tion using the solvent system described in Fig. 2A) by GLC and HPLC after either Me_2SO treatment or methanolic pyrogallol saponification revealed no chromatographic peaks identifiable as ergosterol, ergosterol breakdown product or another sterol.

SERF and ergosterol are radically different compounds as indicated by chromatographic (TLC and HPLC) and solubility properties, yet both satisfy a cellular growth requirement for trace levels of C-5,6 unsaturated sterol. In addition, SERF is not obtained from the growth medium because cholestanol-cycled RD5-R is unable to grow in medium containing only cholestanol (Fig. 1). The results indicate SERF may actually be (i) a direct metabolite of ergosterol, (ii) an induced compound or (iii) functionally activated by trace levels of ergosterol. If SERF were derived directly from ergosterol, it would constitute a new end product of sterol biosynthesis in yeast and, by virtue of its hydrophilic properties, may have non-membrane-associated functions. This would be particularly interesting from an evolutionary standpoint because animal systems derive specific steroid hormones from cholesterol. Regardless, these data indicate that ergosterol is involved in critical, possibly non-membrane-associated, functions in the cell. To the best of our knowledge, these data represent the first evidence that minute quantities of ergosterol are essential for aerobic vegetative growth in yeast and that these amounts of ergosterol are equivalent to functional levels of certain vitamins, hormones and pheromones.

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The Role of Phytosterols in Host Plant Utilization by Cactophilic *Drosophila*

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The Cactus-*Drosophila* Model System of the Sonoran Desert consists of four endemic species of *Drosophila (D. mojavensis, D. nigrospiracula, D. mettleri* and *D. pachea)* and five species of columnar cacti (agria, organpipe, saguaro, cardón and senita). Extensive collection records indicate that each cactus species has only one species of *Drosophila* as the primary resident. The elimination of six of the twenty possible random combinations of *Drosophila* species and cactus species can be attributed directly to phytosterols. *Drosophila pachea* has a strict requirement for Δ^7-sterols such as 7-cholestenol and 7-campestenol. Since Δ^7-sterols are found only in senita cactus, *D. pachea* cannot use agria, organpipe, saguaro or cardón as host plants. The lipid fractions of agria and organpipe are chemically similar and contain high concentrations of several 3β,6α-dihydroxysterols. Larval viability tests using chemical constituents of organpipe cactus demonstrate that the sterol diols are toxic to *D. nigrospiracula* but not to the resident species, *D. mojavensis.* Agria and organpipe are therefore unsuitable as host plants for *D. nigrospiracula.* These results suggest that phytosterols play a major role in determining host plant utilization by cactophilic *Drosophila* in the Sonoran Desert.
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Dietary sterols are of great importance to members of the class Insecta as insects are unable to synthesize them de novo (1). The biological functions of sterols in insects include incorporation into membranes and roles as precursors for steroid hormones (e.g., ecdysone) and, in some cases, as defensive secretions. In addition, phytosterols in the host plants of cactophilic *Drosophila* of the Sonoran Desert appear to play a major role in host plant utilization.

The Sonoran Desert includes most of the Baja Peninsula and a large portion of northwestern Mexico extending into southwestern Arizona. The Cactus-*Drosophila* Model System of the Sonoran Desert consists of four endemic drosophilids which feed and breed in the necrotic tissue of five species of columnar cacti (Table 1). Although several of the *Drosophila* species shift host plants between Baja California and the mainland, extensive rearing records collected over a 10-yr period indicate that there is essentially only one fly species per cactus species. This phenomenon effectively eliminates interspecific competition, an interaction which may be too ecologically expensive to occur in the stressful environment of the desert. Through investigations of the chemical basis of these host plant relationships, we can gain insight into how the relationships are maintained and, perhaps, how they originally evolved.

Phytosterols in one cactus species, senita, now have been firmly established as primary determinants of the host plant specificity of *D. pachea.* The *Drosophila pachea*-senita investigation began in the mid-1960s with the observation that this species of fly bred only in the rotting stems of senita cactus and could not be reared from standard *Drosophila* media unless a cube of senita cactus were added (2,4-7). The unusual sterols in senita, 4α-methyl-Δ^7-cholesten-3β-ol (lophenol) and Δ^7-stigmasten-3β-ol (schottenol), which had been reported by Djerassi (8) suggested that *D. pachea* may have a unique sterol requirement. The initial sterol utilization tests reported that *D. pachea* could use schottenol, lathosterol and 7-dehydrocholesterol, but that $\Delta^{5,7}$-stigmastadien-3β-ol produced infertile females and cholesterol, lophenol, β-sitosterol, stigmasterol, ergosterol and Δ^7-ergosten-3β-ol did not support larval growth (4). *Drosophila pachea,* then, was the first insect species described that could not

TABLE 1

***Drosophila*-Cactus Relationships and Specificities**

	Host Plants[a]		Rearing records[b]
Species	Baja	Mainland	% Resident species
D. mojavensis	Agria	Organpipe	99.6
D. nigrospiracula	Cardón	Saguaro	99.0[c]
D. mettleri	Cardón soaked soil	Saguaro soaked soil	100[d]
D. pachea	Senita	Senita	99.9

[a]Scientific names: *Stenocereus gummosus* (agria), *S. thurberi* (organpipe), *Pachycereus pringlei* (cardón), *Carnegiea gigantea* (saguaro) and *Lophocereus schottii* (senita).
[b]Data modified from Fellows and Heed (2).
[c]May include *D. mettleri.*
[d]Data from Heed (3).

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[1]Deceased.

use cholesterol. Unfortunately, the synthetic schottenol used in the original study later was shown to be contaminated with ca. 40% Δ^7-campesten-3β-ol. Additional experiments have demonstrated that pure schottenol also does not support larval growth of *D. pachea* (9).

A more complete understanding of the *D. pachea*-senita relationship was delayed until the late 1970s and required further characterization of the sterols associated with this plant. Senita cactus apparently is a plant species with an interrupted sterol biosynthetic pathway which results in the accumulation of intermediate forms (10). The absence of Δ^5-sterols and the presence of 4α-methyl, Δ^7- and $\Delta^{8,14}$-sterols as the principal sterols support the statement that the later steps in the pathways to typical phytosterols (campesterol, sitosterol and stigmasterol) are either inhibited or absent in this cactus. In addition to schottenol and lophenol, six other sterols have been identified: Δ^7-cholesten-3β-ol, Δ^7-campesten-3β-ol, α-spinasterol, $\Delta^{8,14}$-cholestadien-3β-ol, 4α-methyl-$\Delta^{8,14}$-cholestadien-3β-ol (locereol) and 24-methylene lophenol. Given the structures listed above, inhibition appears to involve Δ^{14}-reductase, Δ^5-dehydrogenase and 4α-methyl hydroxylase systems. Sterol biosynthetic intermediates have been isolated from rat liver homogenate and cultures of *Chlorella*, bramble cells and yeasts when certain nitrogenous compounds are added to the medium (10). Senita cactus does contain high concentrations of alkaloids, e.g., lophocereine and pilocereine (11), and it was hypothesized that senita alkaloids may inhibit normal phytosterol biosynthesis and cause the observed accumulation of sterol intermediates. However, six species of cactophilic yeasts grown on a complete medium supplemented with a comparable concentration of senita alkaloids produced typical yeast sterols, principally ergosterol with traces of zymosterol (12). No sterol intermediates comparable to those in senita were detected. Alkaloids, then, do not appear to be the inhibitors which interrupt sterol biosynthesis in the cactus, and the cause of this interruption remains to be determined.

Of the eight sterols identified in senita, only two are known to support the growth of *D. pachea* through two generations. These are 7-cholestenol (lathosterol) and 7-campestenol (9). Knowledge of which sterols can and cannot be used by *D. pachea* provides a basis for speculation as to the metabolic deficiencies of this species. Based on a study of sterols in crickets (13), the sequence of double bond changes in the B ring during sterol metabolism in insects has been postulated to be $\Delta^5 \rightarrow \Delta^0 \rightarrow \Delta^7 \rightarrow \Delta^{5,7}$. The overall transformation of $\Delta^5 \rightarrow \Delta^{5,7}$ may be obligatory in insects which ingest Δ^5 sterols because the molting hormones are 6-keto-Δ^7 derivatives. Because *D. pachea* cannot complete its life cycle on cholesterol (Δ^5) or cholestanol (Δ^0), it appears that the $\Delta^0 \rightarrow \Delta^7$ step in the pathway is blocked. This hypothesis is supported by the observation that *D. pachea* does convert dietary lathosterol (Δ^7) to the $\Delta^{5,7}$, i.e., 7-dehydrocholesterol (14).

With respect to the sterol side chain, removal of alkyl groups at C-24 also is a necessary step in the production of ecdysone. Apparently, *D. pachea* lacks the ability to demethylate ergostane derivatives (24β-methyl) or deethylate stigmastane derivatives (24α-ethyl) as it is unable to utilize ergosterol, Δ^7-ergosten-3β-ol or schottenol. Campestane derivatives (25α-methyl), however, can be used. *Drosophila pachea* must be able either to remove an α-methyl group from C-24 or to produce and use a 24α-methyl ecdysone derivative. Makisterone A (22R-2β,3β,14,20,22,25-hexa-hydroxy-5β-campest-7-en-6-one) is a 24α-methyl derivative and has been reported as the main molting hormone in embryos of the milkweed bug (15), an insect that does not dealkylate plant sterols.

This paper deals with the role of phytosterols in the host plant utilization of organpipe and agria cacti. Extensive field and laboratory studies have demonstrated that *D. nigrospiracula* larvae mature in necrotic tissues of saguaro cactus (Table 1) but cannot do so in rotting organpipe and agria tissue. For example, progeny production of *D. nigrospiracula* females on organpipe substrate was less than 5% of their production on saguaro (2). The resident drosophilid for organpipe and agria is *D. mojavensis*.

The chemistry of organpipe has been well-characterized. Approximately 28% of the dry weight of the plant is composed of triterpene glycosides, while lipids make up about 11% (9). The glycosides are glucose-rhamnose tetrasaccharides of oleanolic acid, thurberogenin and queretaroic acid (16). The lipids are composed mainly of C_6 to C_{12} fatty acid esters of nine mono-, di- and trihydroxy triterpenes (17–19) and of five 3β,6α-dihydroxy sterols (20). The major neutral triterpenes are betulin and calenduladiol. In one sample of fresh tissue, hydrolysis of the lipids and compositional analysis gave 2.6% sterol diols, 5.1% neutral triterpenes, 3% medium chain fatty acids and .07% plant phytosterols (cholesterol/campesterol/sitosterol, 1:2:7), all expressed as percent of the cactus dry weight.

The sterol diol category of organpipe consists of five dihydroxy sterols which fall into a logical biosynthetic sequence. These five are cyclostenol (14α-methyl-9,19-cyclo-5α-cholestan-3β,6α-diol), stenocereol (14α-methyl-5α-cholesta-8,24-dien-3β,6α-diol), macdougallin (14α-methyl-5α-cholest-8-en-3β,6α-diol), thurberol (5α-cholesta-8,14-dien-3β,6α-diol) and peniocerol (5α-cholest-8[9]-en-3β, 6α-diol). The putative sequence starts with either cyclostenol or stenocereol and ends with peniocerol. The $\Delta^8 \rightarrow \Delta^7$ isomerization which normally follows in sterol biosynthesis presumably is inhibited by the 6α-OH group of peniocerol, and the biosynthesis terminates with this diol. Alkylation of the side chain also must be prevented because all of the sterol diols have the cholesterol side chain.

Agria cactus has not been characterized as well as organpipe, but appears to be very chemically similar. Approximately 36% of the dry weight of the tissue is triterpene glycosides and about 7% is lipids. Hydrolysis of the triterpene glycosides gave a 2:1 ratio of glucose to rhamnose and a 4:1 ratio of sugars to aglycones. The aglycones are represented by one neutral and two acidic triterpenes, none of which are found in organpipe. Examination of the lipid fraction showed only small amounts of normal phytosterols but a relatively large concentration of sterol diols similar to those in organpipe and of three pentacyclic triterpenes. Like in organpipe, the triterpenes and sterol diols are monoesterified to medium chain fatty acids (9).

The experiments described herein represent an attempt to determine which chemical constituents of organpipe and agria are responsible for the apparent inability of *D. nigrospiracula* to utilize these cacti as larval substrates. Because agria and organpipe are chemically similar, only chemical constituents of organpipe were used.

MATERIALS AND METHODS

Both species of *Drosophila* were obtained from the laboratory of W. B. Heed, Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona. Organpipe chemical constituents were extracted from fresh tissue as follows: cactus tissue was homogenized with an equal volume of MeOH. This slurry was filtered and the residue was extracted exhaustively with $CHCl_3$-MeOH (2:1, v/v). The MeOH and $CHCl_3$-MeOH filtrates were combined, evaporated and distributed between water and ether. Triterpene glycosides were purified by extraction from aqueous solution with n-butanol and precipitated with ether. Aglycones (acidic triterpenes) were obtained by hydrolysis of the saponins with 3N H_2SO_4 in H_2O-PrOH (2:1, v/v) on a steam bath for 24 hr followed by ether extraction. The ether soluble lipids were hydrolyzed with alkali, and betulin and calenduladiol (major neutral triterpenes) subsequently were crystallized from the nonsaponifiable fraction with 95% EtOH. A mixture of sterol diols was obtained from the nonsaponifiable fraction by column chromatography using silica gel.

Medium for testing larval viability was prepared by adding the chemical constituent to homogenized saguaro rot. However, water-insoluble compounds first were dissolved in ether and added to dried saguaro powder. The ether then was evaporated and the powder rehydrated and added to homogenized saguaro rot. In all cases, the resulting mixture was blended to insure homogeneity and divided into six portions in half-pint milk bottles. The quantities of organpipe constituents used were calculated as a percentage of the dry weight of the media. Viability tests for each species were set up in triplicate using 50 first-instar larvae per bottle. Larval density was decided on the basis of a preliminary experiment which showed that 50 larvae per test yielded a higher percent adult emergence than 100, 200 or 300 larvae per test. Larvae were given sufficient time (ca. 30 days) to develop into adult flies. The number of adult flies that eclose in each bottle is a measure of larval viability in the media. Control bottles containing saguaro rot without organpipe constituents were set up for each test of a particular constituent to control for possible differences between batches of larvae or saguaro rot. The data were analyzed using a one-way analysis of variance (ANOVA) with replication (21).

Analyses of fresh tissue, "young" rots and "old" rots of organpipe were performed by blending individual samples (several kg) with two vol of methanol. The mixtures were filtered and the residues extracted with fresh methanol and acetone and dried. The pooled extracts were evaporated, and the aqueous mixtures remaining were acidified with HCl, extracted with ether and evaporated to dryness. The ether extracts were extracted with $NaHCO_3$ solution to remove acids and quantitatively analyzed by gas-liquid chromatography (GLC) (5% OV-101, 250 C) to determine sterol diol content. The bicarbonate solutions were acidified, and the fatty acids were extracted with petroleum ether, methylated and analyzed by GLC (20% DEGS, 180 C).

RESULTS AND DISCUSSION

Average percent larval viabilities of *D. mojavensis* and *D. nigrospiracula* on necrotic saguaro and organpipe homogenate are given in Table 2. From the data in this table it is clear that while *D. mojavensis* does equally well on either substrate, *D. nigrospiracula* suffers an enormous loss of viability when forced to use organpipe as a substrate. This substantiates a previous study (2) and demonstrates that organpipe is indeed an unsuitable host plant for *D. nigrospiracula.*

TABLE 2

Average Percent Viability ± Standard Deviation on Cactus Homogenates

	Average viability	
Substrate	*D. mojavensis*	*D. nigrospiracula*
Saguaro	94.0 ± 4.0	85.3 ± 3.1
Organpipe	93.3 ± 2.3	20.7 ± 2.3
F (df = 1,4)	0.063	855.364
P	n.s.	⩽0.001

Results of one-way ANOVA (F) and probability (P) are given. n.s. = Not significant.

The effects of organpipe constituents on the viability of these two *Drosophila* species are shown in Table 3. In all cases, one-way analysis of variance tests were done on each species separately because comparisons between species for each treatment are not as ecologically relevant as the effects of treatments within a species. Addition of triterpene glycosides to saguaro homogenate had no effect on *D. nigrospiracula,* but the statistical analysis did show a significant effect on *D. mojavensis* at the .05 level. Although the effect is statistically significant, the drop in viability of *D. mojavensis* from 92% (control) to 80% (40% triterpene glycosides) is not particularly severe. This reduction in viability is less than half of the corresponding change in *D. nigrospiracula* (97.3% to 68%), which was not statistically significant. It appears that the significance of the effect of triterpene glycosides on *D. mojavensis* is due mainly to the low variance between replicates within treatments, particularly the ones involving 20% and 40% dry weight triterpene glycosides, and is of minimal biological significance.

No significant effect on viability was observed for either species when triterpene acids (aglycones), crude lipids or the two major neutral triterpenes were added to the saguaro homogenate. The first four compounds in Table 3, therefore, cannot be responsible for the exclusion of *D. nigrospiracula* from organpipe.

Drastic effects on both species were observed when free fatty acids were added to the medium. However, their effect on *D. nigrospiracula* was manifested at a lower concentration compared to *D. mojavensis.* The lowest concentration used (0.5% dry weight) reduced the viability of *D. nigrospiracula* to about 7% that of the control but did not appear to affect *D. mojavensis.* Higher concentrations reduced the viability of both species to zero or near zero.

The addition of free sterol diols to the medium had no significant effect on *D. mojavensis* even at the highest concentrations (10% dry weight). They did, however, affect *D. nigrospiracula* at concentrations of 1% dry weight

TABLE 3

Average Percent Viability ± Standard Deviation on Saguaro Homogenate + Organpipe Constituents

Substrate (Saguaro + constituent)	Average viability	
	D. mojavensis	*D. nigrospiracula*
Triterpene glycosides		
Control	92.0 ± 6.9	97.3 ± 10.3
10% dry weight	94.0 ± 7.2	87.3 ± 21.4
20% dry weight	78.7 ± 2.3	81.3 ± 3.1
40% dry weight	80.0 ± 2.0	68.0 ± 10.4
F (df = 3,8)	6.939	2.653
P	<0.05	n.s.
Triterpene acids		
Control	82.7 ± 12.2	55.3 ± 22.1
2.5% dry weight	74.7 ± 4.2	67.3 ± 14.2
5.0% dry weight	72.7 ± 4.2	64.0 ± 15.6
F (df = 2,6)	1.370	0.370
P	n.s.	n.s.
Crude lipids		
Control	86.0 ± 11.1	83.3 ± 7.0
5% dry weight	82.0 ± 10.0	89.3 ± 1.2
10% dry weight	76.7 ± 4.6	80.7 ± 4.6
F (df = 2,6)	0.804	2.463
P	n.s.	n.s.
Neutral triterpenes: betulin-calenduladiol		
Control	80.0 ± 7.2	65.3 ± 11.7
5% dry weight	82.0 ± 3.5	62.7 ± 10.1
10% dry weight	80.7 ± 2.3	64.7 ± 25.4
F (df = 2,6)	0.135	0.020
P	n.s.	n.s.
Fatty acids		
Control	86.0 ± 9.2	79.3 ± 12.2
0.5% dry weight	85.3 ± 8.1	5.3 ± 4.2
1.0% dry weight	8.0 ± 4.0	0.0 ± 0.0
2.0% dry weight	1.3 ± 1.2	0.0 ± 0.0
F (df = 3,8)	158.003	108.723
P	≪0.001	≪0.001
Sterol diols		
Control	92.0 ± 7.2	68.7 ± 19.6
0.5% dry weight	76.7 ± 9.5	54.7 ± 23.2
1.0% dry weight	78.0 ± 5.3	26.0 ± 19.7
2.0% dry weight	82.7 ± 15.5	4.7 ± 5.0
5.0% dry weight	73.3 ± 8.1	0.0 ± 0.0
10.0% dry weight	87.3 ± 17.0	0.0 ± 0.0
F (df = 5,12)	1.166	11.987
P	n.s.	<0.001

Results of one-way ANOVA (F) and probability (P) are given. n.s. = Not significant.

TABLE 4

Average Percent Dry Weight of Several Constituents of Organpipe During the Rotting Process

Tissue	No. samples	Lipids	Free sterol diols	Free fatty acids
Unrotted	4	16.0	0.43	0.41
"Young" rot	3	19.7	1.23	0.65
"Old" rot	3	23.7	1.87	0.35

or greater, and viability of this species is essentially zero at concentrations of 2% or greater.

These results indicate that only two organpipe constituents, fatty acids and sterol diols, have any toxic effect at reasonably natural concentrations. The explanation as to why these compounds are toxic, when the crude lipid fraction which contains these compounds is not, involves the fact that only small amounts of fatty acids and sterol diols are in a free form in the lipid fraction. In fresh tissue, the majority of these compounds are esterified to each other. The data in Table 3 indicate that the esterified form is not toxic. During the rotting process, however, some of these ester bonds may be broken by the action of microbial enzymes. This leads to an interesting question: just how much of the free C_6-C_{12} fatty acids and 3β,6α-dihydroxy sterols is present in necrotic organpipe tissues? To answer this question, a small number of samples of fresh tissue, "young" rots and "old" rots were analyzed; the results are presented in Table 4. Rots were designated as "young" or "old" based on color. Fresh organpipe tissue is light greenish-yellow, and rots develop through various color stages from orange to brown to black as the microorganisms consume the carbohydrate portion and oxygen converts phenolics to insoluble dark pigments. It can be seen in Table 4 that both the lipid fraction and the concentration of free sterol diols increase during the rotting process. The increase in the lipid fraction is due mainly to the release of the aglycones from the triterpene glycosides by microbial hydrolysis (9). Free sterol diols also are released during rotting by hydrolysis of the fatty acid esters. Free fatty acids, however, do not increase in concentration during rotting even though they are being released by hydrolysis. There are two possible explanations for this lack of increase. First, two frequently encountered cactophilic yeasts, *Candida ingens* and *Pichia mexicana*, produce extracellular lipases, and *C. ingens* can use free fatty acids as carbon sources (22). The growth of these two yeast species would, therefore, reduce the concentration of free fatty acids or prevent their accumulation in necrotic organpipe tissues. Second, free fatty acids are known to be quite chemically reactive. It is possible that the released fatty acids are being complexed with other components of the lipid fraction, e.g., triterpenes. This statement is supported by the observation that effect of fatty acids on both *Drosophila* species was reduced when they were added to saguaro homogenate along with 10% (dry weight) crude organpipe lipids. In the same procedure used to produce the data in Table 3, 2% fatty acids (plus 10% organpipe lipids) had no significant effect on *D. mojavensis* (average percent viability: control = 82.0, 2% = 88.0). Average percent viability of this species on 2% fatty acids without lipids was 1.3 (Table 3). The viability of *D. nigrospiracula* increased from zero on medium with 1% fatty acids to 36.6% on medium with 1% fatty acids plus 10% lipids.

The actual physiological mechanism of the toxic effect of sterol diols on *D. nigrospiracula* is unknown. In a pilot study using axenic cultures, neither species could use organpipe sterol diols as the sole source of dietary sterols because neither could survive for two generations on sterol-deficient medium supplemented with sterol diols (Kircher, H. W., unpublished data). Apparently, *D. mojavensis* has some means of excluding or detoxifying these compounds which is lacking in *D. nigrospiracula*. *Drosophila mojavensis*, which have been reared from an

organpipe necrosis, do not contain sterol diols in their tissues (23).

Also unknown is the relative importance of fatty acids vs sterol diols in the host plant relationships of these two species. Unquestionably, sterol diols are involved as determinants of host plant utilization. Both acids and diols are effective in preventing the development of *D. nigrospiracula* from larvae to adults. The tolerance of *D. mojavensis* and the concentration of these compounds in natural rots is sufficient to explain why *D. mojavensis* and not *D. nigrospiracula* can use necrotic organpipe and agria cactus tissues as breeding sites.

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Selective Sterol Transfer in the Honey Bee: Its Significance and Relationship to Other Hymenoptera

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The honey bee, *Apis mellifera*, is one of only a few species of phytophagous insects known to be unable to convert C-24 alkyl phytosterols to cholesterol. Regardless of the dietary sterols available to worker bees, the major tissue sterol of brood reared by the workers is always 24-methylenecholesterol, followed by sitosterol and isofucosterol. Normally, little or no cholesterol is present in honey bee sterols. The maintenance of high levels of certain sterols is accomplished through a selective transfer of sterols from the endogenous sterol pools of the workers to the developing larvae through the brood food material secreted from the hypopharyngeal and mandibular glands and/or the honey stomach of the workers. The selective uptake and transfer of radiolabeled C_{27}, C_{28} and C_{29} sterols have been studied to correlate these aspects of sterol utilization with the discovery of an unusual molting hormone (ecdysteroid) in honey bee pupae as the major ecdysteroid of this stage of development. The phylogenetic implications of this selective transfer phenomenon in the honey bee and comparison with sterol metabolism in certain other hymenopteran species emphasize the diversity of steroid biochemistry in insects.
Lipids 21, 97–101 (1986).

Phytophagous species belonging to three insect orders are unable to dealkylate and convert C_{28} and C_{29} phytosterols to cholesterol. These include two species of Coleoptera, the Mexican bean beetle, *Epilachna varivestis* (1), and the khapra beetle, *Trogoderma granarium* (2); several species of Hemiptera including the milkweed bug, *Oncopeltus fasciatus* (3,4) and the cotton stainer bug, *Dysdercus fasciatus* (5); and the honey bee, *Apis mellifera*, of the order Hymenoptera (6). The inability to convert 24-alkyl sterols to cholesterol is reflected in the ecdysteroid (molting hormone) metabolism of several of these species, since a C_{28}-ecdysteroid, makisterone A, is the major ecdysteroid during certain stages of development. This is true of last stage nymphs of *O. fasciatus* and four other species in the Pentatomomorpha group of Hemiptera (5,7) as well as the pupa of the honey bee (8). Thus, a C_{28} dietary sterol (e.g., campesterol) serves as a precursor for an ecdysteroid in these phytophagous species, and the usual C_{27} ecdysteroids, such as ecdysone or 20-hydroxyecdysone, are replaced by makisterone A.

We have studied in depth the utilization and metabolism of dietary sterols in the honey bee and discovered a fascinating aspect of sterol utilization in this species. The honey bee is capable of selectively transferring certain sterols to the developing larvae through the brood food. Regardless of the dietary sterols available to the worker bees, 24-methylenecholesterol was always the major component of the sterols isolated from prepupae or adults of the next generation (9). In addition, sitosterol and isofucosterol always were present as the next most abundant of the sterols from prepupae or adults of the next generation, with lesser amounts of campesterol (9). This phenomenon occurred when a chemically defined diet coated with a highly purified sterol or no sterol (9) was fed, when the workers were fed pollen and sucrose in a cage study (Svoboda, J. A., and Herbert, E. W. Jr., unpublished data) and when samples were obtained from free-flying colonies (10). Studies with dietary radiolabeled campesterol, sitosterol or 24-methylenecholesterol coated on the chemically defined diet verified that none of these sterols was metabolized to cholesterol or any other sterol and that the majority of the sterols of prepupae or adults of the next generation was derived from endogenous sterol pools of the nurse bees (6). Thus, it is well-established that this selective transfer of specific C_{28} and C_{29} sterols is functional in the honey bee under all conditions studied.

Several recent studies discussed in this paper have provided additional information on the transfer of sterols from one generation to the next in honey bees. We have examined the sterols of hypopharyngeal glands, mandibular glands and honey stomachs, all of which are involved in the complex feeding system whereby the royal jelly is produced for the brood food (11). The sterols from the corresponding whole insects, royal jelly and pollen samples were compared with those of the previously mentioned organs. Studies on the incorporation of radiolabeled C_{27}, C_{28} or C_{29} dietary sterols coated on a chemically defined diet fed to workers provided additional information on selective uptake and transfer of dietary sterols to the next generation. Finally, we compared the sterols of another phytophagous hymenopteran, the alfalfa leafcutter bee, *Megachile rotundata*, and two species of omnivorous Hymenoptera with those of the honey bee to obtain data on sterol utilization in other species of Hymenoptera.

MATERIALS AND METHODS

Hypopharyngeal glands, mandibular glands and honey stomachs were carefully dissected from 100 adult bees at least two weeks old that had been reared by workers fed an artificial, chemically defined diet (12) coated with 24-methylenecholesterol (>99% pure) at a concentration of 0.1% dry weight and provided with 50% sugar syrup solution ad lib. The glands and honey stomachs were blotted dry and weighed, then stored frozen until analysis for sterol content. The colony was established with about 4,000 newly emerged yellow bees (400 g) plus a mated laying Midnite® queen to provide a genetic marker to easily identify newly emerged dark-colored progeny. The colony, in a small hive, was maintained in a 2 × 2 × 2 m screened flight cage. The adults were held overnight without access to the diet to clear the digestive tract of dietary sterol before dissection. Samples of 25 prepupae and 2i newly emerged "dark" adults were weighed and stored frozen prior to sterol analysis.

Royal jelly samples were obtained from three colonies (1500 g each) of newly emerged yellow bees maintained in five frame swarm boxes in 2 × 2 × 2 m screened flight cages. The colonies were fed sugar syrup and a freshly

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collected mixture of tulip poplar, blackberry and clover pollens. Royal jelly was collected daily from queen cells to coincide with the first six days that queen larvae would be fed. Samples of royal jelly ranging from 1.47 to 4.35 g were collected each day and frozen prior to analysis. Corresponding samples of nurse bees and pollen from this experiment were weighed and stored frozen.

To examine utilization and incorporation of radiolabeled sterols, [4-^{14}C]cholesterol (236 cpm/μg sp act) (The Radiochemical Center, Amersham, England), [2,4-^{3}H]campesterol (380 cpm/μg sp act) (13), 24-[2,4-^{3}H]methylenecholesterol (392 cpm/μg sp act) (13) or [4-^{14}C]sitosterol (258 cpm/μg sp act) (Amersham Corp., Arlington Heights, Illinois) were coated on the chemically defined diet to achieve a concentration of 0.05% dry weight. Experimental duplicate colonies for each diet were established with 300 g of newly emerged bees plus a Midnite® mated laying queen per colony and maintained in hives and flight cages similar to those previously described. Samples of 20 and 15 prepupae from each test were weighed and frozen three and four weeks, respectively, after colony establishment and from 50 to 89 "dark" adults per test were weighed and frozen at the latter time.

For the comparison of sterol utilization in phytophagous and omnivorous hymenopteran species, samples of 25 honey bee prepupae reared in a free-flying field colony foraging primarily on cucumber pollen, 30 newly emerged adult alfalfa leafcutter bees, seven baldfaced hornet *(Dolichovespula maculata)* workers and 31 yellowjacket *(Vespula maculifrons)* workers were weighed and stored frozen until analysis. These samples were all field-collected in the vicinity of our laboratory.

Samples to be analyzed for sterols were homogenized in $CHCl_3$-MeOH (2:1, v/v) with a Virtis or Polytron homogenizer. After partitioning, the $CHCl_3$ phases were combined, dried over Na_2SO_4 and filtered, and the solvent was removed on a rotoevaporator. The crude lipids were saponified and the sterols in the nonsaponifiable fraction were isolated by column chromatography on Acid Grade 1.5 and Neutral Grade II alumina (Woelm, ICN Pharmaceuticals, Cleveland, Ohio) as described previously (9). Fractions from column chromatography were monitored by thin layer chromatography. Gas liquid chromatographic analysis of sterols was accomplished with a Varian model 3700 gas chromatograph equipped with a J & W DB-1 fused-silica capillary column (240 C, 15 m × 0.25 μm film). A Shimadzu C-R1B Chromatopac data processor provided information for qualitative and quantitative analyses of the sterol mixtures. Cholestane was the internal standard used in the determination of relative retention times (RRT) for identification of unknown sterols by comparison of their RRTs with those of authentic standards.

RESULTS

Table 1 summarizes the results of sterol analyses from hypopharyngeal and mandibular glands and honey stomachs of adult bees reared in brood provisioned by workers fed the chemically defined diet coated with 24-methylenecholesterol. The data from sterol analyses of prepupae and intact adults collected at the same time as the glands and honey stomachs are included for comparison. The major sterol in each sample was 24-methylenecholesterol, which comprised >60% of the total in the sterols of hypopharyngeal glands, prepupae and new adults and >50% of the total in the mandibular glands and honey stomachs. Sitosterol and isofucosterol were the next most predominant sterols in each sample, and 5.3–7.7% campesterol was present in each sample. The relative percentages of cholesterol in the sterols from the glands and honey stomachs were several times greater than those from the prepupae and adults.

The sterols of royal jelly samples (Table 2) collected over a six-day period contained 49.0–58.4% 24-methylenecholesterol, whereas the nurse bee sterols had 39.1% and the pollen sterols only 5.9%. Sitosterol was the major pollen sterol and isofucosterol was next in abundance; however, sitosterol and isofucosterol were most predominant after 24-methylenecholesterol in all other samples. Campesterol comprised 4.0–7.7% of the total sterols in the samples summarized in Table 2. No cholesterol was detected in any of the royal jelly, nurse bee or pollen samples analyzed in this experiment.

The sterol content of prepupae and adults reared by workers fed chemically defined diet coated with radiolabeled sterols is summarized in Table 3. The dietary sterol accumulated noticeably in each of the respective prepupal and adult samples. However, 24-methylenecholesterol was the major sterol (59.3–73.5% of total) in every sample, followed by sitosterol and isofucosterol in all but the prepupal and adult samples from the ^{3}H-campesterol-fed colonies. Cholesterol comprised 1.4% or less of the

TABLE 1

Comparison of Relative Percentages[a] of Sterols of Honey Bee Hypopharyngeal and Mandibular Glands, Honey Stomachs, Prepupae and Adults from a Colony Fed Chemically Defined Diet Supplemented with 24-Methylenecholesterol

Sterol	Hypopharyngeal glands	Mandibular glands	Honey stomachs	Prepupae	New adults
Cholesterol	2.0	5.3	4.6	0.5	0.3
24-Methylenecholesterol	63.0	50.7	54.5	62.9	60.3
Campesterol	6.4	7.0	7.7	6.4	5.3
Sitosterol	15.9	18.2	15.8	15.9	16.2
Isofucosterol	10.6	17.2	15.9	12.3	15.2

[a]Columns do not total 100%; only values for these five sterols are included for comparison.

TABLE 2

Relative Percentages[a] of Sterols of Nurse Bees, Pollen and "Queen" Royal Jelly Samples Collected Over a 6-Day Period

Sterol	Nurse bees	Pollen	Royal jelly					
			1-day	2-day	3-day	4-day	5-day	6-day
Cholesterol	NI[b]	NI	NI	NI	NI	NI	NI	NI
24-Methylenecholesterol	39.1	5.9	49.0	54.3	53.6	58.4	54.9	50.8
Campesterol	4.0	6.4	6.6	6.3	7.7	7.5	7.2	6.8
Sitosterol	26.4	44.1	24.6	22.0	22.4	21.9	19.5	19.2
Isofucosterol	24.0	34.6	16.4	11.0	10.0	11.6	9.9	9.6

[a]Columns do not total 100%; only values for these five sterols are included for comparison.
[b]NI: none identified.

TABLE 3

Comparison of Relative Percentages of Sterols from Honey Bee Prepupae (PP) and Adults from Brood Reared by Workers Fed Chemically Defined Diet Coated with Radiolabeled Sterols

Insect sterols	Dietary sterol							
	[^{14}C]Sitosterol		[^{14}C]Cholesterol		[^{3}H]Campesterol		24-[^{3}H]Methylenecholesterol	
	PP[a]	Adult	PP	Adult	PP	Adult	PP	Adult
Cholesterol	1.4	0.3	7.6	6.6	1.3	0.5	1.1	0.4
24-Methylenecholesterol	62.6	55.8	61.9	59.3	65.5	62.5	72.7	73.5
Campesterol	7.9	7.4	6.2	6.7	13.7	14.0	7.3	5.2
Sitosterol	18.1	22.2	14.2	15.9	11.3	13.0	10.7	11.3
Isofucosterol	9.5	13.5	9.5	10.4	7.9	9.8	8.1	8.3

[a]Average of relative percentages of two samples of prepupae from each test.

total sterols in all cases except the samples from the ^{14}C-cholesterol-fed colonies which had 7.6 and 6.6% cholesterol in the prepupal and adult samples, respectively.

The level of incorporation of radiolabeled dietary sterols into the tissues of prepupae and adults in the [^{3}H]-campesterol-fed colony was 41.8 and 56.4 μg/g fresh weight, respectively (Table 4), whereas prepupae and adults from the 24-[^{3}H]methylenecholesterol-fed colony contained 39.1 and 61.6 μg/g fresh weight, respectively, of labeled dietary sterol. The prepupae and adults from both the [^{14}C]cholesterol- and [^{14}C]sitosterol-fed colonies incorporated considerably smaller amounts of dietary sterol than did prepupae or adults from colonies fed either of the two C_{28} sterols.

A comparison of the major sterols of the four species of Hymenoptera is included in Table 5. 24-Methylenecholesterol comprised 37.6 and 34.1% of the sterols of the phytophagous honey bee and alfalfa leafcutter bee, respectively. Sitosterol and isofucosterol were the other most common sterols in both species; 32.2% of the total honeybee sterol was sitosterol and 40.7% of the total leafcutter bee sterol was isofucosterol. The sterols of both species contained comparable levels of campesterol: 9.3% in the honey bee and 10.4% in the leafcutter bee. Less than 1% of the sterols from either species was cholesterol. On the other hand, over 81% of the total sterols from both omnivorous species was cholesterol. The remainder of the sterols from these two species was made up of C_{28} and C_{29} phytosterols, mainly campesterol and sitosterol.

TABLE 4

Incorporation of Radiolabeled Dietary Sterols in Honey Bee Prepupae and Adults

Diet sterol	μg Labeled sterol/g fresh wt	
	Prepupae[a]	Adults
[^{14}C]Sitosterol	23.8	41.4
[^{14}C]Cholesterol	28.3	44.1
[^{3}H]Campesterol	41.8	56.4
24-[^{3}H]Methylenecholesterol	39.1	61.6

[a]Average from two samples of prepupae from each test.

DISCUSSION

There was no appreciable sequestration of 24-methylenecholesterol in the tissues involved in brood food production in the honey bee (Table 1). The relative percentage of this sterol in the hypopharyngeal gland sterols was comparable to the levels in prepupae and new

TABLE 5

Comparison of the Major Sterols of Hymenopteran Species

Sterol	Honey bee[a]	Alfalfa leafcutter bee	Baldfaced hornet	Yellow jacket
Cholesterol	0.9	0.5	86.5	81.5
24-Methylenecholesterol	37.6	34.1	0.6	0.6
Campesterol	9.3	10.4	3.8	2.6
Sitosterol	32.2	13.0	3.7	7.8
Isofucosterol	14.1	40.7	—	—

[a]All samples were from adults except the honey bee sample which was from prepupae. Values are relative percentages of the total sterols.

adults reared in the test colony, whereas there were somewhat lower levels in mandibular glands and honey stomachs. Thus, the selective transfer of this sterol does not involve an accumulation of 24-methylenecholesterol in any of these organs. The levels of sitosterol or isofucosterol also were not unusual in the sterols of these honey bee organs. There was some enrichment of cholesterol levels in all three organs relative to the percentages usually found in intact honey bees at any stage of development. However, there is no explanation at present for these increased levels of cholesterol in the organs that produce nourishment for the brood.

From the data in Table 2, it is apparent that 24-methylenecholesterol is the most abundant sterol incorporated into royal jelly, followed in decreasing order by sitosterol, isofucosterol and campesterol. Cholesterol was not identified in the sterols of pollen fed to this colony or from the nurse bees or any of the royal jelly samples. These data provide further evidence for a selective transfer mechanism, as the levels of 24-methylenecholesterol in all royal jelly samples are so much greater (49% or more) than those found in the sterols of pollen fed to the colony and even considerably greater than the level of this sterol in nurse bees. This indicates that a large percentage of the royal jelly sterols are cycled from endogenous pools of the nurse bees (6) or from selective uptake from pollen.

The data in Table 3 indicate that there was some increased incorporation of the radiolabeled dietary sterol coated on the chemically defined diet in prepupae and adults in each case, as we had seen before with unlabeled sterols (9). However, since 24-methylenecholesterol was the major sterol in each sample, there had to be considerable selective cycling of that sterol as well as others from the endogenous pools of the nurse bees in order to maintain such levels in the next generation. Also, the data in Table 4 indicate that 24-[^{3}H]methylenecholesterol and [^{3}H]campesterol are more selectively absorbed, transferred to brood food and incorporated into tissues of prepupae and adults than either the C_{27} or the C_{29} sterol used in the study, thus ensuring adequate precursor for the C_{28} ecdysteroid, makisterone A (8). The next most highly labeled samples were from [^{14}C]cholesterol-fed colonies, and this C_{27} sterol appears somewhat more preferably passed on to the offspring than the C_{29} sterol, sitosterol. We had seen similar results in an earlier study in which cholesterol was not included (6). In that study, 24-[^{3}H]methylenecholesterol-fed colonies produced offspring with the greatest percentage of labeled sterol, but the workers had been fed labeled diet for a longer period than in the present study.

The comparative data in Table 5 from two species of phytophagous and two species of omnivorous Hymenoptera indicate that the alfalfa leafcutter bee utilizes dietary sterols similarly to the honey bee. It appears that the leafcutter bee is unable to dealkylate 24-alkyl sterols, as is true also for the honey bee (6), and 24-methylenecholesterol is readily incorporated into the larval sterols. The cells of the leafcutter bee are provided with honey and pollen before they are sealed (14), so much of the larval diet is similar to the honey bee brood food through most of the feeding period. The sterols of the two omnivorous species, the baldfaced hornet and the yellowjacket, reflect the sterols of their diet, which would contain high levels of cholesterol. It will be of interest to determine whether the leafcutter bee has adapted to using a C_{28} sterol as a precursor for ecdysteroid production, as was found recently with the honey bee (8), where makisterone A is the major ecdysteroid of the pupa at peak ecdysteroid titer. The sterol utilization of other phytophagous Hymenoptera also will be examined to determine how widespread the selective sterol transfer mechanism is among phytophagous Hymenoptera. A leafcutting ant, *Atta cepholotes isthmicola*, has been reported to contain no cholesterol, and most of its tissue sterols were $\Delta^{5,7}$-24-methylene- and methylsterols (15).

This unique selective transfer mechanism which enables the honey bee to maintain fairly consistent levels of certain sterols such as 24-methylenecholesterol from one generation to the next is still little understood. More definitive studies are needed to follow the passage of injected, labeled sterols through the glands and honey stomachs at various intervals after injection to the brood food and subsequently to the developing larvae. Then the selective permeability properties of the membranes of the organs involved in the overall transfer process should be examined. This may lead to clues as to why a 24-methylenesterol and a 24-methylsterol are transported in preference to a C_{27} or a 24-ethylsterol. Also, because all Hymenoptera evolved from predacious ancestors (16) and these and other phytophagous species have adapted to such unusual means of utilizing dietary sterols, we may gain further insight into certain phylogenetic relationships.

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Evidence for Facilitated Transport in the Absorption of Sterols by *Saccharomyces cerevisiae*

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***Saccharomyces cerevisiae* is known to absorb sterols readily in the absence of air. As shown in this paper, yeast cells also will absorb sterols with and without various double bonds or an alkyl group at C-24 in the presence of air at a concentration (ca. 10% of the gas phase) which is growth-limiting due to limited sterol synthesis. However, if the growth conditions are changed to be fully aerobic, sterol is no longer absorbed to any significant extent even when the sterol in the medium (ergosterol) is the same as that present in the cells. This implies that sterol in the medium does not equilibrate passively with sterol in the plasma membrane and that some sort of facilitated transport, which can be turned on and off, is responsible for the entry of sterol when it occurs as a response to an inadequate endogenous supply of sterol. In agreement with facilitated transport mediated by protein binding, yeast cells in an auxotrophic state for sterol exhibit a high degree of stereoselectivity with respect to the orientation of the side chain around the C-17(20)-bond. For instance, E-17(20)- but not Z-17(20)-dehydrocholesterol is absorbed by cells undergoing limited growth with 10% air.**
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When deprived of oxygen, cells of wild type *Saccharomyces cerevisiae* become auxotrophic for sterol (1–4) and readily will absorb a variety of exogenous sterols including cholesterol, its 24α-methyl, 24β-methyl and 24α-ethyl derivatives, 7-dehydrocholesterol and ergosterol (2–5). Mutants of this species deficient in the 2,3-oxidosqualene cyclase also are sterol auxotrophs and will absorb such sterols as cholesterol and ergosterol even under fully aerobic conditions (6,7). Thus, the ability to absorb sterols is not dependent directly on whether the cells are operating in a respiratory or nonrespiratory mode. Despite this, when the growth conditions of wild type yeast are changed from anaerobic to fully aerobic, the ability of the cells to absorb cholesterol falls virtually to zero (8), presumably because the cells now have an adequate supply of endogenous sterol (8). However, the aerobic exchange of endogenous and exogenous cholesterol has been demonstrated in a number of other well-known cases. It occurred to us, therefore, that the reason aerobic yeast does not permit entry of cholesterol is not because the cells will not absorb sterols per se but because cholesterol does not have the same chemical structure as the sterol (ergosterol) resident in the sterol-rich (7) plasma membrane. This difference might prevent exchange if the plasma membrane were mated structurally to the sterol it contains; that is, a membrane formed with ergosterol might be different from one formed with cholesterol. Some support for this idea comes from reports indicating that the phospholipid content of ergosterol-containing yeast cells is actually different (less unsaturated fatty acid) from the content of cells containing cholesterol (9,10). Therefore, we could explain the inability of cholesterol to enter aerobic yeast cells by the assumption that cholesterol does not fit into a plasma membrane synthesized so as to accommodate ergosterol. If this were the correct explanation for the failure of cholesterol to enter aerobic yeast cells, there should be no impediment to the entry of ergosterol, since exogenous and endogenous sterol would be the same. The main purpose of this paper is to show that while cholesterol, ergosterol and various other sterols can, as expected, be absorbed so long as the amount of air present remains small enough to be growth-limiting, when air is not limiting and constitutes 100% of the gas phase ergosterol does not enter the cells. This contradicts the hypothesis enunciated above, and we believe it implies uptake of sterol is somehow facilitated rather than being a passive interdigitation into the membrane. Several possible mechanisms for this are discussed.

MATERIALS AND METHODS

Yeast culture. All experiments were performed with wild type diploid *Saccharomyces cerevisiae* (ATCC 18790) which was grown in continuous culture at 27 C on a synthetic nitrogen base medium as described previously (11). Cells adapted to oxygen deprivation in the presence of 1.0 mg/l of ergosterol over several years were used for all the experiments. An inoculum (yielding ca. 0.1×10^6 cells/ml in the final medium) from the continuous culture at log phase (ca. 24 hr) was used.

The experiments with a small air supply were carried out by slight modifications of the method presented in our preliminary reports (5,12). In the incubations 1-l vessels were flushed with nitrogen scrubbed with $CrCl_2$ before inoculation (11). The headspace in the vessels was ca. 100 ml. Test steroids dissolved in 2 ml of absolute ethanol were added to Tween-80 (15 ml/l of final medium) and then to the rest of the medium to give a final concentration of 5.0 mg/l. 2,3-Iminosqualene which inhibits 2,3-oxidosqualene cyclase (13) was synthesized according to the literature (14). The inhibitor (at a final concentration in the medium of 50 μM) was added along with the test steroid to the Tween-80 solution before addition to the medium. Tween-80 acted both as an emulsifier and a source of fatty acid. After incubation of the inoculated medium for 68 hr at 28 C with or without a test compound, no growth occurred (4). Then 10 ml of air was added through a septum (12). The incubation was allowed to continue for another 68 hr, which led to eight doublings without added sterol. If sterol was added, growth stimulation was assessed by subtracting the observed value from the control value with no added sterol. Cell counts were made both visually and by the use of a Coulter Counter Model TA_{II} with a population counter in 16 ranges of cell size. In the fully aerobic incubations (carried out in triplicate), no 2,3-iminosqualene was added and air was passed through the medium continuously. Sterols were added in Tween-80 as in the other experiments at a concentration of 5.0 mg/l of final medium (250 ml in each case). The incubations were performed for 68 hr at 28 C.

Sterol analysis. Yeast cells, harvested by centrifugation, were washed with water centrifugally and then

saponified directly in 10% KOH in 95% ethanol at the reflux temperature for one hr, which yielded somewhat more sterol than extraction of the cells followed by saponification of the lipids. The neutral lipids obtained by dilution with water and extraction with ether after the saponification were chromatographed on silica Gel G thin layer plates in ether/benzene (1:9, v/v). The 4-desmethylsterols were eluted with ether and analyzed by gas liquid chromatography (GLC) at 230 C on a Hewlett-Packard gas chromatograph, Model 5840A, equipped with a microprocessor, flame ionization detector and six-ft coiled glass columns packed with XE-60 or SE-30; a QF-1 column was used to differentiate between 5α-stanols and Δ^5-stenols. Cholesterol was the standard for determination of the relative retention time (RRT). A DuPont Zorbax ODS column at 45 C with acetonitrile/isopropanol (80:20, v/v) as the solvent system was used for high performance liquid chromatography (HPLC). The standard for HPLC data was cholesterol, and the rates of movement are given relative to cholesterol (α_c).

Quantitation of the amounts of unlabeled sterols was accomplished by GLC with a standard curve for cholesterol. Since the responses of cholesterol and ergosterol were within a few percent of each other, the differences between the curves for cholesterol and other sterols were ignored, and the cholesterol curve was used as a basis for quantitation of all free sterols. Quantitation of radioactivity was accomplished in Aquafluor or Omnifluor (New England Nuclear, Boston, Massachusetts) for aqueous and nonaqueous samples, respectively, on a Beckman Model LS 7500 microprocessor controlled scintillation counter.

Materials. Cholesterol, 5α-cholestan-3β-ol (cholestanol), lathosterol, sitosterol, stigmasterol and cholest-5-ene were recrystallized commercial samples. Campesterol was isolated from the seeds of *Brassica oleracea* (cabbage) and contained less than 5% of the epimeric 22-dihydrobrassicasterol by ^{1}H-NMR analysis. 7-Dehydrocholesterol was prepared from cholesterol (15), and (E)- and (Z)-17(20)-dehydrocholesterol were synthesized from pregnenolone as described earlier (16) as was 21-norcholesterol (17). Clionasterol was prepared (18) from a mixture of 25(27)-dehydroclionasterol and 25(27)-dehydroporiferasterol, which were extracted from *Kalanchoe daigremontiana* (18). The purity of all samples (>98% unless otherwise noted) was assessed by GLC and in many cases also by HPLC, ^{1}H-NMR and mass spectroscopy (MS).

Radioactive substrates. [U-^{14}C]Ergosterol was prepared by growing wild type diploid *S. cerevisiae* (ATCC 18790) aerobically at 27–28 C in a synthetic nitrogen base medium containing 10% glucose in the presence of 0.5 mCi/l of the sodium salt of sodium [1-^{14}C]acetate with a specific activity of 56.2 mCi/mmol (New England Nuclear). Two one-l batches of growth medium were used. The cells were harvested at 68 hr and extracted with 10% ethanolic KOH. The 4-desmethylsterols were isolated by thin layer chromatography (TLC) using ergosterol as a reference standard. The 4-desmethylsterols were extracted with ether and the [U-^{14}C]ergosterol was separated from zymosterol, 22-dihydroergosterol and traces of other desmethylsterols by repetitive preparative HPLC using a DuPont Zorbax ODS analytical column at 45 C with 20% isopropanol in acetonitrile as the mobile phase. The specific activity of the purified [U-^{14}C]ergosterol was 1.24×10^6 dpm/mg, and it showed virtually a single chromatographic peak. [4-^{14}C]Cholesterol (New England Nuclear) also was purified by repetitive preparative HPLC using the same column and conditions mentioned above. The specific activity of the cholesterol after appropriate dilution with recrystallized commercial cholesterol was 1.25×10^6 dpm/mg.

RESULTS

Although wild type yeast will not grow when cholesterol or other sterol lacking a 24β-methyl group is the only sterol in the cells (4) (see also Table 1, footnote *a*), growth will ensue if small amounts of a 24β-methylsterol, e.g., ergosterol, also are present to function in a regulatory manner (4,7,12). Therefore, to assess the uptake of sterols such as cholesterol we generated ergosterol (and its 22-dihydro derivative) in situ by the addition of a small amount of air. We chose a concentration (10% of the gas phase) which at 68 hr without any steroid gave about one-fifth of the growth obtained in the absence of air with 5 mg/l of ergosterol. Table 1 shows that absorption occurred and was independent of the presence or absence of one or more double bonds, as Δ^0-, Δ^5-, Δ^7-, $\Delta^{5,7}$-, $\Delta^{5,7,22E}$- and $\Delta^{5,17(20)E}$-sterols were absorbed well, giving an average cellular content of 14 fg/cell. No metabolism of the sterols was observed.

All but two of the 24-desalkyl steroids shown in Table 1 were able not only to be absorbed but also to function in place of ergosterol in the "bulk membrane" role (4,12); the exceptions were the steroid with C-22 oriented to the left in the usual view of the molecule (Z-17[20]-dehydrocholesterol) and the hydrocarbon (cholest-5-ene) which lacks an HO-group at C-3. There was no growth response to either of these steroids. While the cells were not extracted in the hydrocarbon case to distinguish between absorbability and functionality due to the hydroxyl group, we did examine the case of the Z-sterol to define the effect of isomerism about the 17(20)-bond. None of the Z-sterol was found in the cells, although seven or so doublings had occurred. Only sterols derived by endogenous synthesis, e.g., ergosterol, were present. This means that the Z-sterol was not absorbed under conditions in which its *trans*-isomer (E-17[20]-dehydrocholesterol) was absorbed quite well (83% as well as cholesterol).

As we have reported elsewhere (19), for wild type yeast under nitrogen with no added air, as the concentration of sterol in the medium rises progressively from 5 mg/l to 100 mg/l, the total sterol (free and esterified) in the cells rises for both cholesterol and ergosterol. However, a much higher level (about fourfold) for cholesterol is reached than for ergosterol. A similar phenomenon has been observed aerobically with the mutant strain FY3, a sterol auxotroph (6). Both wild type (19) and FY3 cells (6) esterify most of the cellular cholesterol when it is more than 40 mg/l in the medium, but no significant esterification of ergosterol occurs. This probably is a reflection of the fact that ergosterol is yeast's natural and preferred sterol. However, the entry of the two sterols into the cells also may be differentially restrained to some extent by the particle size of the sterol aggregates in the medium. We repeatedly have observed that ergosterol-containing media are slightly cloudy, while those containing cholesterol are clear. As seen from Figure 1, increases in

TABLE 1

Steroid Absorption and Growth Response

Steroid added to medium	Actual cell count[a,d] (millions of cells/ml)	Absorbed sterol (fg/cell)	Endogenous 24β-methylsterol[b] (% of total sterol)	Normalized cell count[c]
Ergosterol	118(5)	17.0	100	69[e]
Cholesterol	112(3)	14.2	6	62
5α-Cholestanol	85(3)	13.5	1	45
21-Norcholesterol	97(3)	9.3	3	78
7-Dehydrocholesterol	100(2)	17.9	5	42
Lathosterol	97(3)	13.4	2	54
(E)-17(20)-Dehydrocholesterol	80(3)	11.8	7	47
(Z)-17(20)-Dehydrocholesterol	16(2)	None detected	100	—
Campesterol	107(3)	14.6	6	57
Stigmasterol	104(2)	14.5	5	55
Sitosterol	89(3)	15.7	4	41
Clionasterol	103(3)	16.8	3	47
Cholest-5-ene	21(2)	NE	NE	NE
Control (no steroid)	24(3)	—	100	0

NE: not examined.

[a]Average cell count of *S. cerevisiae* obtained after the addition of 10 ml of air following anaerobic incubation with iminosqualene as described in Materials and Methods. Except for ergosterol, no anaerobic growth was observed prior to air addition (4). Number of experiments is in parentheses. Values for ergosterol were obtained without the addition of air.

[b]The 24β-methylsterol was a mixture of ergosterol and 22-dihydroergosterol.

[c]The normalized cell count is the cell count with the test steroid minus cell count of control divided by the amount of steroid in units of 10 fg.

[d]Data reported in a preliminary communication (12) or for ergosterol elsewhere (4).

[e]Calculated without subtraction of control since no air was added in this case.

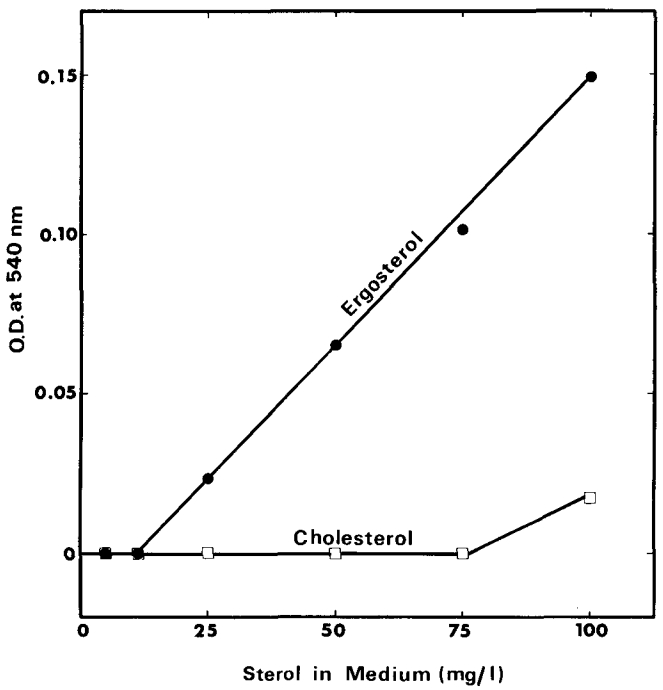

FIG. 1. Effect of sterol structure on reflectance of light at 540 nm. OD = optical density = absorbance. ●, Ergosterol; □, cholesterol. Sterols were emulsified in the medium in the usual way by dissolving them in ethanol, adding the solution to Tween-80 and adding this to the glucose solution containing salts and other nutrients.

the concentration of ergosterol in the medium lead to increased reflectance of photons at 540 nm, indicating increased particle size as the concentration rises, but a similar phenomenon does not occur with cholesterol except to a small extent after 75 mg/l is reached. This might account for some of the differential in uptake at the higher concentrations, as the larger particle size with ergosterol would result in a smaller surface-to-volume ratio and therefore a lower effective concentration and less availability if transfer of ergosterol from inside the particles to the surface were restricted.

The question as to whether ergosterol would enter aerobic cells was addressed first by establishing a control value for cholesterol. As shown in Table 2, when [4-^{14}C]cholesterol was added to an aerobic culture at the same concentration that we use normally under anaerobic conditions, the labeled cholesterol was for all practical purposes unabsorbed. The amount that appeared in the cells based on the radioactivity in the 4-desmethylsterol fraction amounted to only 0.38% of the desmethylsterols and 3.28% of the sterol initially in the medium. It also was possible to see a weak GLC and HPLC peak at RRT 1.02 and α_c 0.99, respectively, which was not observed in the ergosterol-grown culture. This peak also probably represented cholesterol. We quantitated it in the GLC case and found the same amount of cholesterol as observed by radioactive measurement. We believe that the amount of cholesterol found associated with the cells is so small as to be negligible. The observed material may simply have been sticking to the cell surface or reisolated

TABLE 2

Aerobic Absorption of [4-^{14}C]Cholesterol and [U-^{14}C]Ergosterol

	Cholesterol	Ergosterol
Visual cell count (cells/ml)	4.55×10^8	5.18×10^8
Initial label in medium (dpm)	1.56×10^6	1.55×10^6
Cellular neutral lipids (dpm)	1.82×10^5	8.04×10^4
Cellular 4-desmethylsterols (dpm)	5.14×10^4	3.82×10^4
Cellular 4-desmethylsterols (mg)	10.7	10.9
Cellular ergosterol (mg)	9.04	9.9
Cellular ergosterol (fg/cell)	79.5	76.4
Cellular sterol from medium (mg, based on dpm)	0.041	0.03
Cellular sterol from medium (mg, based on GLC)	0.042	—
Ratio of cellular sterol from medium to total cellular 4-desmethylsterols (%)	0.38	0.28
Ratio of cellular sterol from medium to initial sterol in medium (%)	3.28	2.40

from traces of medium associated with the pellet when the cells were harvested.

When aerobic cells were incubated with [U-^{14}C]ergosterol in the medium at the same concentration as in the cholesterol experiment, there was no more uptake than with cholesterol. Only 0.28% of the desmethylsterol was found to be labeled ergosterol from the medium on a weight basis, and only 2.40% of the labeled ergosterol in the medium was associated with the cells.

DISCUSSION

The data of Table 2 show that essentially no sterol (<0.4% of the 4-desmethylsterol) from the medium enters actively growing fully aerobic yeast cells even when the sterol in the medium is the same (ergosterol) as the sterol in the cells. This situation differs drastically from that in which the same cells are grown in a sufficiently reduced amount of air to make them auxotrophic for sterol. Under the latter conditions (Table 1), sterol from the medium actually becomes the major sterol in the cells. We found about one-fifth of the cellular sterol in the latter case was formed by biosynthesis and four-fifths was derived from the medium, in contrast to the fully aerobic cells in which more than 99.6% of the sterol was endogenous. Thus, the cells appear to control whether sterol enters them depending on the cellular need for sterol, and a model in which sterol from the medium freely interdigitates with the phospholipid of the plasma membrane seems excluded by our data. This means that sterol most likely interacts in some manner with something that facilitates its passage into the plasma membrane and from there into the cell. The most likely process is binding to a protein, the concentration or activity of which could in turn be regulated in various ways, thereby controlling sterol absorption. Smith (20,21) suggested many years ago that sterol might be transported into mycoplasmas via glycosylation because steryl glucoside and steryl acetate, putatively by catabolism of the sugar moiety, both were present in addition to free sterol. Such a mechanism is very attractive and could apply to yeast with the enzyme catalyzing the glycosylation (or perhaps the availability of the glycosyl moiety) functioning as a regulator of uptake. Similarly, some other steryl derivative such as an ester might be involved with enzymatic action to control sterol entry. Alternatively, a protein which binds to sterol without catalytic action might be the regulatory agent, and evidence for a sterol carrier protein in yeast already exists (22). Finally, it is conceivable that holes of some sort in the cell wall are opened or closed in response to the cell's varied need for sterol and that, when the channels are open, sterol actually does enter the plasma membrane by passive interdigitation.

Although it is not possible at this time to decide how absorption really occurs, several pieces of information are consistent with the hypothesis of binding to a protein. Our finding (Table 1) that uptake is prevented by the transfer of C-22 from the right (*trans* to C-13) to the left side (*cis* to C-13) of the sterol molecule (E- vs Z-17[20]-dehydrocholesterol, respectively) points to a high degree of stereoselectivity in the absorption process. This is paralleled by inversion at C-20 when it is chiral. Thus, 20(R)- but not 20(S)-sterols are absorbed by cells undergoing limited growth with reduced air (5). The preferred conformation (23) of the former sterols (20R) is stereochemically analogous (C-22 on the right) to the E-$\Delta^{17(20)}$-sterol while the 20(S)-sterols are similar in shape (C-22 on the left) to the Z-$\Delta^{17(20)}$-sterols. Another reason to suggest protein binding has been brought forward by Salerno and Parks (8), who found the rate of uptake follows Lineweaver-Burk kinetics.

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Distribution of Glycosphingolipids of Monkey Small and Large Intestinal Mucosa

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The ganglioside and neutral glycosphingolipid composition of adult monkey small and large intestinal mucosa were characterized and compared. GM_3, GM_2 and GD_{1A} were found to be the principal gangliosides in each of these tissues. Dihexosylceramide was the major neutral glycosphingolipid of both organs. The total content of gangliosides and neutral glycolipids/ceramide, however, was ca. four-fold and two-fold higher, respectively, in small intestinal than colonic mucosa.

While all glycosphingolipids examined contained hydroxy and nonhydroxy fatty acids, the former fatty acids accounted for 60–90% of the total fatty acids in both organs. Sphingosine was the predominant long chain base of ceramide, mono-, di-, tri- and tetrahexosylceramide, whereas phytosphingosine was the major base of GM_3 in both tissues. The results of these studies demonstrate that while many similarities of monkey small and large intestinal glycosphingolipids exist, qualitative and quantitative differences are present along the length of the monkey gut. These differences may be at least partially responsible for certain of the well-recognized variations in normal physiological and pathological processes that occur in these organs.

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The small and large intestines perform many important physiological functions, such as maintenance of normal water and electrolyte balance (1). These organs assume added importance because of the large number of infectious diseases that affect both (2) and the high incidence of malignant transformation that occurs in the large intestine (3).

Glycosphingolipids may be involved in each of these aforementioned processes, including transmembrane transport (4), binding of bacteria and bacterial toxins to intestinal epithelial cells (5,6) and malignant transformation (5,6). Although analyses of the glycosphingolipids of the small (7–15) and large intestine (12,16–18) of several species have been reported, available data in primates is limited. Furthermore, while it is clear that regional variations in many of the normal physiological and pathological processes may exist along the length of the gut (1,3,19), relatively few studies (12,18) have simultaneously examined the distribution of glycosphingolipids in both these organs. The present experiments were undertaken to examine and compare the glycosphingolipid composition of adult monkey small intestinal and colonic mucosa. The results of these investigations are the basis of the present report.

MATERIALS AND METHODS

Materials. The ganglioside standards GM_1, GD_{1A}, GT_{1A} and GT_{1B} were purchased from Supelco (Bellefonte, Pennsylvania). GM_2 isolated from Tay-Sachs brain was a gift from G. Dawson. GM_3 was isolated and purified by the method of Glickman and Bouhours (7) from rat small intestine. Ceramide was purchased from Analabs (North Haven, Connecticut). Mono-, di-, tri- and tetrahexosylceramide purified from human erythrocytes were gifts from S. K. Kundu (20).

Mixtures of hydroxy and nonhydroxy fatty acid standards, EGSS-X 10% on Gas Chrom P and Power-Sil Prep were obtained from Alltech/Applied Sciences Lab Inc. (State College, Pennsylvania). Sphingosine standards and OV-1 3% on Anakron ABS were purchased from Analabs. Phytosphingosine was obtained from Calbiochem (La Jolla, California). Florisil (60–100 mesh) and Fluorescamine (Fluram-Roche) were obtained from Fisher Scientific Co. (Fairlawn, New Jersey).

Lipid extraction. Three adult male monkeys *(Macaca fascicularis)* weighing ca. 4 kg were obtained from Abbott Laboratories (N. Chicago, Illinois). Animals were fasted overnight and killed; their small intestines and colons (minus the cecum) were removed and washed, and the mucosa was scraped off with a glass slide. Each sample was then homogenized in methanol and the lipids were extracted using chloroform/methanol mixtures of increasing polarity (21).

Ganglioside and glycolipid purification. Total lipid extracts were partitioned with water and the lower phase was washed five times with Folch upper phase (chloroform/methanol/water; 3:48:47, v/v/v) to insure complete extraction of gangliosides into the aqueous phase (21). Upper phase gangliosides then were purified by passage through Sep-Pak cartridges as described (22).

The neutral glycolipids were purified by chromatography of the acetylated neutral lipids on a Florisil column (23). The gangliosides and deacetylated neutral glycolipids were analyzed by high performance thin layer chromatography (HPTLC) on Silica-gel 60 precoated plates (E. Merck, Darmstadt, West Germany) in the solvent system chloroform/methanol/water/1% $CaCl_2$ (60:35:7:1, v/v/v/v) (24).

Individual glycosphingolipids were quantified after scraping the silica gel area corresponding to their position following migration. The quantity of sphingoid bases liberated by methanolysis was determined by fluorimetry after reaction of the free bases with Fluorescamine (23). Quantitation of all of the bands of each ganglioside species was performed, using a Beckman densitometer as described by Mullin et al. (24).

Recovery. Known amounts of each ganglioside standard and each neutral glycolipid/ceramide standard were added to the mucosal samples and carried through the extraction and chromatographic procedures to determine recovery of the various glycosphingolipids and ceramide (25). Recoveries for GM_2, GM_3 and GD_{1A} were found to be 82 ± 6%, 87 ± 6% and 72 ± 9%, respectively. Recoveries for ceramide, mono-, di-, tri- and tetrahexosylceramide were 80 ± 7%, 76 ± 8%, 88 ± 9%, 79% ±

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6% and 74 ± 8%, respectively (n = 3). The differences in recoveries for these various lipids were not statistically significant.

Desialylation of gangliosides. Desialylation of each of the purified gangliosides was performed by acid hydrolysis using 0.1 M H_2SO_4 at 80 C for 1.5 hr as described (26). Additionally, purified GM_3 was dried under nitrogen, reconstituted in 100 μl of water and 100 μl of neuraminidase (*V. cholerae,* Calbiochem-Behring Diagnostics) was added. The samples and controls were incubated at 37 C for 15 hr. The reaction was terminated by the addition of 20 vol of chloroform/methanol (2:1, v/v), dried under nitrogen and reconstituted in 100 μl of chloroform/methanol (2:1, v/v). N-glycolyl and N-acetylneuraminic acids were separated on HPTLC plates using a solvent system of n-propanol/water/ammonium hydroxide (60:28:1.5, v/v/v) (7,27) and visualized with resorcinol.

Analysis of carbohydrate moieties of gangliosides and neutral glycolipids. Carbohydrate moieties were identified by gas liquid chromatographic (GLC) analysis of their trimethylsilyl derivatives as described (7,28).

Analysis of fatty acids and long chain bases of glycosphingolipids. Individual glycosphingolipids were hydrolyzed in 1 ml of dry methanolic HCl (0.75 M) at 80 C for 16 hr, followed by extraction of fatty acid methyl esters by hexane (29). The methanolic phase then was alkalinized with aqueous NaOH and the long chain bases were extracted by diethyl ether (30).

Nonhydroxy and hydroxy fatty acids were separated by chromatography on a Florisil column as described (31). In certain experiments, a known amount of C_{21} fatty acid methyl ester was added as an internal standard to quantitate the nonhydroxy and hydroxy fatty acids in the samples (9). Nonhydroxy fatty acid methyl esters were dissolved in a small volume of hexane, while hydroxy fatty acid methyl esters were silylated prior to chromatography (9). Analyses of both nonhydroxy and hydroxy fatty acids were performed on a Hewlett-Packard 5790A gas liquid chromatograph equipped with a flame ionization detector and interfaced with a Hewlett-Packard 3390A integrator as described (9). Peak identification was based on identical retention times with known standards run under the same conditions (9).

Long chain bases free from fatty acids were dissolved in chloroform, washed with 0.1 M NaOH and repeatedly washed with water. The chloroform phase was dried under N_2 and the residue silylated as described (32). The long chain bases then were analyzed by GLC on an OV-1 column operated with temperature programming from 245 to 305 C at 3 C per min (9).

RESULTS AND DISCUSSION

The present data indicate that differences in glycosphingolipids and ceramide exist between adult monkey small intestinal and colonic mucosa. The results of analyses of the content and relative percentages of the gangliosides, neutral glycosphingolipids and ceramide by HPTLC are summarized in Table 1. As shown, the total content of gangliosides was ca. fourfold higher in the small intestinal than colonic mucosa; this organ also possessed about twice as much neutral glycolipid and ceramide as colonic tissue.

The major gangliosides of both small intestinal and colonic mucosa, as assessed by HPTLC, were, in descending order, GM_3, GM_2 and GD_{1A} (Table 1). GM_3 also previously has been shown to be the predominant ganglioside of adult rat small intestinal (7,10) and colonic mucosa (16). In the sheep, however, our laboratory recently has shown that the major ganglioside of both these organs is GD_{1A} and not GM_3 (17; unpublished observations). Analysis of the sugar moieties of these gangliosides by GLC further established their identities (Table 2).

After treatment with neuraminidase and/or acid hydrolysis, sialic acid moieties of the gangliosides of small and large intestinal mucosa were found to contain both N-acetyl and N-glycolyl forms. In the small intestine, N-acetylneuraminic acid was found to be the major sialic acid moiety of GM_3 (78.3 ± 4.6%) and GM_2 (60.3 ± 3.6%) (n = 3). In the colon, however, these two gangliosides contained approximately equal amounts of the two forms (not shown). The major sialic acid moiety of GD_{1A} in both the small (82.8 ± 3.6%) and large (74.1 ± 2.1%) intestines, however, was N-glycolyneuraminic acid (n = 3).

Dihexosylceramide was found to be the principal neutral glycolipid in both small intestinal and colonic

TABLE 1

Content and Relative Percentage of Gangliosides, Neutral Glycosphingolipids and Ceramide from Monkey Small Intestinal and Colonic Mucosa[a]

	Gangliosides					Neutral glycosphingolipids and ceramide			
	S.I. (nmoles NeuAc/mg protein	%	Colon (nmoles NeuAc/mg protein)	%		S.I. (nmoles sphingosine/ mg protein)	%	Colon (nmoles sphingosine/ mg protein)	%
GM_3	13.2 ± 0.8	60.7 ± 1.1	3.5 ± 0.4	61.6 ± 0.6	Ceramide	57.0 ± 3.5	22.4 ± 1.5	45.7 ± 1.5	35.1 ± 1.1
GM_2	7.3 ± 0.6	33.6 ± 0.8	1.5 ± 0.2	26.0 ± 0.3	Monohexosylceramide	55.4 ± 2.8	21.8 ± 1.0	19.3 ± 1.3	14.8 ± 1.0
GD_{1A}	1.2 ± 0.1	5.6 ± 0.2	0.7 ± 0.1	12.4 ± 0.2	Dihexosylceramide	70.2 ± 2.9	27.6 ± 1.0	34.7 ± 1.6	26.6 ± 1.2
					Trihexosylceramide	30.6 ± 1.8	12.0 ± 0.8	8.7 ± 0.6	6.7 ± 0.4
					Tetrahexosylceramide	41.2 ± 1.2	14.1 ± 1.4	21.9 ± 0.6	16.8 ± 0.5
Total	21.7 ± 1.4		5.7 ± 0.6			254.4 ± 1.6		130.0 ± 1.2	

[a]Values represent means ± S.E. of three separate preparations.

TABLE 2

Analysis of Sugar Moieties in Gangliosides and Neutral Glycosphingolipids from Monkey Small Intestinal and Colonic Mucosa[a]

	S.I.		Colon	
	Sugar	Molar ratios	Sugar	Molar ratios
Monohexosylceramide	Gal/Glc	0.1/0.9	Gal/Glc	0.2/0.9
Dihexosylceramide	Gal/Glc	1.1/1.0	Gal/Glc	1.0/1.1
Trihexosylceramide	Gal/Glc	2.0/1.0	Gal/Glc	2.1/1.1
Tetrahexosylceramide	Gal/Glc/GalNac	2.1/1.0/0.9	Gal/Glc/GalNAc	2.1/1.1/1.1
GM_3	Glc/Gal/SA	1.0/1.1/1.0	Glc/Gal/SA	0.9/1.1/1.0
GM_2	Glc/Gal/GalNAc/SA	1.0/0.9/1.1/1.0	Glc/Gal/GalNAc/SA	1.1/1.0/0.9/1.0
GD_{1A}	Glc/Gal/GalNAc/SA	1.0/2.0/1.1/1.9	Glc/Gal/GalNAc/SA	1.0/1.9/1.0/2.1

[a]Glycosphingolipid classes were separated by TLC and trimethylsilyl esters prepared and analyzed by GLC as described in Materials and Methods.

TABLE 3

Percentage of Sphingosine and Hydroxylated (OH) Fatty Acids of Glycosphingolipids Extracted from Monkey Small Intestinal and Colonic Mucosa

	S.I.		Colon	
	% Sphingosine[b]	% OH fatty acids[a]	% Sphingosine	% OH fatty acids
Ceramide	60.3 ± 3.1	63.1 ± 3.9	81.6 ± 4.0	72.9 ± 5.1
Monohexosylceramide	90.1 ± 4.8	88.4 ± 4.6	96.2 ± 4.9	93.4 ± 5.9
Dihexosylceramide	89.7 ± 4.1	91.2 ± 6.0	97.8 ± 3.1	93.6 ± 6.2
Trihexosylceramide	91.2 ± 5.2	N.D.	90.1 ± 5.2	N.D.
Tetrahexosylceramide	88.6 ± 3.7	N.D.	90.3 ± 3.6	N.D.
GM_3	14.1 ± 1.4	70.3 ± 4.7	31.2 ± 1.8	76.4 ± 3.7

[a]Values represent means ± S.E. of three separate preparations and were obtained with the use of methyl heneicosanoate as internal standard.
[b]Values represent means of S.E. of 2-4 determinations of the aldehydes by GLC of three separate preparations.
N.D., not determined.

mucosa (Table 1). Smaller amounts of mono-, tri- and tetrahexosylceramide also were present in each tissue. Additionally, ceramide, a non-sugar-containing compound, was found to be a major component of the mucosal lipids of both organs (Table 1). Earlier studies in rat small intestine (7-9) have shown that mono- and trihexosylceramide were the principal neutral glycosphingolipids, while in rat colon (16) as well as sheep small intestine and colon (17; unpublished observations), mono- and tetrahexosylceramide and not dihexosylceramide were the major neutral glycolipids. These differences support the contention that the pattern of glycosphingolipid composition is species- and tissue-specific (5). Analysis of the sugar moieties of these neutral glycolipids by GLC further confirmed their identities (Table 2).

In order to further elucidate differences between monkey small intestinal and colonic mucosa, the fatty acids and long chain bases of the major ganglioside, GM_3, and the major neutral glycosphingolipids, di- and monohexosylceramide, and ceramide were examined and compared. All samples examined contained both hydroxy and nonhydroxy fatty acids. In agreement with prior studies performed in these organs in a number of other adult species (9,14,16,17), however, hydroxy fatty acids predominated and accounted for 60-90% of the total fatty acids present in both tissues (Table 3). Montanic (28:0) and melissic (30:0) acids were the major hydroxy fatty acids present in small intestinal and colonic GM_3 (Table 4). The former was predominant in the small intestine, whereas the latter was predominant in the colon. Lignoceric (24:0) acid was the major nonhydroxy fatty acid of GM_3 in both tissues (Table 4). In general, the hydroxy fatty acids of GM_3 in both tissues were found to be longer and more saturated than their nonhydroxy counterparts.

The principal hydroxy and nonhydroxy fatty acids of intestinal ceramide were linoleic (18:2) and oleic (18:1) acids, respectively, while in colonic ceramide they were oleic and palmitic (16:0) acids. Stearic (18:0) and montanic acids were the predominant hydroxy and nonhydroxy fatty acids, respectively, of intestinal and colonic monohexosylceramide. As shown in Table 4, however, the predominant hydroxy fatty acid of intestinal and colonic dihexosylceramide was montanic acid, where stearic acid was the principal nonhydroxy fatty acid of this glycolipid in both tissues.

The ceramide backbone of glycosphingolipids in most mammalian tissues is sphingosine (18-sphinganine) (33).

TABLE 4

Fatty Acid Composition of GM_3 and Dihexosylceramide from Monkey Small Intestinal and Colonic Mucosa[a]

	GM_3				Dihexosylceramide			
	S.I.		Colon		S.I.		Colon	
Fatty acids	HFA %	NFA %	HFA %	NFA %	HFA %	NFA %	HFA %	NFA %
16:0	trace	6.6	1.2	3.5	3.1	10.5	trace	trace
16:1	7.2	trace	7.9	trace	trace	trace	trace	trace
18:0	4.3	10.0	2.5	20.1	10.2	52.5	5.8	30.9
18:1	3.2	18.4	trace	4.3	6.4	4.1	13.6	3.6
18:2	trace	trace	trace	trace	4.2	trace	14.2	8.6
20:0	trace	4.3	trace	5.6	trace	trace	trace	trace
20:1	trace	4.2	trace	15.9	trace	trace	4.2	trace
20:2	—	—	—	—	9.3	trace	trace	trace
20:4	—	—	—	—	trace	trace	—	—
22:0	trace	trace	trace	trace	—	—	trace	2.7
22:1	trace	4.4	trace	15.2	trace	trace	trace	6.7
24:0	2.6	36.1	trace	22.6	6.7	7.1	3.8	25.6
26:0	3.4	23.9	7.3	9.7	11.4	4.2	7.4	6.9
28:0	42.2	—	20.8	—	42.5	6.7	43.9	9.2
30:0	31.2	—	50.9	—	—	—	—	—

HFA, Hydroxy fatty acids. NFA, nonhydroxy fatty acids.

[a]Values are means of three separate preparations. S.E. were all less than 5% of the mean value. Trace indicates 2.0% or less of the total.

In certain tissues, however, such as the adult rat small intestine (33) and colon (16), the major base is phytosphingosine (4D-hydroxysphinganine). Several investigators (34-36) previously had suggested an exogeneous origin (dietary or bacterial) for the latter base in the intestine. While this issue remains unsettled, recent studies in the embryonic and adult Japanese quail intestine strongly suggest that in this species, phytosphingosine primarily is synthesized by intestinal tissue (15). In monkey intestinal and colonic mucosa, while sphingosine was found to be the predominant base of ceramide and the neutral glycolipids examined (Table 3), phytosphingosine was the major base of GM_3 in these tissues. These results are interesting since monohexosylceramide serves as a precursor in the biosynthesis of GM_3 via the intermediate dihexosylceramide (8). While speculative, it is possible that the galactosyl- and sialyltransferases involved in the conversion of monohexosylceramide to GM_3 may show base and fatty acid specificity. Thus, the present data might reflect an accumulation of sphingosyl-monohexosylceramide because it is a poor substrate for a transferase relative to phytosphingosyl-monohexosylceramide. Further studies, however, will be necessary to clarify this issue.

In summary, the present studies have characterized the glycosphingolipid composition of monkey small and large intestinal mucosa and have shown that qualitative and quantitative differences exist between these organs. It seems reasonable to suggest that these differences have functional significance and that further studies should clarify the questions of function and mechanism(s) responsible for maintaining these differences in glycosphingolipid composition and content.

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Distribution of Glycosphingolipids and Ceramide of Rat Small Intestinal Mucosa

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Previous studies have suggested that glycosphingolipids may be involved in a number of physiological functions of the small intestinal mucosa. Regional variations in many of these processes exist along the length of this organ. In the present studies, the glycosphingolipid and ceramide composition of the proximal, middle and distal thirds of the rat small intestine were characterized and compared. Mono- and trihexosylceramide were the major neutral glycolipids and hematoside (GM_3), the principal ganglioside of this organ. Monohexosylceramide was the major glycolipid of the proximal segment, whereas trihexosylceramide predominated in the distal segments. The total content of neutral glycolipids, ceramide and gangliosides as well as the content of the individual glycosphingolipids and ceramide were highest in the distal segment, intermediate in the middle and lowest in the proximal segment. Additionally, regional variations were noted in the fatty acid composition of the major glycosphingolipids. These differences in the composition of glycolipids and ceramide along the length of the intestine may be responsible, at least partially, for the regional functional specialization seen in this organ.
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The small intestine performs a number of digestive and transport functions. Regional variation in many of these processes exists along the length of this organ (1,2). In this regard, the small intestine is enriched in glycosphingolipids (3,4), and prior studies have suggested that these substances may be involved in transmembrane transport (4-6) and binding of bacteria and bacterial toxins to intestinal epithelial cells (7,8). In recent years, a number of investigations also have demonstrated alterations in the glycolipid composition of the rat small intestinal mucosa during normal differentiation (9-12) and development (13-15), indicating possible roles for glycosphingolipids in these processes as well.

Although a nonuniform distribution of glycolipids of the small intestine has been shown by immunofluorescence studies (16), to date no studies have been published on the distribution of glycosphingolipids along the length of the rat small intestine. The present investigations were, therefore, undertaken to examine and compare the glycosphingolipid and ceramide compositions of the proximal, middle and distal thirds of the intestinal mucosa. The results of these experiments document that qualitative and quantitative differences in glycosphingolipids and ceramide exist between these segments of the small intestine and may, therefore, be at least partially responsible for the regional functional specialization seen in this organ.

MATERIALS AND METHODS

Materials. The ganglioside standards GM_1, GD_{1A}, GT_{1A} and GT_{1B} were purchased from Supelco (Bellefonte, Pennsylvania). GM_2 isolated from Tay-Sachs brain was a gift from G. Dawson. GM_3 was isolated and purified from rat small intestine by the method of Glickman and Bouhours (9). Ceramide was purchased from Analabs (North Haven, Connecticut). Mono-, di-, tri- and tetrahexosylceramide purified from human erythrocytes were gifts from S. K. Kundu (17). Sphingosine standards and OV-1 3% on Anakron ABS were purchased from Analabs. Phytosphingosine was obtained from Calbiochem (La Jolla, California). Florisil (60-100 mesh) and Fluorescamine (Fluram-Roche) were obtained from Fisher Scientific (Fairlawn, New Jersey). Mixtures of nonhydroxy and hydroxy fatty acid standards, EGSS-X 10% on Gas Chrom P and Power-Sil-Prep were obtained from Alltech/Applied Sciences Lab (State College, Pennsylvania). All other reagents were from Fisher Chemical or Sigma Chemical (St. Louis, Missouri), unless otherwise indicated.

Lipid extraction. Albino male rats of the Sherman strain were maintained on a pelleted diet (Camm Maintenance Rodent Diet) with water and food ad libitum prior to removal of the small intestine. The intestine was divided into proximal, middle and distal thirds and the mucosa of each segment was scraped off with a glass slide. Each mucosa then was homogenized in methanol and the lipids were extracted using chloroform/methanol mixtures of increasing polarity (18).

Glycosphingolipid and ceramide purification and identification by high performance thin layer chromatography (HPTLC). Total lipid extracts were partitioned with water and the lower phase was washed four times with Folch upper phase (chloroform/methanol/water; 3:48:47, v/v/v) to insure complete extraction of gangliosides into the aqueous phase (18). Upper phase gangliosides then were purified by passage through Sep-Pak cartridges as described (19).

The neutral glycolipids and ceramide were purified by chromatography of the acetylated neutral lipids on a Florisil column (20). The gangliosides, deacetylated neutral glycolipids and ceramide were analyzed by HPTLC on Silica-gel 60 precoated plates (E. Merck, Darmstadt, West Germany) in the solvent system chloroform/methanol/water/1% $CaCl_2$ (60:35:7:1, v/v/v/v) (21).

Individual glycolipids and ceramide were quantified after scraping the silica gel area corresponding to their position following migration. The amount of sphingoid bases liberated by methanolysis was determined by fluorimetry after reaction of the free bases with Fluorescamine (20). Quantitation of the various gangliosides was performed using a Beckman densitometer as described by Mullin et al. (21). All of the bands from each ganglioside species were analyzed in the present studies.

Analysis of neutral glycolipids by high performance liquid chromatography (HPLC). Samples and standards first were benzoylated with 10% benzoyl chloride in pyridine at 37 C for 16 hr as described (22). The benzoyl-

ated preparations then each were dissolved in 100 μl of CCl_4 and 20–40 μl injected into the HPLC column.

HPLC analyses were performed as described by Ullman and McCluer (22), using reciprocating pumps (models 6000 and M-45) combined with a universal liquid chromatograph reflector (model U6K) and a solvent programmer (model 660); the equipment was from Waters Associates (Milford, Massachusetts). The chromatographic column was a 50 cm × 2.1 mm (ID) stainless steel tube packed with pellicular silica gel packing (Zipax) (E.I. Dupont de Nemours, Wilmington, Delaware). The column effluent was maintained with a variable wavelength UV spectromonitor (Model 481, Waters Associates, Milford, Massachusetts) and detector output was coupled to a Hewlett-Packard 3390A integrator. Separation of perbenzoylated neutral glycosphingolipids was performed routinely as described (22) using a 10 min linear gradient of 2–17% aqueous ethyl acetate in hexane with a flow rate of 2 ml/min and absorbance measured at 280 nm. After the completion of each run, the gradient was reversed over 2 min and the initial solvent was permitted to flow through the system for at least 5 min before starting another run.

Recovery. Known amounts of each ganglioside standard and each neutral glycolipid/ceramide standard were added to the mucosal samples and carried through the extraction and chromatographic procedures to determine recovery of the various glycosphingolipids and ceramide (23). Recoveries for GM_3, ceramide, mono-, di-, tri- and tetrahexosylceramide were 79 ± 6%, 80 ± 5%, 84 ± 6%, 81 ± 3%, 78 ± 5% and 76 ± 7%, respectively (n = 3). The variations in recoveries for these various lipids were not found to be significantly different.

Desialylation of hematoside. Desialylation of purified hematoside (GM_3) was performed by acid hydrolysis using 0.1 M H_2SO_4 at 80 C for 1.5 hr as described (24). Additionally, purified GM_3 was dried under nitrogen and reconstituted in 100 μl of water, and 100 μl of neuraminidase (*V. cholerae,* Behring Diagnostics) was added. The samples and controls were incubated at 37 C for 15 hr. The reaction was terminated by the addition of 20 vol of chloroform/methanol (2:1, v/v), dried under nitrogen and reconstituted in 100 μl of chloroform/methanol (2:1, v/v). N-glycolyl and N-acetylneuraminic acids were separated on HPTLC plates using a solvent system of n-propanol/water/ammonium hydroxide (60:28:1:5, v/v) (25) and visualized with resorcinol.

Analysis of carbohydrate moieties of hematoside and neutral glycolipids. Carbohydrate moieties were identified by gas liquid chromatographic (GLC) analysis of their trimethylsilyl derivatives as described (9,26).

Analysis of fatty acids and long chain bases of glycosphingolipids and ceramide. Individual glycosphingolipids and ceramide were hydrolyzed in 1 ml of dry methanolic HCl (0.75 N) at 80 C for 16 hr, followed by extraction of fatty acid methyl esters by hexane (27). The methanolic phase then was alkalinized with aqueous NaOH and the long chain bases extracted by diethyl ether (28).

Nonhydroxy and hydroxy fatty acids were separated by chromatography on a Florisil column as described (29). In certain experiments, a known amount of C_{21} fatty acid methyl ester was added as an internal standard to quantitate the nonhydroxy and hydroxy fatty acids in the samples (11). Nonhydroxy fatty acid methyl esters were dissolved in a small volume of hexane, while hydroxy fatty acid methyl esters were silylated prior to chromatography (11). Analyses of both nonhydroxy and hydroxy fatty acids were performed on a Hewlett-Packard 5790A gas liquid chromatograph equipped with a flame ionization detector and interfaced with a Hewlett-Packard 3390A integrator as described (11). Peak identification was based on identical retention times with known standards run under the same conditions (11).

Long chain bases, free from fatty acids, were dissolved in chloroform, washed with 0.1 M NaOH and then repeatedly washed with water. The chloroform phase was dried under N_2 and the residue silylated as described (30). The long chain bases then were analyzed by GLC on an OV-1 column operated with temperature programming from 245 to 305 C at 3 C per min (11). Identification was based on identical retention times with known standards run under the same conditions (11).

RESULTS AND DISCUSSION

Earlier studies have demonstrated that the small in-

TABLE 1

Content and Relative Percentage of Neutral Glycosphingolipids from Different Segments of the Small Intestine Assessed by HPTLC[a]

	Proximal third		Middle third		Distal third	
	Content	%	Content	%	Content[b,c]	%
Ceramide	3.1 ± 0.4	16.1 ± 0.6	6.6 ± 0.8[b]	18.2 ± 0.9	10.7 ± 0.9	17.9 ± 1.0
Monohexosylceramide	9.4 ± 0.8	48.6 ± 1.1	9.6 ± 1.0	23.7 ± 1.1	15.7 ± 0.7	26.2 ± 0.6
Dihexosylceramide	0.6 ± 0.1	3.1 ± 0.1	1.3 ± 0.2	3.6 ± 0.3	2.1 ± 0.2	3.5 ± 0.2
Trihexosylceramide	4.6 ± 0.6	23.9 ± 0.8	14.4 ± 1.6[b]	39.7 ± 1.6	21.7 ± 1.8	36.3 ± 1.7
Tetrahexosylceramide	0.6 ± 0.3	8.1 ± 0.4	5.4 ± 0.8[b]	14.8 ± 0.9	9.6 ± 0.5	16.1 ± 0.4
Total	19.23 ± 1.1		37.27 ± 1.4[b]		59.89 ± 1.5	

[a]Values represent means ± S.E. of six determinations of three separate preparations. Content values are expressed as nmol sphingosine/mg protein.

[b]$p < 0.05$ or less compared to proximal third values.

[c]$p < 0.05$ or less compared to middle third values.

TABLE 2

Content and Relative Percentages of GM_3 and Other Gangliosides from Different Regions of the Small Intestinal Mucosa[a]

	Proximal third		Middle third		Distal third	
	Content	%	Content	%	Content	%
GM_3	5.1 ± 0.5	82.1 ± 0.6	6.7 ± 0.6[b]	91.0 ± 0.5	8.7 ± 0.6[b,c]	89.0 ± 0.5
Others	1.1 ± 0.2	17.9 ± 0.3	0.7 ± 0.2	9.1 ± 0.2	1.1 ± 0.3	11.1 ± 0.2
Total	6.3 ± 0.5		7.4 ± 0.6		9.8 ± 0.5[b,c]	

[a]Values represent means ± S.E. of six determinations. Content values are expressed as μg NeuAc/mg protein.
[b]$p < 0.05$ compared to proximal values.
[c]$p < 0.05$ compared to middle values.

testine of a number of different mammalian species including the mouse (31–34), pig (35), cow (36), dog (37,38) and human (38) possess a complex mixture of glycosphingolipids. In 1973, Forstner and Wherrett (4) published the first extensive description of the glycosphingolipid composition of the rat small intestinal mucosa. These investigators demonstrated that glycolipids accounted for ca. 20% of plasma membrane lipid, which reflected the relatively high content of these substances in the whole small intestine (3). Subsequently, a number of laboratories demonstrated alterations in the glycolipid composition of this organ in the rat during normal differentiation (9–12) and development (13–15). Recently, Breimer et al. (39) also have shown that epithelial and nonepithelial cells of the rat small intestine possess different glycolipids. Although Forstner and Wherrett (4) postulated that variation in the glycolipid composition along the length of the rat small intestine might be responsible for regional transport differences, the present study is the first to characterize and compare the glycosphingolipid pattern in the various regions of this organ in the rat.

The present data indicate that qualitative and quantitative differences in glycosphingolipids and ceramide exist in the various regions of the rat small intestinal mucosa. The results of the analyses of the content and relative percentages of the neutral glycosphingolipids, ceramide and the gangliosides by HPTLC are summarized in Tables 1 and 2. As shown in Table 1, the total content of neutral glycosphingolipids and ceramide increased along the length of the intestine, i.e., distal third > middle third > proximal third. The intestinal ganglioside content demonstrated a similar, albeit less marked, pattern (Table 2).

In agreement with earlier studies performed on the entire rat small intestinal mucosa (9–11), mono- and trihexosylceramide were the major neutral glycolipids and GM_3 was the principal ganglioside of this organ (Tables 1 and 2). In terms of content and relative percentage, monohexosylceramide was the major neutral glycolipid of the proximal segment, whereas trihexosylceramide predominated in the middle and distal segments (Table 1). It should be noted, however, that the content of each neutral glycosphingolipid and ceramide was found to be highest in the distal segment, intermediate in the middle segment and lowest in the proximal segment (Table 1).

TABLE 3

Relative Percentage of N-Acetyl and N-Glycolylneuraminic Acid in Hematoside of Different Segments of Small Intestine[a]

Form of sialic acid	Proximal third	Middle third	Distal third
N-acetyl	20.6 ± 2.1	11.8 ± 1.7	17.6 ± 2.1
N-glycolyl	79.4 ± 3.2	88.4 ± 2.5	81.3 ± 3.6

[a]Values represent means ± S.E. of three separate preparations.

Further analysis of the neutral glycosphingolipids of the various segments by HPLC confirmed that monohexosylceramide (42.1 ± 3.1%, n = 3) was the principal neutral glycolipid of the proximal segment, whereas trihexosylceramide predominated in the middle (40.7 ± 3.2%, n = 3) and distal (31.3 ± 2.9%, n = 3) intestinal segments. As shown in Table 2, while the relative percentage of GM_3 was similar (80–90%) in all regions, the content of GM_3, like the neutral glycolipids, increased distally.

Analysis of the sugar moieties of the glycosphingolipids by GLC further established their identities (not shown). In agreement with Forstner and Wherrett (4), monohexosylceramide in rat intestine contained only glucose and tetrahexosylceramide contained a small amount of fucose. The molar ratios of the sugar moieties of each glycosphingolipid were similar in all regions of the intestine (not shown).

After treatment with neuraminidase as well as after acid hydrolysis, the sialic acid moieties of GM_3 were found to contain N-acetyl and N-glycolylneuraminic acid (Table 3). The latter form, however, predominated in all intestinal segments. This finding confirms earlier studies by Glickman and Bouhours (9) performed in the entire adult rat small intestinal mucosa.

In order to further detect differences between compositions of the glycosphingolipids of the various intestinal segments, the fatty acids and long chain bases of the major ganglioside, GM_3, and the major neutral glycolipids, mono- and trihexosylceramide, were examined and compared. Earlier studies by Bouhours and Glickman (11) showed that the fatty acids of GM_3 and monohexosylceramide were predominantly hydroxylated, whereas

TABLE 4

Hydroxy and Nonhydroxy Fatty Acid Composition of Mono- and Trihexosylceramide and GM_3 from Different Segments of Small Intestinal Mucosa[a]

	Monohexosylceramide						Trihexosylceramide								GM_3			
	Proximal		Middle		Distal		Proximal		Middle		Distal		Proximal		Middle		Distal	
Fatty acids	HFA % (69.3)[b]	NFA % (30.6)	HFA % (70.2)	NFA % (29.3)	HFA % (68.4)	NFA % (31.6)	HFA % (31.0)	NFA % (69.1)	HFA % (29.7)	NFA % (70.4)	HFA % (31.1)	NFA % (68.9)	HFA % (70.3)	NFA % (29.6)	HFA % (73.2)	NFA % (27.0)	HFA % (71.4)	NFA % (28.7)
16:0	20.9	21.3	3.0	18.2	4.0	9.2	20.0	8.2	6.8	10.1	8.9	10.0	20.5	9.3	trace	20.7	11.6	15.1
16:1	—	—	—	trace	—	trace	—	trace	—	—	—	—	trace	—	—	—	—	trace
18:0	15.7	32.7	4.0	17.2	trace	2.1	34.1	26.7	18.7	40.0	9.8	29.7	13.5	6.1	42.4	15.3	8.9	16.3
18:1	17.6	8.0	2.4	14.2	trace	trace	trace	5.3	5.2	—	trace	4.2	trace	7.2	2.9	17.3	4.8	trace
18:2	2.8	5.1	trace	17.3	trace	trace	trace	3.6	—	—	trace	3.2	trace	—	3.5	trace	3.9	trace
20:0	25.2	5.8	trace	—	9.8	4.5	25.0	13.4	17.8	4.2	13.5	4.5	36.0	50.0	8.4	23.8	3.5	2.6
20:4	trace	—	—	—	trace	trace	—	—	—	—	trace	trace	—	—	—	trace	—	—
22:0	8.2	2.7	5.1	12.2	12.4	4.2	5.3	trace	8.4	9.9	13.3	3.0	8.3	16.1	35.4	8.4	trace	trace
24:0	7.2	6.2	71.2	15.1	71.0	80.1	6.2	30.0	27.4	10.2	31.0	35.4	12.9	5.8	3.2	12.4	69.0	61.4
26:0	—	—	2.2	—	trace	trace	—	—	trace	12.6	4.0	trace	trace	trace	trace	trace	—	—
28:0	—	6.9	10.7	trace	—	—	trace	6.3	trace	—	—	—	—	—	—	—	—	—

HFA, hydroxy fatty acids. NFA, nonhydroxy fatty acids.

[a]Values are means of three separate preparations. S.E. were all less than 5% of mean value. Trace indicates 2.0% or less of the total.

[b]Values in parentheses represent the relative percentage of hydroxy or nonhydroxy fatty acids for each glycosphingolipid extracted from different segments of the rat small intestine. Values reported are the means of three separate preparations and were obtained with the use of methylheneicosanate as internal standard. S.E. were all less than 5% of mean values.

trihexosylceramide possessed mainly nonhydroxylated fatty acids in whole rat intestinal mucosa. The data in Table 4 are consistent with these authors' findings and furthermore show no evidence of regional variation in the percentage of hydroxylation of the fatty acids in these glycolipids.

Arachidic (20:0), stearic (18:0) and lignoceric (24:0) acids were the major hydroxy fatty acids of GM_3 in the proximal, middle and distal segments, respectively, whereas arachidic acid was the principal nonhydroxy fatty acid of this ganglioside in the proximal and middle segments, and lignoceric acid predominated in the distal third (Table 4). As shown in Table 4, arachidic acid was also the major hydroxy fatty acid of monohexosylceramide in the proximal segment, while lignoceric acid was highest in the more distal segments. Lignoceric acid also was found to be the predominant nonhydroxy fatty acid of this neutral glycolipid in the distal third, but in the proximal two segments, stearic and palmitic (16:0) acids predominated (Table 4). Stearic acid was the major hydroxy fatty acid of trihexosylceramide in the proximal third, while lignoceric acid predominated in the more distal segments. The latter fatty acid also was found to be the major nonhydroxy fatty acid in the proximal and distal thirds, but stearic acid predominated in this glycolipid in the middle segment (Table 4). It is clear from this data that marked variations in the fatty acid compositions of the major glycosphingolipids occurred along the length of the small intestine.

In contrast to these findings, analyses of the long chain bases of these major glycolipids revealed that phytosphingosine (4D-hydrosphingamine) was the predominant base of GM_3, mono- and trihexosylceramide in all regions of this organ (Table 5). Several investigators have suggested an exogenous origin for phytosphingosine in the intestine (40–42). Recent studies in Japanese quail intestine, however, suggest strongly that at least in that species, this base is synthesized by the intestine (43). If this is also true for the rat, the present data would suggest that all segments of this organ can synthesize phytosphingosine.

In summary, the present studies have for the first time characterized and compared the glycosphingolipid and ceramide content and composition of the proximal, middle and distal thirds of the rat small intestine. The results demonstrate that both qualitative and quantitative differences of these substances exist along the length of the

TABLE 5

Percentage of Sphingosine (S) and Phytosphingosine (P) in Glycosphingolipids of Different Segments of Small Intestinal Mucosa[a]

	Proximal third		Middle third		Distal third	
Glycosphingolipid	S	P	S	P	S	P
Monohexosylceramide	6.9	71.4	7.4	73.6	7.2	72.8
Trihexosylceramide	5.6	74.2	6.6	73.9	6.4	67.8
Hematoside	8.0	71.1	7.1	68.8	7.6	67.4

[a]Each value represents the mean of three determinations of the aldehydes by GLC (11,43). Percentages do not add up to 100% because of other bases as yet unidentified.

intestine. It seems reasonable to suggest that these variations have functional significance and that further studies should clarify the questions of function and mechanisms responsible for maintaining these regional differences in glycosphingolipid and ceramide distribution.

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Comparison of Fatty Acids and Lipids of Smolting Hatchery-Fed and Wild Atlantic Salmon *Salmo salar*

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In Atlantic Canada the Atlantic salmon *Salmo salar* change from the parr stage to the smolt stage while still in fresh water, preparatory to migration to salt water. In some stocks this takes place during the second overwintering. In several hatcheries where the water temperature drops to 0–0.5 C and the ponds ice over, there is a high incidence of erosion of the dorsal and pectoral fins and sometimes of the caudal fin. No disease organism has been identified, and the lesions heal over in most cases. Dietary fatty acids were thought to be a factor. A detailed study of lipid recoveries and classes has shown that in the skins of abnormal fish the total lipid is 7.8% compared to 4.7% in control fish.

Unexpectedly, an analysis of one lot of healthy smolt-stage wild fish showed that whole bodies have only a quarter of the lipid of comparable hatchery fish. Comparison of fatty acids showed that wild fish lipids include a higher proportion of arachidonic acid than those of the hatchery fish. In the latter, linoleic acid is provided readily by diet but the elongation to arachidonic acid evidently does not proceed. These results suggest that the smolt lipid is involved intimately with either the cause of the dermal lesion or is a defense mechanism, possibly mediated through oxygenase activity.

Lipids 21, 117–120 (1986).

Much of the diet work on salmonids and other fish is devoted to maximizing growth (1–12, to cite only a few recent papers). Biochemistry is part of this focus but generally receives less attention than nutrition (see, however, 13–16). Few of the species investigated are exposed to stress comparable to that induced by the overwintering and smoltification process in salmon parr. To quote Folmar and Dickhoff (14), "Until recently, little attention has been paid to the after release or to the important changes that occur during the parr-smolt transformation. It is during this metamorphosis to a salt-tolerant form that the fish are most sensitive to hatchery conditions and are profoundly affected by husbandry practices such as rearing densities, disease therapy and food quality."

The Atlantic salmon *Salmo salar* generally is recognized as an invaluable and yet ever-decreasing fisheries resource. In an attempt to promote the continued return of the adult salmon, Fisheries and Oceans Canada maintains hatching and rearing facilities in the Atlantic provinces and Quebec to rear salmon to the smolt stage, when they can be released to make their way to the ocean. It is widely recognized that hatchery-reared salmonids, although apparently healthy, may be at a physiological disadvantage compared to normal healthy fish in streams of the same watershed. A question arose in Atlantic Canada concerning the nutritional aspects of the "torpedo" (Atlantic salmon) smolt suffering from fin erosion. These fish are held over one winter and smoltify (1+) if large enough, or smoltify after a second winter (2+); the damage is linked to water temperature approaching 0 C during overwintering.

Jezierska et al. (17) examined the fatty acid changes in starving rainbow trout *(Salmo gairdneri).* There was a tendency for saturated and monoethylenic fatty acids to be mobilized and polyunsaturated fatty acids to be retained. However, they examined fatty acids of total lipid. Since all tissue has a base level of about 0.6% phospholipid of typical composition (18), any other lipid of different fatty acid composition (usually triglyceride) only need change in relative proportion to alter the results of fatty acid analyses in such a study. Our long-term research into this problem includes lipid class quantitation, separation and recovery for individual fatty acid analyses. By including wild salmon parr and smolt provided by the cooperation of Fisheries and Oceans Canada we hoped to provide a new data base against which the composition of hatchery fish fed different hatchery diets can be measured. The starting fish analyses provided *Salmo salar* lipid and fatty acid data not to our knowledge available elsewhere and presented herewith.

MATERIALS AND METHODS

Hatchery-reared Atlantic salmon parr (in their second year) were provided from two hatcheries operated by Fisheries and Oceans Canada on the Cobequid and Margaree drainage systems. Wild parr of comparable size were selected from fish electrofished in the Margaree system in November 1984. The diet received ad lib by the hatchery fish from mid-August was a practical diet including fish meal, fish oil, all necessary minerals, etc. It had 12–13% ether-extractable fat. The main recovered fatty acids (as w/w % of diet fatty acids) were saturated, 20.3% (14:0, 4.6%; 16:0, 12.0%); monoene, 52.3% (16:1, 9.0%; 18:1, 14.6%; 20:1, 12.9%; 22:1, 14.5%); and polyene, 27.4% (18:2n6, 9.1%; 18:3n3, 0.9%; 20:4n6, 0.3%; 20:5n3, 5.1%; 22:6n3, 5.5%).

Analyses of tissues followed published procedures for lipid recovery in chloroform/methanol and separation of lipid classes by plate thin layer chromatography (TLC) (19), lipid class quantitation by Iatroscan TLC/FID (20,21) and fatty acid analyses by open-tubular gas liquid chromatography (GLC) on a Supelcowax-10 (bonded carbowax 20M) flexible fused silica column (22–24).

RESULTS

Although a long-term feeding study with varying levels of n-3 polyunsaturated fatty acids is intended, our fall 1984 results for starting fish raised on a "normal" diet and wild fish (Tables 1–3) already indicate three unusual aspects of lipid metabolism. One is the high level of fat in the skin lipids of "abnormal" (i.e., fin erosion) fish com-

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TABLE 1

Lipid Classes of Atlantic Salmon from Normal and Abnormal Whole Bodies and Skins at Maragree Fish Culture Station

Date	% in Fish			
	TL	PL	FS	TG
Nov. 21, 0 (starting)				
Whole bodies				
Normal	6.6 ± 0.20[a]	1.3 ± 0.42	0.3 ± 0.02	5.1 ± 0.02
Abnormal	7.9 ± 0.28	1.2 ± 0.11	0.1 ± 0.05	6.7 ± 0.36
Skins[b]				
Normal	4.7	0.7	0.2	3.9
Abnormal	7.8	1.6	0.3	5.9

[a]Mean ± S.D. (n = 3).
[b]Pooled.

TABLE 2

Lipid Classes of Atlantic Salmon from Whole Bodies and Stomach-Intestine Contents

Date	% in Fish				
	TL	PL	FS	TG	FFA
Nov. 13					
Whole bodies	1.7 ± 0.03[a]	1.0 ± 0.04	0.2 ± 0.02	0.4 ± 0.05	0.03 ± 0.006
Stomach-intestine contents[b]	5.1	2.8	0.3	2.0	—

[a]Mean ± S.D. (n = 3).
[b]Pooled.

pared to the "normal" controls of hatchery trout, a second is the low level of lipid in muscle of wild salmon compared to hatchery stock and third is the importance in the wild fish of n-6 compared to n-3 fatty acids.

DISCUSSION

Very low temperatures are not a problem in Pacific salmonid hatcheries (see, for example, papers in the special issue of *Aquaculture* entitled "Salmonid Smoltification" [13]; only the paper by Saunders et al. [10] addresses this Atlantic problem), and not in all Atlantic area hatcheries. These authors state, "Under most hatchery conditions, even with low (1-2 C) temperatures for much of the winter, low lipid levels in mature male parr would be of much less consequence than for parr in streams." Despite this, levels of fat of the order of 4-8% have been reported in Atlantic hatchery-fed fish (25) and presuppose adequate energy, but do not indicate the optimum availability of fatty acids necessary for organ and membrane function.

The distinctive changes in external markings in the parr-smolt transformation (26) show that changes in the skin are in progress. In some species of fish the subdermal fat is a major fat storage zone. Capelin and mackerel usually have nearly 50% of total fat stored subdermally (18). In salmonids there usually is a deposit of fat in the visceral cavity (18,27), but the proportion of fat in the subdermal region has been found to be 50% more than in muscle of rainbow trout (28). The presence of astaxanthin in diester form in these rainbow trout skin lipids but in free form in the muscle lipid (28) emphasizes the importance of subdermal lipids, their possible biochemical significance and a difference from other lipids of the same fish.

A variety of disease organisms, some of which have proven controllable with dietary modifications, threaten hatchery operations (20,29,30). It is known that the fin erosion sites are subject to bacterial infiltration (Morrison, C., personal communication), but not whether this is a causative agent. The damage to fins does not necessarily lead to immediate Atlantic salmon mortalities. The former fin sites may still show spines but sometimes are smoothly healed over. These fish may be sluggish in behavior but eventually resume feeding and are released in the normal way. Once released they could well be at a disadvantage relative to predators.

The fatty acid analyses (Table 3) show interesting differences between hatchery and wild fish. Thus in phospholipids the total for 18:3n3 and 20:5n3 is the same. The 18:2n6 is twice as high in hatchery fish as in wild fish, but 20:4n6 is at least six times higher in wild fish than in hatchery fish. This enrichment in n-6 fatty acids extends to 22:4n6 and 22:5n6 in wild fish phospholipids. The hatchery fish have ample 18:2n6, as shown by the triglyceride analyses, but do not convert it to 20:4n6. In the wild fish, which must live largely on aquatic insects, the 18:2n6 is somewhat less in triglycerides than in hatch-

TABLE 3

Comparison of Fatty Acid Composition of Cultured and Wild Salmon (wt %)

	Cobequid, Oct. 4			Margaree, Nov. 21			Wild salmon, Nov. 13		
Fatty acid	TL	PL	TG	TL	PL	TG	TL	PL	TG
14:0	3.8 ± 0.09[a]	1.6 ± 0.03	4.1 ± 0.12	4.1 ± 0.15	1.6 ± 0.15	4.4 ± 0.12	1.5 ± 0.01	1.1 ± 0.07	3.1 ± 0.08
16:0	12.7 ± 0.20	17.0 ± 0.77	12.0 ± 0.12	12.9 ± 0.16	18.6 ± 0.88	11.5 ± 0.20	14.2 ± 0.14	18.7 ± 1.04	12.8 ± 0.27
18:0	2.5 ± 0.05	2.7 ± 0.25	2.5 ± 0.00	2.5 ± 0.04	3.3 ± 0.10	2.4 ± 0.05	5.3 ± 0.04	5.2 ± 0.04	5.3 ± 0.05
Σ Saturates	20.7 ± 0.31	23.6 ± 0.93	20.3 ± 0.28	21.1 ± 0.27	25.6 ± 1.29	20.0 ± 0.16	24.4 ± 0.06	27.9 ± 1.14	25.3 ± 0.22
16:1n7	7.6 ± 0.12	2.3 ± 0.10	8.2 ± 0.12	8.2 ± 0.12	2.5 ± 0.16	8.6 ± 0.19	5.5 ± 0.10	2.7 ± 0.18	11.4 ± 0.46
18:1n9	15.9 ± 0.19	8.3 ± 0.57	17.0 ± 0.17	16.9 ± 0.20	8.4 ± 0.22	17.9 ± 0.13	12.6 ± 0.19	9.0 ± 0.57	19.2 ± 0.18
20:1n9+11	10.9 ± 0.18	2.5 ± 0.17	12.2 ± 0.19	10.1 ± 0.05	2.0 ± 0.04	11.8 ± 0.11	0.8 ± 0.05	0.4 ± 0.07	1.2 ± 0.03
22:1n11+13	8.8 ± 0.16	0.7 ± 0.37	9.9 ± 0.44	7.5 ± 0.12	0.6 ± 0.06	9.0 ± 0.21	0.2 ± 0.03	0.1 ± 0.00	0.2 ± 0.03
Σ Monoenes	50.7 ± 0.56	19.6 ± 0.51	55.1 ± 0.47	49.6 ± 0.25	17.8 ± 0.53	54.6 ± 0.31	26.5 ± 0.13	18.0 ± 0.74	40.8 ± 0.16
18:2n6	8.1 ± 0.11	3.7 ± 0.21	8.8 ± 0.04	9.6 ± 0.12	4.0 ± 0.05	10.3 ± 0.11	3.1 ± 0.06	1.7 ± 0.06	5.2 ± 0.16
Dienes	9.2 ± 0.27	4.7 ± 0.25	10.2 ± 0.10	11.1 ± 0.11	5.1 ± 0.06	12.1 ± 0.25	4.5 ± 0.17	2.5 ± 0.06	8.3 ± 0.19
18:3n3	0.7 ± 0.01	0.3 ± 0.03	0.8 ± 0.01	0.8 ± 0.02	0.3 ± 0.00	0.8 ± 0.02	2.2 ± 0.05	1.5 ± 0.05	3.0 ± 0.13
Σ Trienes	1.9 ± 0.09	1.5 ± 0.17	1.8 ± 0.04	1.9 ± 0.02	1.5 ± 0.08	2.0 ± 0.07	4.1 ± 0.07	2.8 ± 0.04	5.1 ± 0.16
20:4n6	0.5 ± 0.07	1.6 ± 0.04	0.3 ± 0.01	0.5 ± 0.01	1.8 ± 0.06	0.3 ± 0.02	8.0 ± 0.06	10.4 ± 0.14	2.8 ± 0.14
22:4n6	0.2 ± 0.04	0.3 ± 0.21	0.2 ± 0.02	0.2 ± 0.08	0.2 ± 0.01	0.2 ± 0.04	1.3 ± 0.05	1.2 ± 0.29	0.8 ± 0.02
Σ Tetraenes	2.4 ± 0.13	3.5 ± 0.23	2.2 ± 0.06	2.3 ± 0.09	3.2 ± 0.08	2.1 ± 0.06	11.4 ± 0.03	13.4 ± 0.26	5.5 ± 0.21
20:5n3	2.5 ± 0.05	7.7 ± 1.11	1.7 ± 0.05	2.1 ± 0.01	6.6 ± 0.23	1.4 ± 0.03	4.6 ± 0.01	5.5 ± 0.10	2.1 ± 0.07
22:5n6	0.4 ± 0.06	0.7 ± 0.55	0.2 ± 0.01	0.4 ± 0.25	0.6 ± 0.03	0.2 ± 0.03	2.0 ± 0.08	2.6 ± 0.21	0.7 ± 0.02
22:5n3	1.0 ± 0.09	1.9 ± 0.62	0.9 ± 0.04	1.1 ± 0.13	1.6 ± 0.09	1.0 ± 0.07	3.3 ± 0.15	3.4 ± 0.39	2.4 ± 0.10
Σ Pentaenes	3.8 ± 0.04	10.4 ± 2.28	2.8 ± 0.07	3.5 ± 0.38	8.7 ± 0.31	2.5 ± 0.08	9.9 ± 0.22	11.5 ± 0.49	5.2 ± 0.17
22:6n3	9.7 ± 0.22	35.1 ± 2.62	6.2 ± 0.20	9.6 ± 0.09	36.2 ± 2.35	5.7 ± 0.07	15.4 ± 0.27	21.8 ± 0.67	4.1 ± 0.16
Iodine value (cal.)	130.2 ± 0.17	239.7 ± 2.12	114.6 ± 1.20	130.4 ± 1.51	234.2 ± 10.54	114.5 ± 0.45	184.9 ± 1.06	212.0 ± 5.98	121.8 ± 1.48
Σ n6	10.2 ± 0.06	7.5 ± 0.43	10.5 ± 0.05	11.9 ± 0.23	7.7 ± 0.07	12.2 ± 0.11	15.8 ± 0.04	16.5 ± 0.61	10.8 ± 0.37
Σ n3	15.6 ± 0.15	46.2 ± 0.72	11.1 ± 0.30	14.8 ± 0.25	45.4 ± 2.61	10.2 ± 0.06	27.2 ± 0.40	33.2 ± 0.86	13.6 ± 0.63

[a]Mean ± S.D. (n = 3).

ery fish. The 20:4n6 is probably available preformed from aquatic insects. In one survey (31) the ratio of 18:2n6 to 20:4n6 in such insects was about 2:1. It appears that wild Atlantic salmon parr have access to adequate supplies of both 18:2n6 and 20:4n6 but selectively accumulate 20:4n6 from the diet. Hatchery fish have ample 18:2n6 but do not convert it to 20:4n6, possibly because of a ready supply of 20:5n3, 22:5n3 and 22:6n3.

Recently, an unexpected report based on observations in a food science laboratory on species-specific aromas in fish has linked these "fishy" aromas to aldehydes (32); later the same group showed that formation of these aldehydes could be suppressed by acetylsalicylic acid and other agents capable of interfering with the capabilities of cycloxygenases (33). The appearance of fin erosion in salmon smolting at low temperatures may reflect some such involvement of metabolic products in polyunsaturated fatty acids.

It is reasonable to ask why this does not occur in the first overwintering of parr. The smolting process is a complex one which stresses the fish severely. There are changes in lipid composition (34), although the skin has not, to our knowledge, been investigated at this stage. Moreover, there are definite differences in enzyme activity in sites such as the liver (35). Our long-term study is as yet incomplete, but indicates that lipids, and possibly the type of polyunsaturated fatty acids, are key components in healthy smolting of Atlantic salmon.

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Retention of Linoleic Acid in Carcass Lipids of Rats Fed Different Levels of Essential Fatty Acids

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Rats of an inbred Sprague-Dawley strain were fed purified diets with low (0.3% of total energy), normal (3%) or high (10%) content of essential fatty acids (EFA) for at least three generations. Two 30-day-old rats with similar weights were chosen from one litter. One was killed; weight increase and food consumption of the other rat was measured for 15 days. Total lipid content and fatty acid composition in total lipid and lipid classes were determined in both rats. Seven pairs of rats from each group were treated in the same way. Calculations based on amount of linoleic acid ingested and retained in the carcass lipids showed that 50% of the ingested linoleic acid was retained in the low EFA rats compared to 10–15% in the normal and high EFA rats.
Lipids 21, 121–126 (1986).

The dietary level of EFA influences the utilization and metabolism of linoleic acid (1,2). Thus, a low level of EFA in the diet leads to low levels of linoleic acid in the tissue lipids. However, studies by Alling and coworkers (3) indicated that rats fed diets with low levels of EFA accumulated more of the dietary linoleic acid in the body fat than did rats fed a diet with a normal EFA content. In previous short-term studies, we have shown that rats fed low levels of EFA (0.3% of total energy) retained more ^{14}C-activity in the carcass from a single oral or intravenous dose of ^{14}C-labeled linoleic acid than rats fed normal (3%) or high (10%) levels (4,5). Using diets similar to those described by Alling and coworkers (3,6), we aimed in the present balance study to measure the retention of dietary linoleic acid in the body fat during a longer period in rats fed balanced diets with a low, normal or high content of EFA.

MATERIALS AND METHODS

Diets. The rats were fed pelleted, purified diets supplying 0.3% (low), 3% (normal) or 10% (high) of total energy as EFA (Table 1). The fatty acid composition of the diets is shown in Table 2. The ratio between linoleic and linolenic acid was 4:1 in both the normal and high EFA diets. The contribution of EFA from the fish protein and wheat starch preparations in the low EFA diet also was determined. One g of either fish protein or wheat starch was hydrolyzed with 7 M hydrochloric acid after addition of ethanol. The mixture was heated on a steam bath for one hr and the homogenate was extracted with ethyl ether and ethyl ether/petroleum ether (1:1, v/v). Methyl esters were prepared as described below and quantitatively determined by gas liquid chromatography (GLC), using heptadecanoic acid (17:0) and Δ7,10,13,16-docosatetraenoic acid (22:4ω6) as internal standards. The ratio between linoleic acid and total ω3 acids was ca. 7:1 in the low EFA diet.

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Rats. Rats from an inbred Sprague-Dawley strain were used (3,6). Females were mated at the age of 90 days. On the third or fourth day after birth, each litter was reduced to six animals, usually three males and three females. The dam and her offspring were housed together for 25 days. The pups then were separated from the mother and fed the experimental diets. Male rats from the fourth generation or more on the experimental diets (second for the high EFA group) weighing within ±2 SD of the mean representative of the given diet and age were selected. Two rats aged 30 days and with similar weights were chosen from one litter. One was killed immediately (30-day reference). The other (45-day) was placed alone in a cage and food consumption and weight were measured for 15 days. The rat then was killed. Gastrointestinal contents of the 30- and 45-day-old rats were removed and the carcasses were frozen until lipid analysis was performed. Seven pairs of rats from each dietary group were treated in the same way.

Quantitative determination of lipids. Samples of the diets were extracted with chloroform/methanol (C/M) (2:1, v/v) and the lipid content was determined gravimetrically according to Folch et al. (7). The frozen carcass was thawed for 15–30 min at room temperature and cut into thin (3–5 mm) slices with a knife. The slices were put into a 2-l Erlenmeyer flask containing 1 l C/M (1:1, v/v). After standing overnight with magnetic stirring, the solvent

TABLE 1

Composition of Diets

	Low EFA	Normal EFA	High EFA
Energy percent EFA	0.3	3.0	10.0
Energy percent fat	20	20	20
Energy percent protein	15	15	15
Constituents, g/kg diet			
Fish protein	175	175	175
Wheat starch	608	608	608
Sucrose	51	51	51
Fat mixture	91	91	91
Hydrogenated tallow	91	67.2	16.8
Sunflower seed oil	—	28.3	57.0
Linseed oil	—	5.5	17.2
Salt mixture[a]	39	39	39
Vitamin mixture[b]	2.0	2.0	2.0
Choline chloride	2.0	2.0	2.0
Cellulose	35	35	35

[a]USP 17 + per kg of salt mixture 0.088 g $KAl(SO_4)_2 \cdot 12\ H_2O$ 0.28 g NaF; 0.009 g $NaAsO_2$; 0.022 g $Na_2B_4O_7 \cdot 10\ H_2O$ and 0.0031 g $Na_2MoO_4 \cdot 2\ H_2O$. Selenium content was analyzed and found to be 0.46 mg/kg in all diets.

[b]Vitamins in 1 kg of diet: retinol 300 μg; ergocalciferol 12.5 μg; thiamin 50 mg; riboflavin 20 mg; pyridioxine 20 mg; nicotinamide 200 mg; panthotenic acid 100 mg; p-aminobenzoic acid 100 mg; menaquinone 10 mg; biotin 1 mg; folic acid 5 mg; cyanocobalamin 0.005 mg; myoinositol 1,000 mg, and tocopheryl acetate 500 mg.

TABLE 2

Fatty Acid Composition of Diets

Fatty acid	Wt % fatty acids		
	Low EFA	Normal EFA	High EFA
14:0	1.7	1.4	0.5
16:0	27.2	23.2	11.0
18:0	64.3	53.4	18.9
20:0	2.7	2.3	0.9
22:0	0.8	0.7	0.7
Σ Saturated	97.9	81.7	32.0
18:1ω9	0.7	5.0	16.9
18:2ω6	1.3	10.8	41.5
18:3ω3	tr	2.5	9.6
ω6/ω3	7[a]	4.3	4.3
mg 18:2/100 g diet	117	898	3490

[a]Based on separate analyses of fish protein and wheat starch.

was filtered into a 2-l flask. The slices were re-extracted with 500 ml C/M for 5–6 hr and homogenized in a Waring blender; the homogenate again was extracted with 500 ml C/M. The solvent was filtered into the flask and the volume adjusted to 2 l. The total lipid content in an aliquot of the extract then was determined gravimetrically after the extract had been freed from nonlipid contaminants by phase partition (8). The remainder of the carcass was hydrolyzed with 7 M hydrochloric acid after addition of some ethanol. The mixture was heated on a steam bath overnight or until the carcass was dissolved. Ten ml of this homogenate was extracted with 25 ml ethyl ether and 30 ml ethyl ether/light petroleum (1:1, v/v). The solvent then was evaporated and the lipid-like material weighed. This material represented 2–5% of the total carcass lipid. The quantity of fatty acids in this residue also was determined by GLC using heptadecanoic acid (17:0) and Δ7,10,13,16-docosatetraenoic acid (22:4ω6) as internal standards (see below) and was found to represent 1–2% of the total carcass fatty acids. The fatty acid composition of the homogenate did not show any important differences compared to the fatty acid composition of the C/M extract.

For quantitative determination of lipid classes, aliquots of the C/M extracts of the reference and experimental rats on the respective diets were pooled. Lipid phosphorus was assayed by a Bartlett method (9). Both cholesterol esters (10) and triglycerides (11) were determined colorimetrically.

Separation of lipids. The lipids of the pooled C/M extracts were separated into lipid classes by thin layer chromatography (TLC) (12). Portions of the cleaned lipid extracts containing 5–10 mg lipid were applied as 15-cm broad bands on 20 × 20 cm thin layer plates coated with 0.2 mm silica gel 60 (E. Merck AG, Darmstadt, West Germany). The plates were developed with 100 ml light petroleum/diethyl ether/glacial acetic acid (85:15:1, v/v/v). The plates were sprayed with water and the bands containing total phospholipids (PL) and triglycerides (TG) were transferred to glass tubes with Teflon screw caps. The tubes were dried overnight in a vacuum desiccator over P_2O_5.

Fatty acid analysis. The TG fatty acids were transmethylated with 1 ml of 2 vol% sulphuric acid in dry methanol at 100 C for 1 hr. The fatty acids of the PL were transmethylated with 1 ml of 0.1 M sodium methylate (12), whereas the fatty acids in the diet and carcass lipids were transmethylated using 14% BF_3 (13). The methyl esters were injected into a Hewlett-Packard 5830A gas chromatograph equipped with a flame ionization detector, a 25 m capillary column coated with P 1000 (Supelco, Bellefonte, Pennsylvania) and a Hewlett-Packard 18835B capillary inlet system. Helium was used as the carrier gas (2 ml/min). The column temperature was programmed as follows: 1 min at 100 C, then 30 C/min to 170 C and finally 4 C/min up to 210 C. The peaks were identified by comparing retention times with those of commercial GLC reference standards (Nu-Chek-Prep, Elysian, Minnesota) and quantified with a Hewlett-Packard electronic integrator model 18850A.

Identification of 18:2 isomers. Fatty acid methyl esters from the pooled C/M extracts of the low EFA rats were fractionated according to unsaturation by TLC on Silica Gel G plates containing 12.5% silver nitrate (14). The fractions containing monoenes and dienes were scraped off and gas-chromatographed. The dienes were further fractionated according to chain length by preparative scale gas chromatography (GC) (14). The 18:2 fraction was dissolved in hexane and subjected to ozonolysis. A sample of the reaction mixture, containing aldehydes and aldehyde esters was then analyzed by GC (14).

Calculation of linoleic acid retention in carcass. The amount of linoleic acid ingested by the 45-day-old rat was calculated from food consumption and fatty acid composition of the diets. The amount of fatty acids in the linoleic acid series retained in the carcasses was calculated from the lipid content × wt percent ω6 acids. By subtracting the amount in the 30-day-old reference rat from that in the 45-day-old rat, the retention of ingested linoleic acid by the tissues could be calculated.

Statistical analyses. One-way variance analysis was used to test whether any differences between the groups existed. If significant differences were found ($P < 0.05$), the Student-Newman-Keuls' test was used to compare the different groups. A paired t-test was used to detect any differences between the 30- and 45-day-old rats on each diet (15).

RESULTS

Body weight and fat content. Weight and food consumption data of the experiment are shown in Table 3. For two rats on the high EFA diet, the weight increase was substantially lower than for the other high EFA rats. The reason for this is not known, but could be due to some infection. These rats were excluded from the subsequent analyses. The initial weights of the 30-day-old reference and experimental rats were similar within each diet group. The 30-day-old rats of the low EFA group weighed significantly less than the normal EFA rats, whereas there were no significant differences between the normal and high EFA groups. At the end of the experiment, the low EFA and the normal EFA groups still differed in weight. The 45-day-old rats of the high EFA group weighed significantly less than those of the normal EFA group. The weight increase was lower in both the low and

TABLE 3

Weight and Food Consumption Data[a,b]

EFA level in diet	Reference rats, 30-day weight (g)	Experimental rats				
		30-day weight (g)	45-day weight (g)	Weight increase (g)	Food consumption (g)	Feed efficiency[c]
Low (n = 7)	64.5 ± 2.8**	64.6 ± 4.3**	133 ± 7.4**	68.7 ± 4.7*	213 ± 16	0.32 ± 0.03
Normal (n = 7)	78.4 ± 4.1	78.4 ± 4.3	156 ± 10	77.1 ± 8.2	226 ± 29	0.34 ± 0.02
High (n = 5)	79.8 ± 6.9	79.2 ± 6.4	142 ± 12*	63.1 ± 8.6**	171 ± 21*	0.37 ± 0.02*
	75.5	74.3[d]	104	29.7	135	0.22
	78.5	77.4[d]	98	20.4	110	0.19

[a]Results are mean ± SD; n = number of animals.

[b]Differences from normal EFA group: **, $P < 0.01$; *, $P < 0.05$. Absence of asterisk in the low EFA or high EFA row indicates a non-significant ($P > 0.05$) difference from the normal EFA group.

[c]g Weight increase per g food consumed.

[d]These rats grew poorly and were excluded from further analyses.

TABLE 4

Lipid Concentration and Fatty Acid Composition in Total Carcass Lipids from 30- and 45-day-old Rats[a]

Fatty acid	Low EFA group (n = 7)		Normal EFA group (n = 6)		High EFA group (n = 5)	
	30-day	45-day	30-day	45-day	30-day	45-day
			wt% fatty acid			
10:0	0.7 ± 0.6	trace[b]	1.5 ± 0.2	trace	1.6 ± 0.3	trace
12:0	2.4 ± 0.7	trace	3.0 ± 0.4	trace	2.8 ± 0.6	trace
14:0	4.4 ± 0.6	2.2 ± 0.2	4.2 ± 0.5	2.3 ± 0.1	3.2 ± 0.5	1.9 ± 0.2
16:0	30.6 ± 0.5	29.6 ± 0.9	27.8 ± 0.7	30.6 ± 0.8	19.5 ± 0.6	24.1 ± 1.6
16:1ω7	9.0 ± 0.6	10.5 ± 0.6	5.2 ± 0.6	8.4 ± 0.5	2.2 ± 0.3	3.9 ± 0.5
18:0	7.8 ± 0.7	7.4 ± 0.7	9.4 ± 0.6	7.9 ± 0.3	7.3 ± 0.4	8.0 ± 0.5
18:1ω9	41.1 ± 0.9	45.6 ± 0.9	31.7 ± 0.9	37.9 ± 1.0	23.2 ± 0.4	25.8 ± 0.6
18:2ω6	1.5 ± 0.2	1.8 ± 0.2	12.0 ± 1.4	8.5 ± 0.9	31.2 ± 0.9	28.8 ± 2.6
18:3ω3	trace	trace	1.4 ± 0.2	1.2 ± 0.2	4.9 ± 0.5	4.3 ± 0.4
20:3ω9	1.5 ± 0.2	1.5 ± 0.3	trace	trace	trace	trace
20:4ω6	1.1 ± 0.2	1.2 ± 0.3	2.9 ± 0.3	2.2 ± 0.2	2.6 ± 0.1	2.5 ± 0.1
22:6ω3	trace	trace	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.05	0.7 ± 0.4
Σ ω6	2.6 ± 0.3	3.0 ± 0.5	14.9 ± 1.6	10.7 ± 1.0	33.8 ± 0.8	31.3 ± 2.5
Σ ω3	trace	trace	2.4 ± 0.3	2.1 ± 0.2	6.4 ± 0.4	5.0 ± 0.5
ω6/ω3	—	—	6.2 ± 0.2	5.2 ± 0.4	5.3 ± 0.3	6.3 ± 0.5
			Lipid in carcass (mg/per g)			
	77.5 ± 9.4	66.7 ± 10.9	70.6 ± 6.3	65.4 ± 5.6	83.7 ± 4.1	74.4 ± 4.4

[a]Values are mean and SD; n = number of animals.

[b]Trace = < 0.1%.

high EFA group than in the normal EFA group. The food consumption was lower in the low EFA and high EFA groups than in the normal EFA groups but the differences were significant only between the two latter groups. The feed efficiency was higher in the high EFA group (Table 3).

For each dietary group, the fat concentration of the 30-day-old rats was higher than that of the 45-day-old rats (Table 4). The differences were significant ($P < 0.05$) only for the high EFA group. The 30-day-old rats from the high EFA group had a significantly higher body fat content ($P < 0.05$) than the rats of the normal EFA group, but at the end of the experiment there were no significant differences between the groups. The differences in fat content could be attributed to variations in the TG concentration.

Fatty acids. The fatty acid composition of the carcass fat was related to the differences in the dietary levels of EFA (Table 4). The differences were most pronounced for linoleic acid and monounsaturated fatty acids. The 18:2 fraction in the carcass lipids of the low EFA rats contained about 90% linoleic acid. Small amounts of 18:2ω7 (6.0%) and possibly 18:2ω9 (1.8%) also were detected.

The fatty acid composition in the TG and PL was determined in pooled samples, as the variations in the total fatty acid composition were small within the groups

TABLE 5

Fatty Acid Composition in Phospholipids from Pooled Total Carcass Lipids

	Low EFA		Normal EFA		High EFA	
Fatty acid	30-day	45-day	30-day	45-day	30-day	45-day
			Wt% fatty acid			
16:0	19.8	19.5	22.0	22.7	21.5	21.9
16:1	5.8	4.8	1.7	2.5	1.1	1.3
18:0	17.6	18.2	20.1	18.6	22.3	21.6
18:1	27.1	25.7	13.7	16.0	11.1	10.9
18:2ω6	6.4	6.6	13.2	14.2	17.4	17.6
18:3ω3	0.9	0.7	0.6	0.6	0.8	0.7
20:3ω9	8.8	9.8	0.9	1.0	0.1	n.d.[a]
20:3ω6	1.1	1.2	1.4	1.3	1.1	1.1
20:4ω6	7.0	8.1	15.0	12.7	13.7	14.3
20:5ω3	1.1	tr[b]	0.6	0.9	0.4	0.4
22:4ω6	0.6	0.6	1.4	0.9	1.2	1.2
22:5ω6	1.2	1.2	0.9	0.8	0.3	0.6
22:5ω3	0.2	0.4	2.0	1.6	2.8	2.4
22:6ω3	2.4	3.3	6.6	6.4	6.2	6.1
Σ ω6	16.3	17.7	31.9	29.9	33.7	34.8
Σ ω3	4.6	4.4	9.8	9.5	10.2	9.6
20:3ω9/20:4ω6	1.3	1.2	<0.1	<0.1	<0.1	<0.1
			Phospholipids in carcass (μmol/g)			
	16.1	14.5	16.3	14.9	17.3	13.3

[a]n.d., Not detected.
[b]tr = <0.1%.

(Table 4). In the triglycerides, the levels of saturated, monoenoic, linoleic and linolenic acids were closely related to those found in the total carcass lipids. Apart from traces of arachidonic acid, no other long chain PUFA could be detected in the TG.

The PL showed a different and more stable fatty acid composition (Table 5). The level of saturated fatty acids was similar in all groups. Eicosatrienoic acid, 20:3ω9, was detected only in the PL and the level decreased with increasing amounts of dietary EFA. The triene/tetraene ratio was 1.2–1.3 in the low EFA group and below 0.1 in the other groups. Other markers of EFA deficiency, e.g., monoenes, also decreased with increasing levels of EFA in the diet. The level of linolenic acid was uninfluenced by the dietary supply, but the percentage of the long chained metabolites 22:5ω3 and 22:6ω3 was higher in the normal EFA and high EFA groups than in the low EFA rats. The level of 22:5ω6 was increased in the low EFA group, reflecting the low supply of ω3 acids.

Linoleic acid retention. Data on the amount of linoleic acid retained in the carcass lipids of the rats are given in Table 6. A much higher percentage of the ingested linoleic acid was retained in the carcass lipids of the low EFA rats compared to the normal EFA or high EFA rats.

DISCUSSION

In a study of this type, it is very important to have rats with minimal variations in body fat content and fatty acid composition. For all diets, the initial weight of the experimental rats in most cases did not differ by more than 1 g. The breeding system resulted in small variations in body fat content and fatty acid composition. This strongly justifies the assumption that the body fat content and fatty acid composition of the 30-day-old experimental rats were similar to those of the 30-day-old reference rats.

As expected, the low EFA rats weighed significantly less than the normal EFA rats, both at 30 and 45 days of age. The 30-day-old high EFA rats were selected to weigh within ±2 SD of the standard mean of the normal rats at that age, since no growth curve yet had been established for the high EFA group. However, at 45 days of age, the high EFA rats weighed 9% less ($P < 0.05$) than the normal EFA rats (Table 3). To test if this was due only to chance, weight development was followed up to 90 days of age for a larger group of high EFA rats. The results showed that these rats weighed 10–15% less ($P < 0.05$) than the normal EFA rats at corresponding ages (Becker, W., unpublished observations).

At least two reasons for the retarded growth of the high EFA rats can be given. One obvious contributing factor was the lower food intake in the high EFA compared to the normal EFA group. In a subsequent similar experiment (Becker, W., unpublished observations), the food consumption of the high EFA group also was lower than that of the normal EFA group, but only by 10%, compared to 30% in the present study. This lower food intake could have been due to presence of oxidation products, since elevated peroxide values (40–80 meq/kg) were found in this diet after longer periods of storage. However, Kaunitz and collaborators (16,17) fed rats diets with 20% by weight of different mildly oxidized fats containing 10–324 meq/kg peroxides for periods up to 96 wk

TABLE 6

Deposition of ω6 Acids in Carcass Lipids[a,b]

EFA level in diet	ω6 acids			% of Retained ω6 acids in:[c]	
	Consumed (g)	Deposited (g)	Retention (%)	PL	TG
Low (n 5 7)	0.25 ± 0.02	0.12 ± 0.02	48.9 ± 8.6**	63	37
Normal (n = 6)	2.59 ± 0.33	0.27 ± 0.11	10.3 ± 3.9	65	35
High (n = 5)	5.97 ± 0.73	0.96 ± 0.27	15.9 ± 4.0	22	78

[a]Values are means and SD; n = number of animals.

[b]Differences from the normal EFA group: **, $P < 0.01$. Absence of asterisk in the low EFA or high EFA row indicates a nonsignificant ($P > 0.05$) difference from the normal EFA group.

[c]Calculated from pooled lipid extracts. PL, phospholipid; TG, triglyceride.

and found no negative effect on growth or food consumption in rats fed these rancid fats compared to rats fed the corresponding fresh fats.

Another, and in our opinion more important, explanation is that the high EFA diet had a specific stimulatory effect on the brown adipose tissue. In separate studies, it was found that these rats have higher levels of thermogenin in the brown fat adipose tissue compared to rats fed the other diets (18) and a higher energy expenditure following a subcutaneous injection of norepinephrine (19). These data strongly suggest that an increased capacity for nonshivering thermogenesis could be a major reason for the lower weight of these animals.

The EFA content of the carcass lipids correlated well with the dietary level. This was most clear in the TG. In the PL, the percentage of individual or total ω6 and ω3 acids was no more than twice as high in the normal EFA rats as in the low EFA rats, despite a 10-fold higher dietary supply of EFA to the normal EFA rats. In the high EFA group, the level of linoleic and total ω6 and ω3 acids was similar to that of the normal EFA group. This probably is due to the fact that the ratio between, rather than absolute amounts of, linoleic and linolenic acids in the diet, which was the same in these two latter diets, regulates their subsequent metabolism (1,2,20).

It is known that the lipids of rats fed fat-free diets contain isomeric unsaturated fatty acids that are uncommon in normal rats (21). Sand and coworkers (21) fed weanling EFA-normal rats a fat-free, casein-sucrose diet and analyzed the occurrence of unsaturated isomers in the carcass lipids after different periods on this diet. After two months on this diet, only about half of the 18:2 fraction was linoleic acid, the rest being a mixture of 18:2ω7, 18:2ω9 and 18:2ω10. After six mo, the linoleic acid accounted for only about 20% of the 18:2 fraction. Our own analyses showed that linoleic acid constituted almost 90% of the 18:2 acids, indicating that the other isomers are formed only after long-term fat-free feeding or when the supply of EFA is lower than 0.2–0.3% of total energy.

The results of this study show that rats fed a diet with a marginal EFA content retain more of an ingested amount of linoleic acid than rats fed sufficient or high amounts of EFA. This is in agreement with earlier observations by Alling et al. (3). They fed similar diets, containing 0.07, 0.75 or 3.0% of total energy as EFA, to rats and calculated that rats from the 0.07% group retained ca. 50–70% of the linoleic acid fed compared to 5–20% in the other two groups. In our study, the retention was about 50% in the low EFA group and around 10% in the normal EFA group.

Previous studies with rats fed the same diets also showed that the carcass retention of a single oral dose of 1-^{14}C-linoleic acid was significantly greater in rats fed the low EFA diet than those fed the normal EFA or high EFA diets (4). Further studies also showed that the larger retention seen in the low EFA rats was due to a higher incorporation of ^{14}C into the PL than into the TG, whereas the opposite was found in the normal and high EFA groups (22). Similar observations were made by Catala and Brenner (23), who found in fat-deficient rats a specific incorporation of oral unlabeled linoleic acid into the PL of the liver and other viscera during the first 48 hr of administration, whereas the incorporation into the TG was small in these tissues.

Data from the present study also show that in the low EFA rats more of the retained ω6 acids was deposited in the PL and less in the TG, whereas in the high EFA rats the opposite was found (Table 6). In contrast to the results from the ^{14}C-tracer study (22), however, there were no differences between the low EFA and normal EFA group in this respect. The reason for this discrepancy is not clear, but it could be that the long-term incorporation of ω6 acids at this dietary level is different from that of a single tracer dose. One possible explanation of the present results is that the level of linoleic acid in the normal EFA diet (2.2% of total energy) is below the optimum for the growing rat and that most of the retained dietary linoleic acid is needed for the synthesis of membrane PL.

In conclusion, the results of the present balance study and of the experiments with labeled linoleic acid agree with respect to the prinipal differences between the dietary groups, e.g., the low EFA rats retained more of a given dose of linoleic acid in their tissues than rats fed the other two diets.

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Formation of Complexes Between Lecithin and Apovitellenin I, An Avian Egg-Yolk Apoprotein

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In a study of lipid-protein interactions in egg yolk, it was found that L-α-dipalmitoyl lecithin gave two distinct noncovalent complexes (A and B) with apovitellenin I, an apoprotein in the major yolk lipoprotein. Interaction took place under widely varied conditions, and yolk lecithin gave similar complexes. Complex A, which was formed within minutes, consisted of round particles of about 9 nm diameter. Complex B, which was formed more slowly, consisted of larger particles, possibly resembling curved discs, with diameter of 30–40 nm. The preparation and some properties of these complexes are described. It is suggested that they may be suitable for an extensive study of phospholipid-protein interactions in yolk.
Lipids 21, 127-131 (1986).

About 60% of the dry yolk of hen's eggs is a low density lipoprotein (YLP) (12% protein, 25% phospholipid, 60% triacyl glycerols plus other lipids; density 0.95 g/ml [1,2]), which is largely responsible for the commercial properties of yolk (e.g., [3]) and probably for many of its biological functions. The structure of this lipoprotein and the nature of the noncovalent interactions between the lipids and apoproteins therefore are of interest but have not been elucidated.

Of particular interest is the difference between lipoproteins of yolk and blood. Studies on the lipoproteins of mammalian blood (see for example reviews [4–6]) indicate that interactions of phospholipids and apoproteins are important for lipoprotein structure and function. Avian egg yolk apoproteins do not, however, correspond to those of mammalian blood. In particular, the main low molecular weight apoprotein of YLP, apovitellenin I, whose precursor is apo VLDL-II in laying hen's blood (7), has no mammalian counterpart. Studies by Jackson et al. (8) have shown that hen's apo VLDL-II binds dimyristoyl lecithin and that a helical, amphipathic binding site on the protein may be involved.

We have found that apovitellenin I has a high affinity for lecithin and that two distinct complexes can be prepared. We suggest that these complexes have great potential for the study of the molecular details of phospholipid-protein interactions in YLP. We describe here their preparation and isolation and some of their properties, including electron microscopic appearance, calorimetric behavior and composition. Some aspects of this work have been reported in abstract form (9,10).

EXPERIMENTAL

Materials. The yolk proteins, hen's apovitellenins I and II, were isolated from the lipid-free apoprotein mixture of the YLP of Australorp hens *(Gallus domesticus)* by gel filtration chromatography in urea (11). Apovitellenin I also was isolated from eggs of the Pekin duck *(Anas platyrhynchos)* by the same procedure. Each protein was purified by hydrophobic chromatography (12) and freeze-dried. Absence of other proteins was checked by gel electrophoresis in detergent (12). Apoprotein concentrations were determined from the absorbance at 280 nm (11).

L-α-dipalmitoyl lecithin was from either Sigma Chemical Co. (St. Louis, Missouri) or Calbiochem-Behring (San Diego, California). The purity was checked by thin layer chromatography. Yolk lecithin was isolated from hen's egg yolk by the procedure of Singleton et al. (13).

Interactions of lecithin and proteins. Conditions for the interaction of lecithin and apovitellenin I were not critical, provided that the temperature was above the transition point of the lipid (41 C for dipalmitoyl lecithin) and the salt concentration was low (<0.05 M). Apovitellenin II failed to interact. For Figures 1 to 5 the following standard procedures were used:

Complex A: Dipalmitoyl lecithin (8 mg in 0.4 ml ethanol) was injected in 0.1 ml increments into a solution of hen's apovitellenin I (8 mg in 3.6 ml 0.02 M sodium chloride, pH 3.5 to 4.0, 45 C), and the mixture was stirred for 10 min at 45 C. Complex B: The same conditions were used as for complex A but 28 mg of lecithin were used and the mixture was left for three hr at 45 C. The mixture remained cloudy.

The inclusion of ethanol speeded up the formation of complex A but was not essential; it could be omitted if the lecithin was spread by evaporation as a thin film on the side of the reaction flask before adding the apoprotein solution.

Complexes indistinguishable from A and B were produced under other conditions; e.g., in 0.02 M sodium chloride at pH 4 to 7, in 0.16 M sodium chloride pH 7 and in 6 M urea pH 3.3 and 7. Apovitellenin I is not soluble above pH 4, except in the presence of urea or above ionic strength 0.05. Even below these values it was dissolved best in 0.02 M hydrochloric acid and the pH raised with 0.1 M sodium hydroxide. Above pH 4 precipitated apovitellenin I was redissolved by interaction with lecithin.

Chromatography of lecithin-protein complexes. The following procedure, which was relatively rapid and gave reasonable separation, was used for the experiments described here with the exceptions noted below. A large column was used compared to the amount of sample applied to help prevent clogging by unreacted lipid, most of which was retained at the start of the column.

The lecithin-protein mixture in 4.0 ml of solvent was applied to a column (60 × 2.2 cm) of Sephadex G 150 (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with unbuffered 0.03 M sodium chloride at 12 ml/hr, 21 C. Complexes were detected by their optical absorbance at 280 nm, measured either continuously or on individual fractions of 3 ml. For the curves given in Figure 1 the latter method was used. The void volume was determined using a high molecular weight YLP fraction or high molecular weight blue dextran. Globular proteins were used as molecular weight standards. A correction was ap-

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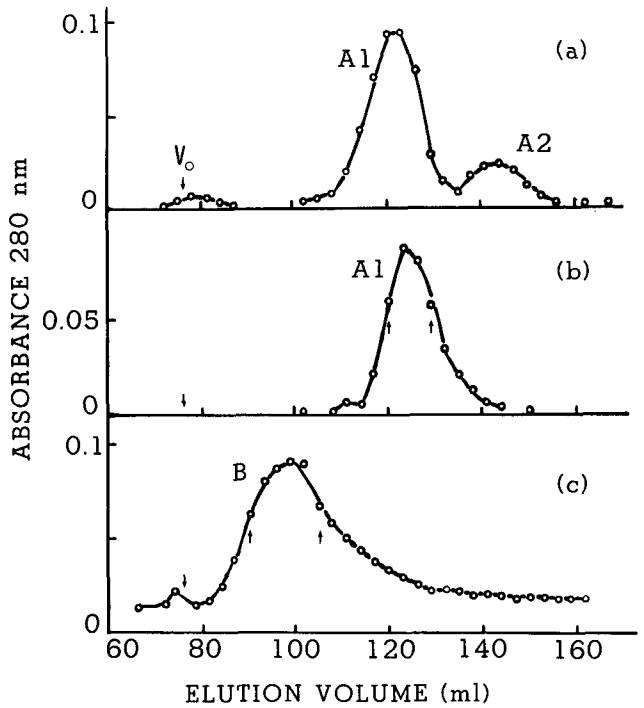

FIG. 1. Gel filtration chromatography of complexes of dipalmitoyl lecithin and hen's apovitellenin I on a column of Sephadex G 150 as described in Experimental. The void volume is indicated by V_o. (a) Lecithin (8 mg in 0.4 ml ethanol) and apovitellenin I (8 mg in 3.6 ml 0.02 M sodium chloride, pH 4.0) were mixed at 45 C and applied after stirring for 10 min. (b) Peak A1 (110 to 130 ml) was concentrated by ultrafiltration and reapplied. (c) Lecithin (25 mg in 0.4 ml ethanol) and apovitellenin I (8 mg in 3.6 ml 0.02 M sodium chloride, pH 4.0) were incubated for three hr at 45 C before applying to the column. In (b) and (c), samples of complex between the arrows were used for tests.

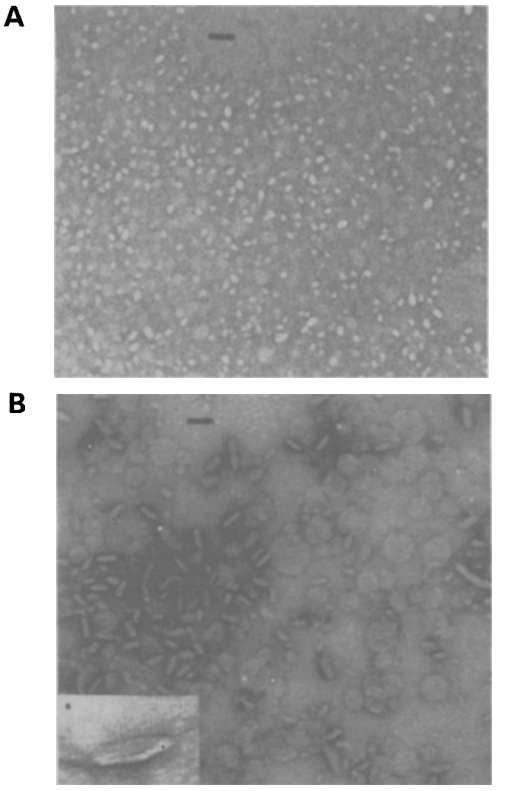

FIG. 2. Electron micrographs of negatively stained complexes of lecithin and hen's apovitellenin I. (a) Complex A, sample from peak A1, between arrows, Fig. 1b. (b) Complex B, showing elongated and circular shapes, from between arrows peak B, Fig. 1c. Magnification 98,000. The bars represent 50 nm. (b) Inset. An example of an elongated particle of complex B (arrow) showing "rolled-up" appearance. Magnification 230,000.

plied in estimating the particle weights of complexes to take into account the density of lecithin in the complex.

Larger columns were used to obtain material for replicate analyses and particle weights were checked on longer columns (90 × 2.2 cm), which also were used to obtain the yields of complex B. For experiments in which 6 M urea was used, the column also was eluted with this solvent.

To determine yields of complexes, all the fractions in a chromatographic peak were pooled, concentrated by ultrafiltration (Diaflow membrane Y10, Amicon Corp., Lexington, Massachusetts) and dialyzed into water. The mixture then was dried at 103 C and the complex weighed to 0.02 mg or, for small samples, to 0.001 mg. The composition of the complexes was determined from the phosphorus content of the dried material, measured by the method of Fiske and Subbarow (14) which was used to calculate the percentage of lecithin. Protein was obtained by difference.

Physical methods and analyses. To determine particle shapes and sizes, a Siemens Elmiskop I electron microscope was used. The samples (0.02 to 0.2% suspensions) were negatively stained with 0.2% uranyl acetate on a carbon-coated nitrocellulose grid and viewed at a magnification of 33,000. Particle sizes were measured on enlarged photographs by means of a cathetometer.

Differential scanning calorimetry was used to measure the heat-absorbing transitions of lecithin and complexes as a function of temperature, as described in (15) with the difference that the calorimeter was modified to take sealed sample tubes containing 0.8 ml of liquid. Solvent was used as reference and lecithin that had been treated in the same way as mixtures was used as control. The rate of temperature increase was 0.5 C/min.

For density-gradient centrifugation, a linear gradient (total volume 32 ml) was prepared from sucrose (70% w/v in 0.03 M sodium chloride) and 0.03 M sodium chloride by means of a Pharmacia Gradient Mixer Model GM 1. The sample (3 to 4 ml) was layered on top of the gradient in a 37 ml tube of a Beckman SW 27 rotor (Beckman Instruments Inc., Palo Alto, California). The tube was centrifuged for 48 hr at 25,000 rpm, 10 C. Afterwards, the gradient was divided into fractions (1.5 ml) by pumping from the bottom of the tube. The optical absorbance of each fraction was measured at 280 nm and solution densities were measured on a density meter (Model DMA 02C, Anton Paar KG, Graz, Austria) at 20.0 C.

CD spectra were obtained at room temperature using a Jobin-Yvon Dichrograph IV (Longjumeau, France) at a scan rate of 30 nm/min and a sample cell with 0.1 mm lightpath. Sample solutions were filtered through 0.45 μm microfilters into the cell immediately prior to a run. Calibration of the Dichrograph was performed at 304 nm using a solution of epiandrosterone in dioxane (1.25 mg/ml) in accordance with the specifications given by Jobin Yvon. The CD readings at every 1.0 nm of increased

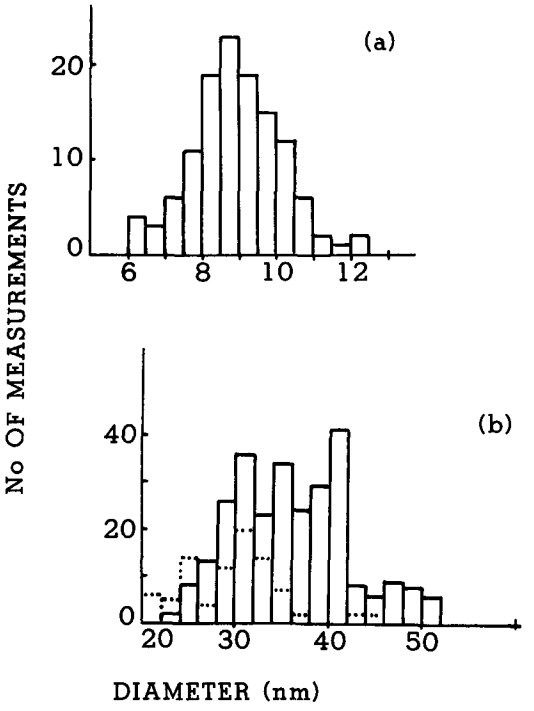

FIG. 3. Histograms showing distribution of particle sizes from electron micrographs of complexes. (a) Complex A. (b) Complex B; solid line, diameters of circular particles; dashed line, major axis of elongated particles.

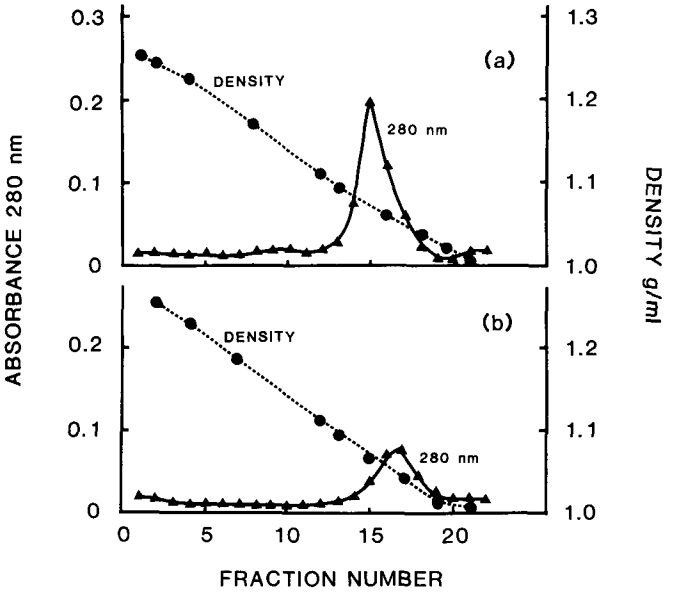

FIG. 4. Density-gradient centrifugation of complexes of lecithin and apovitellenin I. The gradient of sucrose-0.03 M sodium chloride was prepared and centrifuged as described in Experimental. The fractions were 1.5 ml each. Left-hand scale (triangles) gives optical absorbance at 280 nm. Right-hand scale (solid circles) gives density of selected fractions. (a) Complex A, concentrated sample from peak A1, Fig. 1b. (b) Complex B, a sample from peak B, Fig. 1c.

wavelength were recorded on-line in a computer. Each spectrum recording was cycled six times, and the mean value at each nm was calculated. From these data the helix content of proteins and complexes were estimated by the computer following the procedure of Chang et al. (16).

RESULTS

Figure 1a shows the gel filtration chromatography of a mixture of equal weights of dipalmitoyl lecithin and hen's apovitellenin I in 0.02 M sodium chloride after brief interaction at 45 C as described in Experimental. The material in peak A1 is referred to as complex A. The fractions of this peak (eluted from 110 to 130 ml) were pooled and concentrated by ultrafiltration to 5 ml. The concentrate then was applied to the same column. Figure 1b shows that it gave a single peak. The material in peak A2 did not survive rechromatography. It evidently was unstable and it has not been investigated further.

Purified complex A, such as the material in the upper part of peak A1 (Fig. 1b between the arrows), was concentrated and used for several tests. According to the electron microscope (Fig. 2a), complex A consisted of irregular particles with a narrow range of sizes (Fig. 3a). On density-gradient centrifugation, complex A gave a single peak (Fig. 4a).

According to differential scanning calorimetry (Fig. 5), the large transition peak of dipalmitoyl lecithin (L) was absent after interaction with hen's apovitellenin I to form complex A, although there was a very small peak at 38 C. The isolated complex did not give a peak in the range 20–50 C. With another yolk apoprotein, hen's apovitellenin II, there was no displacement of the lecithin

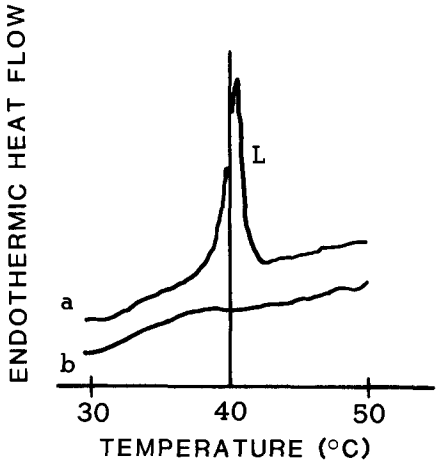

FIG. 5. Differential scanning calorimetry. Measurements were made on samples in 0.8 ml of 0.02 M sodium chloride containing 10% (v/v) of ethanol. (a) Control; dipalmitoyl lecithin (1.6 mg), heat-treated as in (b). (b) Apovitellenin I (1.6 mg) plus 1.6 mg dipalmitoyl lecithin mixed at 45 C for 15 min, cooled and then measured. L refers to the lecithin peak.

peak and its area was reduced by only 9%. Above ionic strength 0.05, complex A formed a precipitate with no change in the calorimetric pattern, but in concentrated salt (50% saturated ammonium sulfate), decomposition was indicated by the return of the lecithin pattern.

Calculations based on measurements of circular dichroism (Table 1) show that the proportion of α-helix in apovitellenin I in 0.02 M sodium chloride pH 4 invariably increased as a result of interaction with dipalmitoyl lecithin under the conditions used to produce complex A. Reduction of the disulfide group in hen's

TABLE 1

Effect of Complex Formation with Dipalmitoyl Lecithin on Helix Content of Apovitellenin I[a]

	Helix content (%)[b]	
	Prior to interaction	After interaction
Hen's apovitellenin I	62	84
Hen's apovitellenin I, reduced form[c]	57	72
Duck's apovitellenin I	42	66

[a]Estimated from circular dichroic spectra using the model of Chang et al. (16).

[b]The reproducibility of the CD spectra corresponds to a standard error of less than ±1 in these percentages.

[c]Reduced by treatment with 0.5 M mercaptoethanol and 0.001 M ethylenediaminetetraacetic acid.

TABLE 2

Data for Isolated Complexes of Dipalmitoyl Lecithin and Hen's Apovitellenin I

Property	Complex A	Complex B
Lecithin (% dry sample)[a]	44.6 ± 1.3	55.1 ± 2.2
Mean diameter of round particles (see Fig. 3) (nm)	8.7	30–40
Density (g/ml)		
Measured[b]	1.11	
Calculated[c]	1.15	1.11
Particle weight (daltons)		
From gel filtration	0.9×10^5	
Calculated from particle size	2.3×10^5	

[a]The variations (standard errors) were among different preparations: 13 for A (range 40 to 52) and 11 for B (range 46 to 63).

[b]From density-gradient centrifugation.

[c]Assuming densities of 0.93 and 1.33 g/ml for the lecithin and protein.

apovitellenin I decreased the percentage of α-helix and diminished the effect of lecithin. Duck's apovitellenin I, which initially had less helix than hen's apovitellenin I, also was affected by lecithin. Other properties of complex A are summarized in Table 2.

Complex A was formed rapidly under the conditions used for Figure 1a and the yield could not be increased by using a higher proportion of lecithin. This could imply that there is a limiting size to the complex. By contrast, when a mixture of apovitellenin I and a three- or fourfold excess of lecithin was left at 45 C for three hr, the electron microscope showed that much larger particles were present and the chromatographic pattern contained a new peak (peak B, Fig. 1c). The material from the top of peak B (between arrows) consisted predominantly of large particles (Fig. 2b) that were identical in appearance to those in the mixture before chromatography. This material is referred to as complex B. Complete separation from complex A was achieved on a longer column (see Experimental). Complex B was altered by ultrafiltration or prolonged storage, as it did not give the same chromatographic pattern afterwards. The nature of the changes has not been examined. Studies on this complex were therefore confined to material that had been chromatographed only once.

Figure 2b clearly shows that particles of complex B have two shapes, circular and elongated, although density-gradient centrifugation (Fig. 4b) on the isolated complex gave a single band. The dimensions of particles of complex B, which are indicated by the histograms (Fig. 3b) are surprisingly large and not consistent with the position of elution in Figure 1c. Some of the elongated particles have a rolled-up appearance (Fig. 2b inset) which may provide a clue to their chromatographic behavior.

Solutions of complex B that had been concentrated by ultrafiltration did not give a peak between 30 and 50 C in the scanning calorimeter, although, as already mentioned, the complex was altered by concentration. Circular dichroic measurements on complex B so far have been prevented by the cloudiness of the solutions. Some other properties of complex B are summarized in Table 2.

The samples used in Figures 1 to 5 were prepared by the standard methods described in Experimental, i.e., 0.02 M sodium chloride, pH 3.5–4, plus ethanol. Complexes A and B could be prepared under a variety of other conditions, although the yields differed. This is indicated in Table 3, which shows that highest yields were obtained for interaction in 6 M urea. Table 3 also shows that complexes could be prepared from the reduced form of apovitellenin I and from duck's apovitellenin I, as also indicated in Table 1 for complex A. Furthermore, dipalmitoyl lecithin could be replaced by yolk lecithin to achieve interaction at a lower temperature.

DISCUSSION

Our results show that the egg yolk apoprotein, apovitellenin I, forms two distinct complexes (A and B) with dipalmitoyl lecithin. Evidence for interaction at the molecular level in these complexes depends on the absence of a thermal transition at the usual temperature for lecithin (Fig. 5). Furthermore, for complex A, there was a marked increase in helicity of the protein (Table 1), as expected from studies on the interactions of other apoproteins with phospholipid (17). Another yolk apoprotein, apovitellenin II, gave no evidence for complex formation, so interaction with lecithin is not a general property of yolk proteins.

Because of the low solubility of apovitellenin I except under disaggregating conditions, most of our results were from complexes prepared at low ionic strength at relatively low pH. Nevertheless, apovitellenin I has such a high affinity for lecithin that complexes could be prepared even in 6 M urea pH 3.3. Complex A also could be formed in 0.16 M sodium chloride pH 7, when the protein was present initially as a precipitate. It therefore is possible that such a complex could be formed under physiological conditions.

Hen's apovitellenin I consists of two identical polypeptide chains each with 82 residues (M_r ca. 9500), joined at residue 75 by a disulfide group (18). Apovitellenin I from duck's eggs has no disulfide (18). The reduced form of hen's apovitellenin I and duck's apovitellenin I formed complexes with lecithin (Tables 1 and 3). Both the initial helix content and that after interaction with lecithin were

TABLE 3

Yields of Complexes Isolated by Column Chromatography[a]

Protein (apovitellenin I)[b]	Lecithin	pH	Temp. (°C)	Solvent system[c]	Yield, weight %[d]	
					Complex A	Complex B
Hen (SS)	DPL[e]	4.0	45	0.02 M NaCl	48(5)	44(3)
Hen (SS)	DPL	4.0	45	0.02 M NaCl*	51	48
Hen (SS)	DPL	6.8	45	0.16 M NaCl	51	
Hen (SS)	Yolk	4.0	20	0.02 M NaCl	36	30
Hen (SS)	DPL	3.3	45	6 M urea 0.02 M NaCl	81(2)	83(3)
Hen (SH)	DPL	3.8	45	0.02 M NaCl	40	41
Duck	DPL	3.8	45	0.02 M NaCl	41	60

[a]See Experimental.

[b]SS indicates the disulfide form; SH indicates the reduced (−SH) form.

[c]All solvents contained ethanol (10% v/v) except the one with an asterisk.

[d]Where more than two values were obtained averages are given and standard errors are in parentheses.

[e]DPL indicates dipalmitoyl lecithin.

lower for these proteins than for the disulfide form of the hen's protein (Table 1), thus suggesting that the disulfide group increases the proportion of helix. As is evident from Table 1, however, the absence of a disulfide bridge appears to have no general effect on the proteins' ability to become more helical on complex formation.

The size and shape of complex B have not been determined unequivocally by our data because of the discrepancy between the elution pattern of gel filtration chromatography (Fig. 1) and the appearance in the electron microscope (Fig. 2). From its electron microscopic appearance we suggest that complex B consists of disc-shaped particles. In this respect it would resemble some of the interaction products of phospholipids and other apoproteins (e.g., 19). There is, however, the difference that the particles of complex B had no tendency to form stacks. In fact, the elongated shapes seldom occurred in parallel pairs. In explanation we suggest that the discs are not parallel-sided but are curved, and that the elongated particles represent curved discs that have curled up from opposite edges. Enlarged electron micrographs of the elongated particles sometimes have a rolled-up appearance (Fig. 2).

The proposed ability of apovitellenin I to form curved discs with lecithin suggests further that one of its roles in the lipoprotein is the organization of lecithin molecules on the curved surface. It should be easier to explore the molecular details of this process with a model system such as complex B than with the whole lipoprotein.

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Effect of Total Parenteral Nutrition with Linoleic Acid-Rich Emulsions on Tissue ω6 and ω3 Fatty Acids in the Rat

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The effect of total parenteral nutrition (TPN) with lipid emulsions containing ca. 48% 18:2ω6 plus 8% 18:3ω3 (Intralipid®) or 76% 18:2ω6 plus 0.5% 18:3ω3 (Liposyn®) on the fatty acid composition of liver and plasma triglyceride and phospholipid (PL) was studied in the rat. Plasma PL showed a clear influence of the egg PL present in the infused lipid, suggesting replacement of endogenous lipoprotein PL. The incorporation of 20 and 22 carbon ω6 and ω3 fatty acids was reduced in liver phosphatidylcholine of rats infused with either Intralipid or Liposyn. Phosphatidylethanolamine was much less affected and showed reduced 20:4ω6 and 20:5ω3 in the Liposyn group only. Reduction in levels of long chain essential fatty acid metabolites was compensated for by an increased content of saturated fatty acids. There was no accumulation of 18:2ω6 or 18:3ω3 in liver PL. The similarity between the effects of Intralipid and Liposyn supports the conclusion that 18:2ω6, not 18:3ω3, was responsible. The data suggest that intravenous administration of high levels of 18:2ω6 in parenteral lipid reduces desaturation/elongation of essential fatty acids but does not competitively inhibit esterification of other fatty acids into PL.
Lipids 21, 132-138 (1986).

It has been recognized for many years that the content and composition of lipid in the diet is reflected in the fatty acyl composition of the body's triacylglycerol (TG) store (1). In recent years a growing body of research has shown that the composition of structural PL in many tissues such as heart, liver and kidney, and in specific subcellular organelles including mitochondria, endoplasmic reticulum and sarcolemma, is influenced by the lipid substrate supplied in the diet (2). The PL class and fatty acid composition and cholesterol content of biomembranes are critical determinants of membrane physical properties and have been shown to influence a wide variety of membrane-dependent functions such as integral enzyme activity, membrane transport and receptor function (2-4). The ability to alter membrane lipid composition and function in vivo by diet, even when essential fatty acids (EFA) are adequately supplied, demonstrates that metabolic control over the membrane environment is not absolute.

In addition to their role in membrane lipid, the essential dietary ω6 and ω3 fatty acids also serve as the obligate precursors for the synthesis of eicosanoids (5,6). The rate and balance of production of the different eicosanoids, particularly prostaglandins (PG), is known to be altered by changing the fatty acid composition of the diet (7-11). The ω6 or ω3 fatty acid substrate for eicosanoid production is derived from tissue PL. Thus, the potential influence of diet on tissue PL extends to both membrane structure-function relationships and the local synthesis of eicosanoid products.

The intravenous administration of parenteral fat emulsions is used widely in TPN to supply EFA and deliver concentrated energy in a relatively small volume of isotonic solution. The most widely used products are based on vegetable oil TG, usually from soybean oil (Intralipid, also known as Nutralipid® in Canada) or safflower oil (Liposyn). They contain very high levels of linoleic acid, 18:2ω6, and markedly different 18:2ω6/18:3ω3 ratios due to the presence of ca. 8% and 0.5% 18:3ω3 in Intralipid and Liposyn, respectively. Usually one emulsion is given as the sole source of fat in the TPN regime, giving direct venous entry of a fatty acid composition substantially different from that encountered in a normal human diet. Since the composition of fat in the oral diet can influence tissue structural PL components, membrane-associated functions, PG synthesis (2-4,7-11) and PG-related physiological events (7,12-14), it is logical that TPN with these highly polyunsaturated fatty acid emulsions would have similar effects. These studies, therefore, were conducted to investigate the effect of complete TPN using different content and composition of parenteral fat on hepatic PL fatty acid composition. In addition, the effects of TPN on plasma TG and PL and liver TG fatty acids also were determined. The TPN system used previously established procedures in which infusions were done at a constant and continuous rate for seven days (15).

MATERIALS AND METHODS

Total parenteral nutrition. Male Wistar rats (175-225 g) were prepared for TPN via jugular vein infusion as described in detail elsewhere (15). The system used in this laboratory permitted the rats complete mobility while parenteral nutrient solutions were infused at a constant and continuous rate for seven days. Three groups of six rats each were infused with a basal essential nutrient solution containing amino acids, vitamins and minerals, based on NRC requirements for the rat, and dextrose to give ca. 2/3 calories as described previously (15) and later modified (16). Remaining calories were supplied by infusion of 10% Intralipid (Nutralipid, Pharmacia [Canada], Dorval, Quebec), 10% Liposyn (Abbott Labs., Montreal, Quebec) or 25% dextrose (Table 1). EFA were supplied to the 25% dextrose group as a bolus of two ml Intralipid once every second day. Control rats were sham-operated, attached to the infusion apparatus and given free access to rat chow (Ralston Purina, St. Louis, Missouri). The fatty acid composition of rat chow and of triglyceride and phospholipid from the emulsions used is given (Table 2). Lipid infusion was stopped four hr before termination of the studies.

Analytical procedures. After seven days of TPN, the rats were removed from the infusion apparatus and weighed; venous blood samples were taken for separation of plasma by centrifugation. The rats were killed quickly and livers were excised and rinsed in ice-cold saline. Total plasma and liver lipid were extracted (17). Plasma unesterified and esterified cholesterol was determined by gas liquid chromatography (GLC) as described by others (18), using a Varian 6000 GLC equipped for packed and

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TABLE 1

Nutrients Received During 7 Days of TPN

Group	K calories/24 hr/rat					
	Oral (chow)	Amino acid solution[a]	25% Dextrose[b]	10% Liposyn[c]	10% Intralipid[d]	Total
Control	67 ± 2[e]	—	—	—	—	67 ± 2
TPN + 25% dextrose	—	39 ± 2	19 ± 0	—	—	58 ± 2
TPN + 10% Liposyn	—	38 ± 2	—	18 ± 1	—	56 ± 2
TPN + 10% Intralipid	—	39 ± 1	—	—	18 ± 1	56 ± 2

[a]All rats receiving TPN were infused with a basal solution described in Materials and Methods.
[b]25% Dextrose infused in addition to basal solution.
[c]10% Liposyn infused in addition to basal solution.
[d]10% Intralipid infused in addition to basal solution.
[e]Data given represent means ± SE, n = 6 for all groups.

TABLE 2

Major Fatty Acids of Triglyceride from Rat Chow and of Triglyceride and Phospholipid from Parenteral Lipid Emulsions

Fatty acids (% wt/wt)	Triglyceride			Phospholipid	
	Chow	10% Liposyn	10% Intralipid	10% Liposyn	10% Intralipid
16:0	19.4	7.5	9.5	26.4	31.1
16:1	0.9	<0.1	0.6	0.6	0.9
18:0	7.0	3.0	4.0	15.6	13.1
18:1	28.6	12.2	23.0	27.6	27.4
18:2ω6	33.6	75.6	48.5	16.3	13.5
18:3ω3	2.6	0.5	8.3	0.4	0.6
20:3ω6				0.4	0.2
20:4ω6				6.6	3.5
22:5ω6				2.0	<0.1
22:5ω3				0.2	0.4
22:6ω3				1.7	7.7
Total (mg/gm)	4.3	—	—	—	—
(mg/dl)	—	10.8	10.3	1.1	1.3

capillary column operation and a Varian Vista 402 chromatography data system. Total TG and PL were separated from plasma and liver lipid by thin layer chromatography (TLC); liver PL was separated further into individual PL classes using established methods of two dimensional TLC (19). Fatty acids in separated lipids were analyzed as their methyl esters (19) using the above instrumentation with a SP 2330 column (30 m × 0.25 mm ID). Operating conditions were as follows: the injection port was set at 240 C, the FID at 260 C and the oven temperature was programmed from 170 C to 200 C at 5 C/min after an initial hold time of 5 min. Helium was used as the carrier gas (1 ml/min) with the inlet splitter set at 10:1.

Data handling. Peak areas and wt percent fatty acid or cholesterol composition and μg values were calculated, based on known internal standards, by the data system. For liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the sum of the positively identified saturated, monounsaturated and ω6 and ω3 series fatty acids were computed for each chromatogram. The unsaturation index (UI) was calculated as Σ_k^a = (number of double bonds in a) × (wt percent occurrence of a for each fatty acid in a group of k fatty acids) (19). Significant differences among treatment groups were determined using analysis of variance and Duncan's multiple range test.

RESULTS

Body weight gain over the seven-day experimental period was 29.5 ± 3.2 g, 5.2 ± 3.2 g, 7.7 ± 4.4 g and 8.3 ± 1.9 g for control rats and rats receiving TPN plus 25% dextrose, 10% Nutralipid or 10% Liposyn, respectively. The difference in body weight between control and TPN rats can be explained largely by the weight of the gut and gut contents in the orally fed as compared to the intravenously fed animal. The gastrointestinal tract from pylorus to anus was 29.5 ± 1.3 g in orally fed and 11.41 ± 0.7 g in intravenously fed rats.

Plasma and liver lipid composition. Plasma TG levels in the rat were not influenced by continuous intravenous delivery of all nutrients for seven days, irrespective of

TABLE 3

Plasma and Liver Lipid Composition of Rats Following 7 Days of TPN

Group	Triglyceride	Cholesterol			Phospholipid
		Total	Free	Esterified	
Plasma (mg/dl)					
Control	50.4 ± 3.9	66.8 ± 7.3	12.9 ± 1.7	51.8 ± 5.3	104.1 ± 6.1
TPN + 25% dextrose	42.7 ± 0.4	50.4 ± 4.9	13.2 ± 0.6	37.2 ± 4.2	114.2 ± 17.6
TPN + 10% Liposyn	46.4 ± 4.7	59.9 ± 6.5	17.7 ± 0.4[a]	41.4 ± 2.4	108.8 ± 6.7
TPN + 10% Intralipid	48.7 ± 9.6	60.9 ± 9.5	18.6 ± 0.9[a]	43.1 ± 3.8	106.1 ± 13.0
Liver (mg/g wet wt)					
Control	5.1 ± 0.6	2.2 ± 0.2	1.8 ± 0.2	0.4 ± 0.1	18.2 ± 0.7
TPN + 25% dextrose	15.4 ± 2.8[a]	2.8 ± 0.4	1.1 ± 0.2[a]	1. ± 0.2[a]	18.2 ± 1.2
TPN + 10% Liposyn	8.9 ± 2.4	1.9 ± 0.3	1.5 ± 0.2	0.4 ± 0.1	16.0 ± 1.9
TPN + 10% Intralipid	5.8 ± 0.5	2.4 ± 0.1	1.5 ± 0.3	0.7 ± 0.1	17.3 ± 0.9

Data given represent means + SE, n = 6 for all groups

[a]Values are significantly different ($p < 0.05$) from the control value.

[b]Values are significantly different ($p < 0.01$) from the control value.

TABLE 4

Major Fatty Acids of Rat Plasma Triglyceride Following 7 Days of TPN

Fatty acids (% wt/wt)	Control	TPN		
		+ 25% Dextrose	+ 10% Liposyn	+ 10% Intralipid
16:0	17.6 ± 0.2	19.4 ± 1.9	19.9 ± 1.7	22.1 ± 0.3[b]
16:1	0.7 ± 0.1	2.2 ± 1.2	0.5 ± 0.3	0.9 ± 0.4
18:0	4.3 ± 0.3	4.7 ± 0.9	5.4 ± 0.9	4.3 ± 0.6
18:1[c]	20.1 ± 0.4	33.5 ± 5.8[a]	17.3 ± 1.8	21.4 ± 1.3
18:2ω6	37.5 ± 0.7	17.8 ± 5.2[b]	39.7 ± 5.5	30.9 ± 1.4[b]
18:3ω6	0.4 ± 0.7	0.4 ± 0.2	0.9 ± 0.5	0.4 ± 0.3
18:3ω3	1.9 ± 0.1	0.5 ± 0.2[b]	<0.1[b]	1.9 ± 0.4
20:1ω9	1.4 ± 0.5	2.5 ± 1.8	4.4 ± 1.2[a]	2.4 ± 0.6
20:3ω9	<0.1	0.5 ± 0.3	1.1 ± 0.3[b]	<0.1
20:3ω6	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
20:4ω6	3.3 ± 0.4	5.4 ± 1.0	4.8 ± 1.0	3.8 ± 0.4
20:5ω6	2.5 ± 0.4	2.2 ± 1.0	0.4 ± 0.1[b]	1.4 ± 0.6
22:4ω6	0.6 ± 0.1	0.5 ± 0.3	1.2 ± 0.2[a]	0.6 ± 0.2
22:5ω3	1.6 ± 0.2	1.2 ± 0.4	0.4 ± 0.1	1.2 ± 0.5
22:6ω3	4.0 ± 0.7	6.3 ± 1.6	1.1 ± 0.3[b]	5.1 ± 1.0

Data given represent means ± SE, n = 6 for all groups; for experimental details see Materials and Methods.

[a]Values within a line are significantly different ($p < 0.05$) from control.

[b]Values within a line are significantly different ($p < 0.01$) from control.

[c]Values for 18:1 represents all isomers.

whether lipid was used as a significant source of calories (Table 3). The infusion of Intralipid or Liposyn resulted in significant elevation of plasma-free cholesterol but had no effect on plasma cholesteryl ester or PL levels.

The controlled system of TPN developed in this laboratory specifically for use in the rat did not result in any abnormality of gross lipid composition of the liver when lipid was included to supply ca. 1/3 of total calories given (Table 3). The system essentially free of lipid calories containing additional dextrose, however, did result in significant elevation of hepatic TG and cholesteryl esters, although unesterified cholesterol was reduced.

Fatty acid composition of plasma triglyceride and phospholipid. Rats receiving TPN with 25% dextrose and lipid supplied as 1 ml Nutralipid every second day had reduced levels of the essential 18:2ω6 and 18:3ω3 fatty acids in plasma TG, although longer chain desaturation/elongation products of 18:2ω6 and 18:3ω3 were maintained at levels similar to control values (Table 4). Levels of the monounsaturate 18:1ω9 were elevated in this group. TPN with Liposyn resulted in decreased 18:3ω3, 20:5ω6 and 22:6ω3 and increased 20:1ω9 and 22:4ω3 levels in plasma TG when compared to sham-operated controls. The use of Intralipid, on the other hand, increased plasma TG 16:0 and led to reduced 18:2ω6

TABLE 5

Major Fatty Acids of Rat Plasma Phospholipid Following 7 Days of TPN

Fatty acids (% wt/wt)	Control	TPN		
		+ 25% Dextrose	+ 10% Liposyn	+ 10% Intralipid
16:0	21.6 ± 1.2	20.4 ± 0.9	20.5 ± 0.7	25.3 ± 0.9[a]
16:1	1.1 ± 0.3	1.9 ± 0.4	1.1 ± 0.5	0.2 ± 0.2[a]
18:0	21.6 ± 0.9	22.7 ± 1.0	23.8 ± 0.6	21.1 ± 1.0
18:1[c]	7.0 ± 0.3	9.5 ± 1.0	10.2 ± 1.7	13.8 ± 1.9[b]
18:2ω6	26.4 ± 1.0	12.4 ± 1.9[b]	20.1 ± 0.6[b]	17.6 ± 0.9[b]
18:3ω6	0.5 ± 0.2	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.0
18:3ω3	0.4 ± 0.1	0.7 ± 0.1	0.2 ± 0.0	0.7 ± 0.1
20:3ω9	ND[d]	1.2 ± 0.3[b]	0.7 ± 0.0[b]	0.7 ± 0.1[b]
20:4ω6	15.6 ± 0.9	17.7 ± 0.8	16.3 ± 1.2	12.7 ± 1.6
20:5ω6	0.5 ± 0.0	0.7 ± 0.2	<0.1[b]	<0.1[b]
20:5ω3	<0.1	0.5 ± 0.2[b]	0.6 ± 0.1[b]	0.4 ± 0.0[b]
22:4ω6	0.4 ± 0.1	0.4 ± 0.0	0.6 ± 0.1	0.3 ± 0.0
22:5ω6	0.1 ± 0.1	0.7 ± 0.2[a]	0.9 ± 0.2[b]	<0.1
22:5ω3	0.6 ± 0.0	0.6 ± 0.1	0.4 ± 0.0[b]	0.7 ± 0.0
22:6ω3	4.1 ± 0.5	6.5 ± 0.7[a]	4.8 ± 0.5	7.2 ± 0.2[b]

Data given represent means ± SE, n = 6 for all groups; for experimental details see Materials and Methods.
[a]Values within a line are significantly different ($p < 0.05$) from control.
[b]Values within a line are significantly different ($p < 0.01$) from control.
[c]Values for 18:1 represents all isomers.
[d]ND, not detected.

TABLE 6

Major Fatty Acids of Rat Liver Triglyceride Following 7 Days of TPN

Fatty acids (% wt/wt)	Control	TPN		
		+ 25% Dextrose	+ 10% Liposyn	+ 10% Intralipid
16:0	30.2 ± 1.5	29.0 ± 3.4	26.6 ± 1.4	30.7 ± 5.2
16:1	2.4 ± 0.5	3.6 ± 0.9	1.1 ± 0.2[a]	1.0 ± 0.3[a]
18:0	4.9 ± 0.3	3.0 ± 0.4[b]	4.6 ± 1.1	5.8 ± 0.9
18:1[c]	23.7 ± 1.2	30.2 ± 3.1	14.8 ± 1.3[b]	22.0 ± 1.1
18:2ω6	21.3 ± 2.0	14.6 ± 4.3	36.4 ± 3.0[b]	22.6 ± 4.8
18:3ω6	0.2 ± 0.0	0.5 ± 0.2	0.3 ± 0.1	0.3 ± 0.0
18:3ω3	0.8 ± 0.3	1.0 ± 0.4	0.2 ± 0.0	1.8 ± 0.4
18:4ω3	0.9 ± 0.2	0.4 ± 0.3	1.0 ± 0.2	0.9 ± 0.2
20:1ω9	1.2 ± 0.3	4.9 ± 2.3	2.3 ± 0.3[a]	3.4 ± 0.6[b]
20:3ω6	1.1 ± 0.2	0.3 ± 0.1[b]	0.5 ± 0.1[a]	0.6 ± 0.1[a]
20:4ω6	1.6 ± 0.2	1.1 ± 0.4	2.2 ± 0.3	1.0 ± 0.1[a]
20:5ω6	1.6 ± 0.7	0.5 ± 0.2	0.7 ± 0.2	0.2 ± 0.9
22:4ω6	0.4 ± 0.1	0.4 ± 0.0	0.8 ± 0.3	0.4 ± 0.0
22:5ω6	0.2 ± 0.2	0.2 ± 0.4	0.4 ± 0.1	0.1 ± 0.1
22:5ω3	0.6 ± 0.3	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
22:6ω3	0.6 ± 0.1	1.3 ± 0.5	1.0 ± 0.3	0.6 ± 0.2

Data given represent means ± SE, n = 6 for all groups; for experimental details see Materials and Methods.
[a]Values within a line are significantly different ($p < 0.05$) from control.
[b]Values within a line are significantly different ($p < 0.01$) from control.
[c]Values for 18:1 represents all isomers.

(Table 4) despite the high level (48.5%) of 18:2ω6 in the emulsion itself (Table 2).

Analysis of the fatty acid composition of plasma PL from the TPN + 25% dextrose group showed that, as in the TG fraction, levels of long chain metabolites of 18:2ω6 and 18:3ω3 were preserved at equivalent or greater control levels while 18:2ω6 itself was decreased (Table 5). PL fatty acids from rats infused with Liposyn showed decreased 18:2ω6 and 20:5ω6 levels and increased 20:5ω3, 22:5ω6 and 22:5ω3 levels. The infusion of Intralipid similarly reduced 18:2ω6 and 20:5ω6 and increased 20:5ω3 in plasma PL compared to the control group. In addition, this emulsion increased 16:0, 18:1ω9 and 22:6ω3. All three TPN regimes led to a measurable quantity of 20:3ω9 in the plasma PL fraction; this was not found in control rats.

Fatty acid composition of liver triglyceride. The effect of TPN containing lipid to supply essential fatty acid only (TPN + 25% dextrose group) had little effect on the liver TG fatty acid pattern (Table 6). However, infusion of Liposyn, containing ca. 75% of its TG fatty acid as 18:2ω6, caused significant accumulation of this fatty acid together with a concomitant decrease in 18:1 levels. The infusion of Intralipid, which contain about 50% 18:2ω6, did not have this effect.

Fatty acid composition of liver phospholipids. The effect of the three TPN regimes on liver PC and PE fatty acids is shown in Tables 7 and 8. TPN with 25% dextrose did not alter the fatty acid composition of liver PC but was associated with a reduction in the 18:2ω6 and 18:3ω3 content of PE. The infusion of Liposyn or Intralipid, on the other hand, had a much greater influence on PC than PE. Both parenteral lipid emulsions reduced 18:1ω9, 20:3ω6, 20:4ω6, 20:5ω3, 22:5ω3 and 22:6ω3 and increased 16:0, leading to a significant reduction in total ω3 fatty acids and in the UI of this PL. TPN with Liposyn also reduced total ω9 fatty acids in PC while Intralipid increased total saturated fatty acids (Table 7).

In PE, levels of ω6 and ω3 fatty acids were decreased only by the use of Liposyn. Infusion of this emulsion reduced 18:3ω3, 20:4ω6 and 20:5ω3 levels. These changes, however, did not lead to significant alteration of PE total ω6, ω3 or UI values (Table 8). TPN with either Liposyn or Intralipid increased the level of 18:0 in PE, while Liposyn also decreased 18:1ω9.

DISCUSSION

An important clinical concern in administration of parenteral lipid emulsion is not only the provision of EFA but also the extent to which the composition of lipid supplied may perturb either fatty acid metabolism or incorporation into structural lipids involved in the maintenance and function of biomembranes or eicosonoid synthesis. Liposyn and Intralipid contain very high levels of C18 polyunsaturated fatty acids and are given intravenously, usually as sole source of lipid in TPN. This study reports the effect of seven days infusion of these emulsions on plasma and tissue lipid composition in the rat.

The effect of the TPN lipid was dissimilar for plasma and liver PL. Plasma levels of 18:2ω6 were reduced despite infusion of emulsions rich in this fatty acid. In contrast to liver, however, levels of longer chain ω6 and ω3 metabolites in plasma PL were maintained (Tables 5 and 7). This finding, and the differences between the two emulsion products, may relate to the composition of the emulsion PL. Both Liposyn and Intralipid contain 10 g egg PL/100 ml, although of different composition with respect to ω6 and ω3 fatty acids (Table 2). Previous studies (17) with infusion of Intralipid via the rat tail vein have reported 70% replacement of endogenous plasma lipoprotein PL by egg yolk PL within 24 hr. Data from the present studies indicate a similar replacement. Thus, plasma PL 18:2ω6 levels were reduced by the infusion of Liposyn or Intralipid, which contained 16% and 14% 18:2ω6 in their PL, respectively, in comparison to the higher level of ca. 26% of this fatty acid in control rat plasma PL. The emulsion TG 18:2ω6 content of both products was much higher, suggesting a primary influence of the PL rather than TG fraction. Similarly, the replacement of 20 and 22 carbon chain fatty acids by the infusate PL for plasma PL can explain, at least in part, the differences in these fatty acids of plasma PL which occurred with TPN and between TPN with Liposyn and Intralipid. These findings are important in their demonstration that plasma PL fatty acid composition is not a useful indicator of tissue EFA status during nutritional support with parenteral lipid.

In the liver, infusion of high levels of 18:2ω6 (Liposyn) or 18:2ω6 plus 18:3ω3 (Intralipid) reduced the percentage of 20 and 22 carbon ω6 and ω3 present in PL. This was more pronounced in PC than in PE. Levels of 20:4ω6 and 22:6ω3 in PC were reduced markedly by infusion of either emulsion. They were replaced by higher levels of saturated fatty acids rather than, as might have been anticipated, by 18:2ω6 and/or 18:3ω3 from the infusate lipid. In agreement with this, Martins et al. (18) documented that 10 days of TPN with 30% calories as Intralipid did not lead to accumulation of 18:2ω6 or 18:3ω3 in rat liver total lipid; both fatty acids, however, were increased in subcutaneous and epididymal fat. These studies, therefore, imply that reduced levels of 20 and 22 carbon EFA derivatives in liver PL cannot be explained by competitive esterification of the high percentage of 18 carbon EFA given in the infusate lipid. Whether a steady state response of liver PL fatty acid composition to the TPN lipid was achieved in these studies is unknown. The fatty acyl components of tissue PL are known to turn over rapidly and independently. In this regard, previous studies with different oral diets indicated that maximal diet fat influence on most rat tissue PL fatty acids was achieved by 11 days of treatment (19). The pattern of fatty acid changes found in present studies may suggest inhibition of hepatic Δ6-desaturase by intravenous administration of high levels of the precursor 18:2ω6. This hypothesis requires further experimental support. Interestingly, only Liposyn, which has a substantially higher level of 18:2ω6 than Intralipid, reduced 20:4ω6 levels in hepatic PE.

Little information is available on the effects of parenteral lipid on tissue PL in the human. Postmortem investigations of human infants following TPN with lipid have reported normal 18:2ω6 plus 20:ω6, but markedly reduced 20:4ω6/18:2ω6 ratio in lung, liver, skeletal muscle, renal medulla and cortex and red blood cells (19). Whether this was due to lipid infusion is unclear. Infants who received TPN were premature while control infants were of normal gestation. As the premature infant may have a functionally immature capacity for desaturation of 18 carbon fatty acids (20), EFA metabolism and incorporation into tissue lipid would be expected to have differed in the TPN and control infants irrespective of the route of lipid administration or infusate content of 18:2ω6.

The presence of high levels of 18:3ω3 in Intralipid is of theoretical concern in the further metabolism of ω6 fatty acids and their availability for synthesis of PG and other eicosanoids. The substrate preference of Δ6-desaturase for 18:3ω3 over 18:2ω6 has been well-established both in vitro and in vivo (21). Thus, for example, an oral diet containing linseed oil with 62% 18:3ω3 suppressed rat liver 20:4ω6 levels and altered hepatic PGF-2α synthesizing capacity (22). This has been explained by reduced availability of 20:4ω6 and competitive inhibition by 20:5ω3 for the PG synthetase complex. The

TABLE 7

Major Fatty Acids of Rat Liver Phosphatidylcholine Following 7 Days of TPN

Fatty acids (% wt/wt)	Control	TPN		
		+ 25% Dextrose	+ 10% Liposyn	+ 10% Intralipid
16:0	22.5 ± 1.4	24.0 ± 1.0	29.4 ± 2.3[a]	26.4 ± 1.0[a]
16:1ω9	0.3 ± 0.0	0.6 ± 0.2	0.4 ± 0.1	0.5 ± 0.2
18:0	20.8 ± 2.2	19.8 ± 1.0	29.2 ± 5.3	28.4 ± 2.6
18:1ω9	9.3 ± 0.9	12.0 ± 1.4	5.3 ± 1.2[a]	7.6 ± 0.5[a]
18:2ω6	14.6 ± 1.0	12.3 ± 1.6	15.8 ± 2.4	16.1 ± 1.4
18:3ω3	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
20:3ω9	0.4 ± 0.1	0.2 ± 0.0	0.8 ± 0.2	0.7 ± 0.1
20:3ω6	1.6 ± 0.3	1.0 ± 0.2	0.8 ± 0.1[a]	0.3 ± 0.1[b]
20:4ω6	19.2 ± 1.6	18.6 ± 1.7	11.4 ± 1.7[b]	12.6 ± 1.0[b]
20:5ω3	1.2 ± 0.2	0.8 ± 0.1	0.4 ± 0.1[b]	0.4 ± 0.1[b]
22:4ω6	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.1[a]	0.2 ± 0.0
22:5ω6	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.3	0.0 ± 0.0
22:5ω3	1.0 ± 0.2	0.5 ± 0.1	0.1 ± 0.0[b]	0.3 ± 0.1[a]
22:6ω3	6.1 ± 0.3	5.9 ± 0.5	2.5 ± 0.5[b]	3.4 ± 0.5[b]
Σsat	43.3 ± 3.4	43.8 ± 0.9	58.6 ± 6.2	54.8 ± 3.2[a]
Σω9	9.7 ± 1.0	12.4 ± 1.3	5.7 ± 1.1[a]	8.1 ± 0.5
Σω6	36.9 ± 3.1	33.2 ± 2.8	29.4 ± 4.0	29.4 ± 1.5
Σω3	7.3 ± 0.5	6.7 ± 0.6	3.6 ± 0.6[b]	4.4 ± 0.4[b]
UI	172 ± 13	160 ± 9	114 ± 17[a]	123 ± 8[b]

Data given represent means ± SE, n = 6 for all groups; for experimental details see Materials and Methods.
[a]Values within a line are significantly different ($p < 0.05$) from control.
[b]Values within a line are significantly different ($p < 0.01$) from control.
[c]UI, unsaturation index as described in Materials and Methods.

TABLE 8

Major Fatty Acids of Rat Liver Phosphatidylethanolamine Following 7 Days of TPN

Fatty acids (% wt/wt)	Control	TPN		
		+ 25% Dextrose	+ 10% Liposyn	+ 10% Intralipid
16:0	21.5 ± 1.3	19.6 ± 0.9	22.3 ± 1.4	18.9 ± 0.4
16:1ω9	0.5 ± 0.1	0.7 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
18:0	25.9 ± 1.9	24.0 ± 1.2	33.1 ± 1.9[a]	31.9 ± 1.6[a]
18:1ω9	6.7 ± 0.5	6.9 ± 0.8	4.4 ± 0.8[a]	5.8 ± 0.5
18:2ω6	7.1 ± 0.5	4.9 ± 0.7[a]	9.4 ± 1.4	7.5 ± 0.4
18:3ω3	0.2 ± 0.0	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.2 ± 0.1
20:3ω9	0.4 ± 0.0	0.2 ± 0.1	1.4 ± 0.6	0.5 ± 0.1
20:3ω6	0.8 ± 0.1	0.7 ± 0.3	1.1 ± 0.4	0.5 ± 0.2
20:4ω6	21.5 ± 1.5	24.3 ± 1.6	16.9 ± 0.9[a]	20.0 ± 0.5
20:5ω3	0.8 ± 0.2	0.8 ± 0.1	ND[d]	0.5 ± 0.1
22:4ω6	0.4 ± 0.0	0.6 ± 0.1	0.4 ± 0.2	0.8 ± 0.0[b]
22:5ω6	0.2 ± 0.1	0.5 ± 0.2	0.2 ± 0.3	0.1 ± 0.0
22:5ω3	1.6 ± 0.4	1.2 ± 0.1	0.6 ± 0.4	1.1 ± 0.1
22:6ω3	9.6 ± 1.6	12.9 ± 0.8	6.6 ± 0.8	9.1 ± 0.9
Σsat	47.4 ± 3.0	43.6 ± 1.6	55.3 ± 3.1	50.8 ± 1.3
Σω9	7.1 ± 0.5	7.6 ± 1.0	4.6 ± 0.8[a]	6.3 ± 0.4
Σω6	30.1 ± 2.0	31.2 ± 2.0	28.1 ± 2.5	28.9 ± 0.7
Σω3	12.3 ± 2.0	14.9 ± 0.9	7.5 ± 0.9	11.1 ± 1.0
UI	186 ± 20	214 ± 12	148 ± 10	172 ± 8

Data given represent means ± SE, n = 6 for all groups; for experimental details see Materials and Methods.
[a]Values within a line are significantly different ($p < 0.05$) from control.
[b]Values within a line are significantly different ($p < 0.01$) from control.
[c]UI, unsaturation index as described in Materials and Methods.
[d]ND, not detected.

current studies suggest that 18:3ω3 as 8% total fatty acid in Intralipid is unlikely to be of concern, based on the similar effects observed with Intralipid and Liposyn (ca. 0.5% 18:3ω3).

Several studies have suggested that the dietary intake of 18:2ω6 and the 18:2ω6/18:3ω3 ratios are determinants of tissue PG synthesis (7-10,13,25). Because only a very small portion of tissue total 20:4ω6 is needed for PG production in vivo, it is possible that reduced tissue incorporation of this fatty acid, such as observed in these studies, does not lead to limitation of specific PL eicosanoid precursor pools. However, reduced urinary PGE-M excretion in human neonates during TPN with Intralipid has been reported (22) and may suggest reduced 20:4ω6 availability in these infants. Clearly, the effects of parenteral lipid on this aspect of EFA metabolism require further experimental consideration.

In conclusion, these studies raise several issues concerning the effects of infusions of polyunsaturated fatty acid-rich emulsions during TPN. These include: (i) the effect of replacement of endogenous plasma lipoprotein PL with egg PL on plasma lipoprotein metabolism; (ii) the effect of infusion of high levels of 18:2ω6, or 18:2ω6 plus 18:3ω3, on pathways of EFA desaturation, elongation and incorporation into structural lipids, and (iii) the influence of altered tissue PL composition on the maintenance of membrane lipid structure and associated metabolic and physiological functions, and on the availability of PL-bound 18:3ω6, 20:4ω6 or 20:5ω3 as precursors for eicosanoid production.

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Effects of Essential Fatty Acid Administration on Cardiovascular Responses to Stress in the Rat

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This study examined the effects of 18:2(n-6), 18:3(n-6), 20:4(n-6) and 18:3(n-3) on cardiovascular responses to isolation stress in male rats. Group-acclimated rats were fasted for 2 days, then placed on a fat-free diet. Two wk later animals were divided into six groups (six animals per group) and given eight-wk intraperitoneal osmotic pumps releasing 1.47×10^{-7} mol/hr of either olive oil (OL), or of 18:2(n-6), 18:3(n-6), 20:4(n-6) or 18:3(n-3) in OL. Another group received dummy pumps. Two wk after pump implantation, animals were isolated for four wk. Blood pressure (BP), heart rate and body weight were followed before and during stress. Following the stress period, animals were assessed for cardiovascular reactivity to norepinephrine (NOR) and angiotensin (ANG).

Prior to isolation, 18:3(n-6) lowered BP vs OL ($p < 0.01$). Stress increased BP within 24 hr in all groups except 18:3(n-6) and 20:4(n-6). Treatment with 20:4(n-6) vs OL prevented the BP rise ($p < 0.001$) only for the first two wk of stress. Administration of 18:3(n-6) vs OL prevented any BP increase over the four-wk stress period ($p < 0.001$). Stress increased heart rate in all groups except 20:4(n-6). Heart rate was lowered by 18:3(n-6) vs OL ($p < 0.01$) before and during stress. Vascular reactivity to NOR was unaffected by treatment, but OL and 18:3(n-6) decreased responses to ANG infusion. These data suggest that 18:3(n-6) supplementation attenuates cardiovascular responses to chronic stress, and that Δ6- and Δ5-desaturase activity are inhibited during chronic psychological stress. *Lipids 21,* 139–142 (1986).

Social isolation has been reported to produce a reversible elevation in BP in rats (1–3). Furthermore, the administration of 18:3(n-6) has been demonstrated to suppress the development of borderline hypertension during a two-wk period of social isolation stress in group-acclimated male Sprague-Dawley rats (4). It is not known, however, whether the effects of 18:3(n-6) in this model are mediated via its conversion to 20:3(n-6) and to 1-series prostaglandins (5), or via the subsequent conversion of 20:3(n-6) to 20:4(n-6) and its derivatives (6).

In vitro studies have indicated that stress hormones such as epinephrine and corticosteroids inhibit the activity of both the Δ6- and Δ5-desaturase enzymes, reducing the conversion of 18:2(n-6) to 18:3(n-6) and 20:3(n-6) to 20:4(n-6), respectively (7,8). Should this situation also occur during stress in vivo, it would suggest that n-6 fatty acids other than 18:3(n-6) or 20:3(n-6) would be unable to suppress the cardiovascular response to stress. However, if stress does not suppress Δ6- and Δ5-desaturase activity in vivo, 18:2(n-6), which is abundant in the diet, should also be able to lower BP during stress.

The purpose of the present study was to evaluate the effects of essential fatty acid administration on cardiovascular responses to chronic psychosocial stress in rats in order to determine (i) which n-6 fatty acid is responsible for the attenuation of the blood pressure responses to stress; (ii) whether or not Δ6- and Δ5-desaturase activity in the rat is inhibited during stress, and (iii) whether or not the n-3 fatty acid, 18:3(n-3), produces cardiovascular effects similar to those of the n-6 series.

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MATERIALS AND METHODS

Animals. The study used 36 adult male Wistar Kyoto rats purchased from Taconic Farms (Germantown, New York) at six wk of age. Prior to the start of the study, all animals were group-housed, four per cage, for a five-wk acclimation period. During the course of the study all animals were maintained in a temperature- (21 ± 1 C) and light-cycle-controlled (14 hr light, 10 hr dark) room.

Blood pressure determination. Systolic BP was measured indirectly using the tail cuff technique (4,9). Animals were prewarmed for 8 min in a 35 C incubator and then placed in a conical cloth holder during the measurement. Each recorded BP value represents the mean of 10 determinations taken over a two-min period after stabilization of the tracing. Prior to the study, all animals had three BP determinations made to accustom them to the procedure.

During the determination of cardiovascular reactivity to NOR and ANG, BP was monitored directly via an ortic cannula inserted abdominally to the level of the renal artery.

Administration of test agents. Fatty acids were administered by means of eight-wk, constant flow (1.5 μ/hr) osmotic minipumps (modified Alzet 2ML4) implanted under halothane anesthesia at the start of the study. OL was used as the vehicle for fatty acids and was shown by gas chromatography to contain 0.4% 18:3(n-3) and 9.6% 18:2(n-6). OL has been shown to have no effect on resting BP at the rate of administration used (4). In order to assess cardiovascular reactivity, 4.30 μg NOR and 1.25 μg ANG (ED_{50}) were injected intra-arterially in a volume of 0.10 ml.

Protocol. Following the five-wk group acclimation period, all animals were fasted for two days and then placed on fat-free diets (ICN, Montreal, Quebec, Canada) for the remainder of the study. Two wk after initiation of the fat-free diet, animals were divided into six groups of six animals each and implanted with pumps releasing 1.47×10^{-7} mol/hr (≅0.4 mg/hr) of either OL or of 18:2(n-6), 18:3(n-6), 20:4(n-6) or 18:3(n-3) in OL. The sixth group received dummy pumps (DUM) to control for the OL administration. All animals remained group-housed for a two-wk control period after pump implantation. Following this, animals were placed in individual Nalgene cages for a four-wk period of isolation stress. BP and heart rate (HR) were measured two wk, one wk and 24 hr prior to isolation and after 24 hr and 1, 2, 3 and 4 wk of isolation.

One day following the week-4 measurement, animals were anesthetized with halothane and arterially cannulated as described above. Following separate injections of NOR and ANG (Sigma, St. Louis, Missouri), BP and HR responses were monitored using a Coulborne

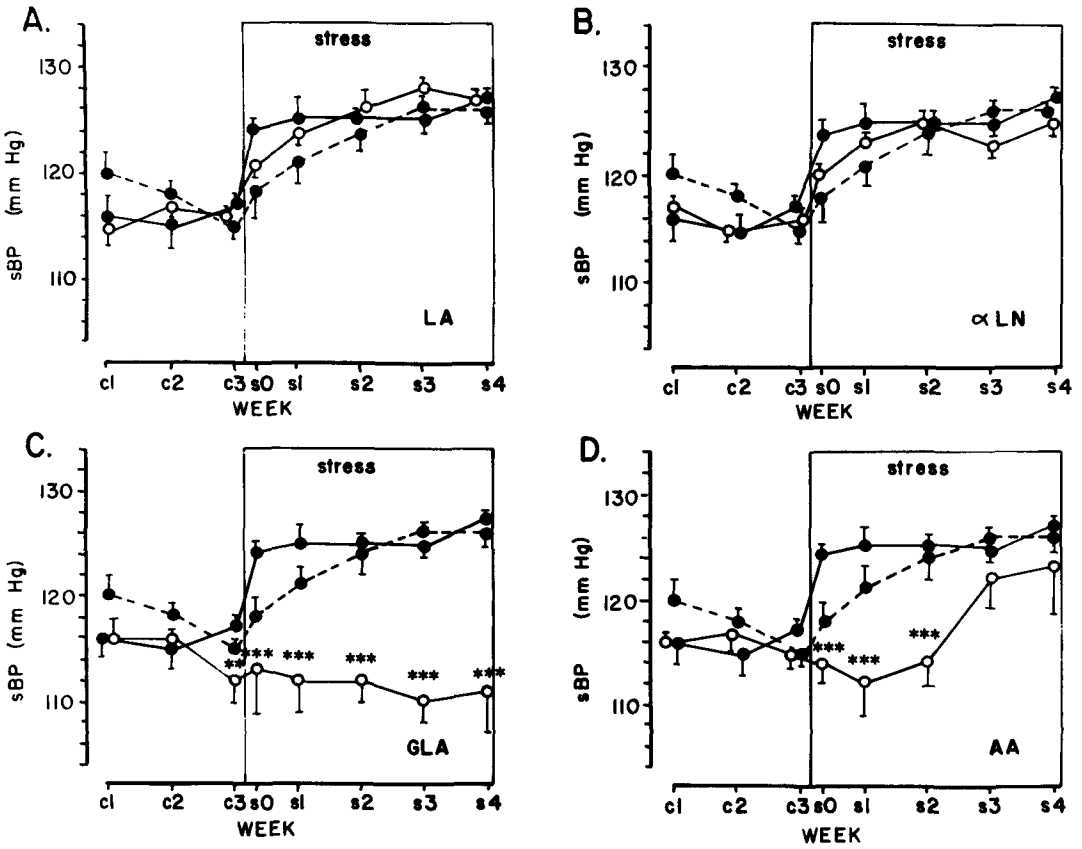

FIG. 1. Systolic blood pressure (sBP) of adult male Wistar Kyoto rats (n = 6/group) over a two-wk period prior to and during a four-wk period of social isolation stress. Measures were taken at weekly intervals as well as 24 hr prior to (c3) and 24 hr following (s0) isolation. Control groups in all four panels represent OL-treated (●—●) and DUM-treated (● - - - ●) animals. Test substances (○—○) are 18:2(n-6) (LA, Fig. 1A), 18:3(n-3) (αLN, Fig. 1B) 18:3(n-6) (GLA, Fig. 1C) and 20:4(n-6) (AA, Fig. 1D). All values represent $\bar{x} \pm$ S.E. **, $p < 0.01$ vs OL. ***, $p < 0.001$ vs OL.

physiograph to assess cardiovascular reactivity.

Statistics. BP and HR were analyzed using a 2-way ANOVA. Where a significance of $p < 0.05$ was attained, specific points of difference were determined using planned orthogonal comparisons (10). Vascular reactivity was analyzed using Student's t-test.

RESULTS

BP responses to stress. Systolic BP responses to stress are illustrated in Figure 1. Social isolation of the OL and DUM control groups produced a significant ($p < 0.001$) increase in BP of ca. 10–12 mm Hg vs prestress, which stabilized after two wk of isolation. Prestress BP was not affected by the administration of any of the fatty acids except 18:3(n-6), which significantly decreased BP ($p < 0.01$) after two wk of control period administration.

Neither 18:2(n-6) nor 18:3(n-3) significantly altered the BP response to four wk of stress vs DUM and OL groups. In contrast, 20:4(n-6) vs OL significantly attenuated the BP response to stress over the first two wk of the stress period ($p < 0.001$), eliminating any stress-related increase during this time. Over the last two wk of the stress period, however, BP in the 20:4(n-6) group increased to control group levels. Administration of 18:3(n-6) significantly reduced BP over the entire stress period vs OL ($p < 0.001$) completely eliminating any BP increase in response to stress.

HR responses to stress. The HR responses to stress are shown in Table 1. The 18:3(n-6) significantly decreased control period resting HR vs OL ($p < 0.01$), whereas the other fatty acids were without effect prior to stress. Exposure to chronic stress significantly increased HR in all groups except 20:4(n-6), although the temporal patterns of HR increases varied between groups. DUM animals had significantly higher HR than did the OL group after two ($p < 0.001$) and three ($p < 0.01$) wk of stress. Treatment with 18:2(n-6) vs OL also led to increased HR at weeks 1 ($p < 0.01$), 2 and 3 ($p < 0.001$). In contrast, 18:3(n-6), 20:4(n-6) and 18:3(n-3) groups demonstrated lower HR than the OL group after 24 hr of isolation ($p < 0.001$, $p < 0.01$, respectively), and higher HR vs the OL group after one wk of stress ($p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively). After that time, 20:4(n-6) vs OL increased HR at week 2 of stress ($p < 0.001$), whereas 18:3(n-6) vs OL decreased after weeks 3 and 4 ($p < 0.01$).

Vascular reactivity to NOR and ANG. Cardiovascular responses to exogenous NOR and ANG are shown in Table 2. Infusion of the saline vehicle had no effect on BP or HR in this model. None of the fatty acids tested had any effect on the systemic responses to NOR administration. In contrast, the HR response to ANG was

TABLE 1

Effects of Fatty Acid Administration (n = 6/group) on HR Responses to Isolation Stress in Male Wistar Kyoto Rats ($\bar{x} \pm$ S.E.)

Time	DUM	OL	18:2(n-6)	18:3(n-6)	20:4(n-6)	18:3(n-6)
C3	348 ± 8	338 ± 11	345 ± 3	322 ± 7[b]	350 ± 9	340 ± 9
S0	362 ± 9[d]	362 ± 11[e]	372 ± 5[f]	333 ± 11[c]	347 ± 12[b]	343 ± 10[b]
S1	348 ± 9	334 ± 9	353 ± 9[b]	350 ± 11[a,f]	360 ± 15[c]	358 ± 11[c,d]
S2	375 ± 8[c,f]	342 ± 3	375 ± 15[c,f]	353 ± 7[f]	343 ± 7	366 ± 15[c,f]
S3	368 ± 9[b,e]	344 ± 12	363 ± 12[c,d]	323 ± 9[b]	348 ± 8	355 ± 9[d]
S4	362 ± 10[d]	352 ± 12[d]	360 ± 9[d]	330 ± 9[b]	362 ± 5	360 ± 9[e]

Measurements were taken 24 hr prior to isolation (C3), 24 hr post isolation (S0) and weekly over the next 4 weeks of isolation (S1-S4).

[a]$p < 0.05$ vs OL.

[b]$p < 0.01$ vs OL.

[c]$p < 0.001$ vs OL.

[d]$p < 0.05$ vs C3.

[e]$p < 0.01$ vs C3.

[f]$p < 0.001$ vs C3.

TABLE 2

Cardiovascular Responses of Male Wistar Kyoto Rats Receiving Dummy Pumps (DUM) or Infusions of Either OL or of 18:2(n-6), 18:3(n-6), 20:4(n-6) or 18:3(n-6) in OL (n = 6/Group) to Intra-Arterial Infusions of NOR (4.30 μg) and ANG (1.25 μg), Following 4 Wk of Isolation Stress

	OL	DUM	18:2(n-6)	18:3(n-6)	20:4(n-6)	18:3(n-3)
NOR						
Duration of response (sec)	314 ± 33	287 ± 59	302 ± 37	381 ± 52	327 ± 49	346 ± 56
HR response (min^{-1})	50 ± 16	41 ± 11	57 ± 8	40 ± 9	29 ± 15	45 ± 11
Δs BP (mm Hg)	40 ± 5	44 ± 6	34 ± 2	44 ± 6	33 ± 5	41 ± 9
Δd BP (mm Hg)	39 ± 5	44 ± 6	35 ± 2	44 ± 6	34 ± 4	38 ± 8
ANG						
Duration of response (sec)	287 ± 18	217 ± 37	194 ± 16	199 ± 27	339 ± 61	229 ± 28
HR response (min^{-1})	9 ± 9	73 ± 29[a]	90 ± 25[b]	16 ± 11	37 ± 20	38 ± 15
Δs BP (mm Hg)	21 ± 1	33 ± 4[a]	36 ± 3[b]	25 ± 2	34 ± 4[a]	36 ± 4[b]
Δd BP (mm Hg)	21 ± 2	32 ± 4[a]	40 ± 5[c]	25 ± 2	41 ± 3[c]	38 ± 4[a]

[a]$p < 0.05$ vs OL.

[b]$p < 0.01$ vs OL.

[c]$p < 0.001$ vs OL.

significantly greater in DUM ($p < 0.05$) and 18:2(n-6) groups ($p < 0.01$) in comparison to the OL group. In addition, the systolic and diastolic BP responses to ANG were greater in DUM ($p < 0.05$), 18:2(n-6) ($p < 0.01$ and $p < 0.001$, respectively) and 20:4(n-6) ($p < 0.01$ and $p < 0.05$, respectively) groups in comparison to the OL group. The response of the 18:3(n-6) group was not significantly different from the OL group.

DISCUSSION

In the present study, 18:3(n-6) administration decreased BP in unstressed animals after two wk. This is in contrast to a previous report in the literature in which a similar dose of 18:3(n-6) had no effect on resting BP (4). It is possible that the difference in response to 18:3(n-6) during the resting condition reflects genetic differences in fatty acid metabolism and BP regulation between the Wistar Kyoto and Sprague-Dawley strains. Supporting this is the fact that resting BP in the former strain is approximately 10–13 mm Hg lower than that reported for the latter (4). In addition, the BP response to isolation stress in Sprague-Dawley rats was 15–20 mm Hg (4), compared to 10–12 mm Hg response in the Wistar Kyoto strain. Another possible reason for the difference is that animals in the present study were maintained on a fat-free diet for two wk prior to the initiation of 18:3(n-6) administration, whereas the previous reports maintained animals on standard rat chow (4). This explanation is not likely, however, as animals showed no overt symptoms of essential fatty acid deficiency in the present study, and 18:3(n-6) administration in the control period should have corrected any subclinical deficiency which might have resulted from the fasting and fat-free diet.

A second observation in the present study was that only 18:3(n-6) and 20:4(n-6) attenuated the BP responses to stress. Furthermore, the suppressive action of 20:4(n-6) on BP was only present during the first two wk of stress,

in contrast to the maintained effect seen with 18:3(n-6). This suggests that after two wk of stress there was a shift in 20:4(n-6) metabolism which eliminated its antihypertensive action. This shift may have been either from a derivative of 20:4(n-6) exhibiting vasodepressor actions (11,12) to one with vasopressor activity (12,13), or from an extrarenal to an intrarenal site of action (13).

The rat normally has relatively high Δ6- and Δ5-desaturase activity compared to the human, so that 18:2(n-6) and 18:3(n-6) are rapidly converted to 20:4(n-6) (6,15). Furthermore, it has been reported that the development of hypertension in both the rat and human is accompanied by a decrease in Δ6- and Δ5-desaturase activity (16–18). In the present study, the differential responses to equimolar amounts of the various n-6 fatty acids suggested that there was little, if any, conversion of 18:2(n-6) to 18:3(n-6) and of 18:3(n-6) to 20:4(n-6) during psychosocial stress. This agrees with earlier reports from studies that stress hormones inhibit Δ6- and Δ5-desaturase activity (7,8). In light of this, 18:3(n-3) may not have demonstrated any antihypertensive activity because it also requires Δ6-desaturase for metabolism. Further studies on the effects of 18:4(n-3) and 20:5(n-3) on BP during stress must be conducted before a possible stress-attenuating action of n-3 fatty acids can be excluded.

The suppressive effect of 18:3(n-6) on BP during stress appears to be independent of its effects on vascular reactivity to NOR and ANG. Although OL and 18:3(n-6) similarly reduced cardiovascular response to ANG in comparison to the DUM group, this does not explain the differential stress response to 18:3(n-6) vs OL. While the effect of 18:3(n-6) on reactivity to ANG may be mediated via its conversion to prostaglandin E_1 (19), these results also suggest that OL itself may have an effect on vascular responsiveness to circulating pressor hormones. The OL effects may be mediated by its high content of oleic acid.

In the present study, 18:3(n-6) administration significantly depressed both resting and stressed HR in comparison to OL and DUM groups. This coincided with the initial reductions in BP during resting and stress conditions. As 18:3(n-6) does not alter cardiovascular reactivity to NOR, these data suggest that it may act via decreasing central sympathetic activity. In a previous study on Sprague-Dawley rats, however, 18:3(n-6) had no effect on HR and was believed not to act on central sympathetic activity (4). The HR data also suggest that OL had a suppressive effect on HR during stress, as it suppressed the HR response to stress in comparison to DUM treatment.

Thus, the results of the present study suggest that 20:4(n-6) acutely, and 18:3(n-6) chronically, prevents BP increases resulting from psychological stress in rats and that the mechanism of action does not result from altered vascular reactivity to NOR and ANG. Furthermore, OL, 18:2(n-6) and 18:3(n-3) may exert independent effects on these parameters, even in the absence of a BP effect. These findings may have implications for dietary approaches to stress reduction in animals and humans.

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Acetate and Mevalonate Labeling Studies with Developing *Cuphea lutea* Seeds

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Cuphea seeds contain large amounts of medium chain (C_8 to C_{14}) fatty acids, mainly as triacylglycerols. The biosynthesis of these lipids was studied in vivo by incubating developing *Cuphea lutea* seeds with labeled acetate. Incorporation of label into triacylglycerols and into medium chain fatty acids occurred principally during the period of endogenous lipid deposition, but some label was encountered in these products even during seed dehydration. At this later stage palmitate and oleate were the dominant labeled fatty acids. During the period of rapid endogenous lipid deposition acyl lipids other than triacylglycerols were minor labeled components. The labeling patterns were consistent with the Kennedy pathway for triacylglycerol biosynthesis. The fatty acid composition of the acyl-CoA pool was similar to the total lipid fatty acid composition, but the acyl-ACP pool contained relatively more short chain acyl groups. Squalene was labeled from acetate throughout the period of seed development, but labeled sterols were not detected. Using [2-^{14}C]mevalonic acid lactone as substrate, squalene was the principal labeled product. Small amounts of label were found in free sterols. However, in terms of mass, free sterol dominated over squalene. The possibility of two independent sites of isoprenoid biosynthesis in the developing embryo is discussed.
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Saturated, medium chain (8:0 to 14:0) fatty acids (MCFA) are found in the seed oils of a number of plant genera, especially tropical flora (1). These fatty acids, particularly lauric acid, are important commodities and are produced commercially from coconut and palm kernel oils. The biosynthesis of MCFA in developing seeds has never been explained satisfactorily (2,3). By contrast, two mechanisms of MCFA biosynthesis have been discovered in the lactating mammary gland of mammals. These involve termination of chain extension by a thioesterase which is independent of the fatty acid synthetase complex (4) or by a transacylase which is an integral part of the fatty acid synthetase complex (5). In order to investigate the mechanism of MCFA biosynthesis in plants we have turned to developing cuphea seeds. The genus *Cuphea* offers a large number of species with a range of oil compositions (6,7) and currently is being investigated for its agronomic potential (8). Cuphea offers a steady, year-round supply of developing seed tissue, and though the seeds are rather small they very actively synthesize lipid. We have been able to obtain active in vitro preparations from these seeds (2). In this paper we describe in detail our studies on the in vivo labeling patterns of *Cuphea lutea* seeds. During the study with labeled acetate we noticed high levels of labeled squalene, which we had not encountered before with other developing seeds. As there appeared to be scant literature on the biosynthesis of squalene and sterols during seed development we pursued this topic also.

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MATERIALS AND METHODS

Seed material. Cuphea seed material was the gift of F. Hirsinger, Department of Crop Science, Oregon State University, Corvallis. *Cuphea lutea* was chosen as the experimental plant as it was self-pollinating and gave good seed set in the growth chamber and the greenhouse. For the detailed developmental studies growth chamber plants were used, with a 16 hr day, 50% humidity, 25 C day and night temperature growth regime. Flowers were tagged when the petals first opened. For more routine experiments seeds were harvested from greenhouse plants according to the description given in Results and Discussion. Safflower seeds were harvested from greenhouse plants 12 to 16 days after flowering. Embryos from these seeds were in the early part of the lipid deposition phase.

Radiochemicals. [2-^{14}C]Acetate (sp. act. 55.9 Ci/mol), [2-^{3}H]acetate (sp. act. 3290 Ci/mol) and [2-^{14}C]R-mevalonic acid lactone (sp. act. 53.0 Ci/mol) were purchased from Amersham (Arlington Heights, Illinois).

Incubations and lipid extractions. A typical assay would contain 20 halved, maturing *Cuphea lutea* seeds in 0.5 ml of 50 mM 2-(N-morpholino)ethanesulphonic acid, pH 6.0. The incubation was started by the addition of 5 μCi [2-^{14}C]acetate (89.4 nmol, 179 μM) or 2 μCi [2-^{14}C]mevalonate (37.7 nmol, 75.5 μM) and run for up to 4 hr in a shaking water bath at 27 C. To terminate the incubation the seeds were rinsed several times in water and rapidly separated into seed coat and embryo, and the embryos were heated at 80 C in 1 ml of isopropanol. Lipids were extracted by homogenization of the tissue in hexane/isopropanol (3:2, v/v), addition of aqueous sodium sulphate to give phase separation, and re-extraction of the aqueous phase and insoluble residues with hexane/isopropanol (7:2, v/v) as described by Hara and Radin (9).

In assays where the purpose was to measure the labeled acyl-CoA and acyl-ACP pools, seeds were halved and dissected prior to the incubation. Incubations were run with 20 dissected embryos and 1 mCi [2-^{3}H]acetate (304 nmol). The reaction was terminated by very rapid rinsing of the embryos with water and immediate homogenization in 2 ml of isopropanol/50 mM phosphate buffer, pH 7.2 (1:1, v/v). After centrifugation of the debris the supernatant was acidified with 0.05 ml of glacial acetic acid. At this point the extraction scheme of Mancha et al. was followed (10). The method gave 80–85% recovery of acyl-ACP in the ammonium sulfate pellet when exogenous [1-^{14}C]stearoyl-ACP was added.

Lipid analysis. Thin layer chromatographic (TLC) separations of neutral lipids were carried out using silica gel plates developed halfway twice with hexane/diethyl ether/acetic acid (60:40:2, v/v/v) and then fully with hexane/diethyl ether (90:10, v/v). TLC separations of polar lipids were carried out using half development with hex-

ane/diethyl ether/acetic acid (50:50:2, v/v/v), followed by a full development with acetone/benzene/acetic acid (100:25:10, v/v/v), and finally a half development with chloroform/methanol/water (65:35:2, v/v/v). Mass was detected by light staining in iodine vapor. Radioactivity generally was detected by a radio-TLC scanner, but when higher resolution was required autoradiography was used.

Fatty acids were analyzed by saponification of the lipid in 2 ml of 5% KOH in aqueous ethanol for 1 hr at 80 C. After acidification and addition of water (5 ml) fatty acids were extracted into diethyl ether (3 × 2 ml); the ethereal extract was washed with water (2 × 3 ml) and dried over anhydrous sodium sulphate. The sample was then evaporated to dryness under nitrogen with evaporative cooling keeping the temperature below 0 C. Methyl esters were prepared using ethereal diazomethane, and [^{14}C]fatty acid methyl ester analysis was performed by radio-GLC using a 6 ft × 4 mm 10% DEGS-PS column temperature programmed from 100 C to 190 C at 10 C/min. Heptanoic and pentadecanoic acids were used as calibrants for the procedure. Recovery of fatty acids of chain length of C_{10} or greater was quantitative and recovery of octanoic acid was acceptable (70–80%), while the very short chain fatty acids were essentially lost.

For analysis of ^{3}H labeled acyl-CoA and acyl-ACP pools, these fractions were saponified as described above in the presence of 50 μg each of octanoic, decanoic, lauric, myristic, palmitic and stearic acids. After extraction the free fatty acids were purified by silica gel TLC and derivatized to produce phenacyl esters. The fatty acids were heated at 90 C for 1 hr in 0.2 ml of acetonitrile containing 2-bromoacetophenone (10 μmol), 18-crown-6 macrocyclic polyether (0.5 μmol) and triethylamine (15 μmol) (11). On cooling the acetonitrile solution was evaporated to dryness and the sample suspended in a small volume of acetone. Individual fatty acid phenacyl esters were separated by reversed phase TLC using Whatman LKC18F plates and developing with methanol/water (95:5, v/v). Individual components were located under UV light, scraped off and eluted with acetone and the radioactivity was assayed.

Analysis of endogenous squalene and sterols was performed by gas liquid chromatography (GLC) using internal standards (tetracosane for squalene and cholesterol for sterols) added during the lipid extraction. Squalene was quantitated by injection of the total lipid extract on a 4 ft × 2 mm 1% Dexisil-300 column, temperature programmed from 150 C to 350 C at 5 C/min. Free sterols were eluted from the 4-desmethyl, 4-monomethyl and 4,4′-dimethyl sterol region of neutral lipid silica gel TLC plates (12) and quantitated by capillary GLC using a 30 m × 0.25 mm SE-30 column temperature programmed from 250 C to 300 C at 10 C/min. Total sterols were determined by saponification of the lipid sample by refluxing in 5% KOH in ethanol for 2 hr and extracting the unsaponifiables in diethyl ether. The unsaponifiable fraction then was examined by capillary GLC. Acetylation of free sterols was achieved by standing overnight at room temperature in acetic anhydride/dry pyridine (1:1, v/v), then evaporating the reagents under nitrogen. Silylation was achieved by heating overnight at 80 C in dry pyridine/N-methyl-N(*tert*-butyldimethylsilyl)trifluoroacetamide/*tert*-butyldimethylchlorosilane (100:99:1, v/v/v), then evaporating the reagents under nitrogen.

RESULTS AND DISCUSSION

The rate of endogenous lipid deposition. Cuphea lutea seeds contain a large amount of neutral lipid. Average figures for total seed dry wt, embryo dry wt and total seed lipid were 2.9 mg, 1.8 mg and 0.95 mg per seed, respectively. Because the neutral lipid is deposited in the embryo, lipid represents 53% of the dry weight of the embryo. As *Cuphea lutea* seeds exhibit very rapid maturation the rate of lipid deposition is very high, being greater than any other developing oilseed we have examined. Greenhouse-grown *Cuphea lutea* plants exhibited a rate of seed lipid deposition of 40 mg/day/g fresh wt, which compares with values of 15.5, 12.7, 7.5 and 2.75 mg/day/g fresh wt, respectively, for greenhouse-grown safflower, rapeseed, meadowfoam and soybean. A value of about 17 mg/day/g fresh wt for safflower was estimated from the data of Ichihara and Noda (13) and of 35 mg/day/g fresh wt for *Cuphea procumbens* from the data of Slabas et al. (14). *Cuphea lutea* exhibits a useful morphological marker during seed development, which saves tagging for routine experiments. The seed capsule splits midway through the lipid deposition phase, and the placenta with its five seeds rises. The seed coat is still green and will not begin to brown for another two days. Seeds are harvested at the stage when the capsule first begins to split.

Acetate uptake data. Acetate uptake was linear up to about four hr when incubating halved seeds (Fig. 1). Separation of the seed coat from the embryo prior to incubating the embryo with labeled acetate resulted in nonlinear kinetics. Optimum acetate incorporation occurred over a pH range of 4.5–6.0. Acetate incorporation was proportional to the number of seeds incubated up to about 30 seeds per 0.5 ml assay (120 mg fresh wt). Acetate concentration curves showed biphasic behavior with a

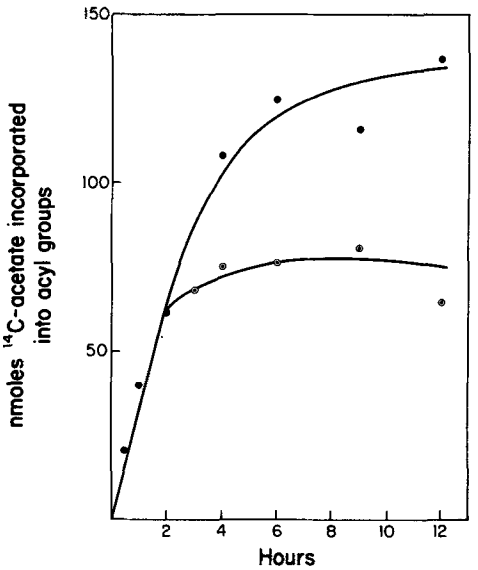

FIG. 1. Time course (● — ●) and pulse chase (○—○) kinetics for [^{14}C]acetate incorporation into fatty acids by developing embryos of *Cuphea lutea*. Acetate concentration was 0.72 mM and the assay tube contained 18 halved seeds in 0.25 ml of buffer. For the pulse chase at 2 hr the seeds were rinsed three times in water, then once in 2 mM cold acetate, before continuing the incubation in fresh buffer.

tendency to plateau at concentrations above 2 mM, at rates of the order of 1.5 nmol/hr/embryo. Thus the level of acetate incorporation in these in vivo experiments is two orders of magnitude less than C_2 utilization required to sustain endogenous lipid synthesis (180 nmol/hr/embryo). This is to be expected in the light of previous studies on developing oilseeds. In seeds which contain long chain fatty acids, such as jojoba, meadowfoam, nasturtium and rapeseed, [^{14}C]acetate labeling shows two distinct metabolic pools (15–18). The pool utilized for chain elongation has a 10–50-fold greater specific activity than the pool utilized for de novo fatty acid synthesis, so acetate label for de novo fatty acid synthesis is diluted out extensively. This rationalization can be applied to cuphea to explain, at least in part, the discrepancy between endogenous and assay rates of lipid synthesis.

TABLE 1

Incorporation of [^{14}C]Acetate into Seed Coat and Embryo Lipids of Developing *Cuphea lutea* Seeds

	Seed coat	Embryo
Rate of acetate incorporation (nmol/hr/seed)	0.1	0.34
Percentage ^{14}C distribution within lipid classes		
Hydrocarbons	16	13
Triacylglycerols	6	57
Diacylglycerol fraction[a]	10	11
Polar lipids	48	18
Endogenous fatty acids (μg/seed)	6.5	125
Percentage ^{14}C in total lipids present as fatty acids	46	76
Percentage ^{14}C and (percentage mass) distribution within acyl groups		
8:0	0 (0)	8 (1.0)
10:0	3 (6)	25 (24.5)
12:0	6 (7.5)	19 (42.5)
14:0	20 (2.5)	5 (11.5)
16:0	29 (14)	5 (3)
18:0	7 (6)	2 (1)
18:1	16 (11.5)	23 (8)
18:2	11 (45)	7 (8)
18:3	1 (6.5)	0 (0)
Other	7	6

[a]The diacylglycerol fraction contains several labeled compounds with R_f values similar to the 1,2-diacylglycerol standard. In the case of the embryo only 2–4% of the total label, not 11%, is actually acyl lipid, presumably diacylglycerol.

[b]Incubation conditions as described in Materials and Methods.

Product analysis. Table 1 shows the product analysis from a typical experiment. Labeling of both seed coat and embryo occurs, but the distribution of label is very different. The embryo produces large amounts of labeled triacylglycerols and MCFA. The [^{14}C] fatty acid pattern found in the embryo is close but not identical to the endogenous fatty acid composition. There is a tendency for shorter chain fatty acids to show increasing specific activities. The endogenous fatty acid profile of the seed coat does show 16% MCFA, but in absolute amount this represents only 1% of the mass of the MCFA in the embryo and is likely to arise from cross-contamination during the separation process.

Labeling of the hydrocarbon fraction in the embryo ranged between 7 and 22% in five experiments. From a preparative scale incubation the labeled hydrocarbon fraction was isolated by silica gel TLC. This fraction then was examined by radio-GLC (1% Dexisil-300 column, isothermal, 280 C) and C_{18} reversed phase TLC. Both techniques showed a single labeled compound. The compound was purified to apparent radiochemical purity by high performance liquid chromatography (HPLC) (elution with methanol/THF [9:1, v/v] using a 4.6 mm × 25 cm C_{18} reversed phase column). The resulting compound was identified as squalene by gas chromatography-mass spectroscopy (GC-MS) (EI). An authentic sample of squalene then was used as a standard to confirm the elution behavior of [^{14}C] compound as squalene by silica gel TLC, C_{18} reversed phase TLC and radio-GLC on 1% Dexisil-300 and 10% SP-2330 stationary phases.

Table 2 shows the [^{14}C] fatty acid distribution in lipid classes of the maturing embryo. Triacylglycerol, the dominant species, is enriched in MCFA. The 1,2-diacylglycerol

TABLE 2

Composition of ^{14}C-Labeled Acyl Lipid Fractions after [^{14}C]Acetate Incorporation into Developing Embryos of *Cuphea lutea* Seeds

	Lipid fractions				
Acyl group	Total lipids	Triacylglycerols	Diacylglycerols	Polar lipids	Phosphatidylcholine
8:0	5.5	6.5	11	0	1
10:0	37	48	20	4	7
12:0	24	29	9	3	6
14:0	7	7	4	6	9
16:0	9.5	5	11	31	21
18:0	4	1	11	14	12
18:1	7	2.5	3	21	27
18:2	2	1	8	6	8
18:3	0	0	0	1	1
20:0	3	1	16	9	6
22:0	1	0	6	5	2

Incubation conditions as described in Materials and Methods.

fraction contains MCFA (44%), normal chain fatty acids (C_{16} and C_{18}, 33%) and long chain fatty acids (C_{20} and C_{22}, 22%), and therefore probably represents several metabolic pools. Neutral lipid TLC with multiple development and autoradiography highlighted several overlapping bands in the diacylglycerol region of the plate. Free sterols, which run very close to 1,2-diacylglycerols, could not be detected. Within the total polar lipid fraction, which accounted for 9–24% of the label incorporated depending on the experiment, phosphatidylcholine represented 30–40% of the radioactivity. Both the total polar lipid and the phosphatidylcholine fractions showed small but reproducible amounts of label in MCFA. The level of ^{14}C-labeling in other polar lipid bands such as phosphatidylethanolamine, phosphatidylglycerol and phosphatidic acid was insufficient to conduct fatty acid analyses.

The composition of the labeled acyl-CoA and acyl-ACP pools is shown in Table 3. These experiments were facilitated by the use of high specific activity [2-^{3}H]acetate labeling. Developing safflower seeds were run as a control. The acyl-ACP pool of developing *Cuphea lutea* seeds showed a build-up of MCFA, particularly C_8, but does not mirror the labeled fatty acid composition (Tables 1 and 2). Most of this pool was depleted in a one-hr pulse chase. Calculations based on the rate of acetate incorporation into embryos and the cpm in the acyl-ACP pool suggested that the turnover time for the acyl-ACP pool was of the order of five sec or less. As the label in the acyl-ACP pool in cuphea decreased from 15,000 to 3,000 cpm in the one-hr chase period, we assume that the pulse chase did not remove all available ^{3}H substrate, particularly since the labeling kinetics show that about 20% of the label could be expected to move through to lipids in the one-hr chase period (Fig. 1). The [^{3}H] acyl-CoA pool in developing *Cuphea lutea* seeds resembled quite closely the end product fatty acid composition, presumably because it is an acceptor pool for the final products of fatty acid synthesis. Surprisingly, when we examined the labeled acyl-ACP pool of developing safflower seeds (high oleate variety), we found appreciable levels of MCFA. This is different from the previous findings of Sanchez and Mancha, who observed that palmitate, stearate, oleate and, to a lesser extent, myristate were the principal components of the endogenous acyl-ACP pool in developing olives and soybean seeds (19). Interpretation of the results in Table 3 in terms of a mechanism for MCFA biosynthesis is somewhat difficult. We do not know the endogenous acyl-ACP pool sizes and acyl compositions, nor do we have detailed specificity data on potential chain termination mechanisms. However, since the labeled acyl-ACP pool in cuphea differs in acyl composition from the labeled acyl-CoA and lipid pools it is likely that there is a controlling acyl specificity for some step in the ACP-track:CoA-track switching mechanism (20) which will determine fatty acid chain length in the oil. This conclusion is even more obvious for the safflower control and is presumed to be a consequence of the specificity of the acyl-ACP thioesterase (21).

Pulse chase experiments. Table 4 gives details of representative time points from the experiment shown in Figure 1. In the four-hr chase period following a two-hr labeling period, the 1,2-diacylglycerol and monoacylglycerol pools were significantly depleted, while label in triacylglycerols increased slightly. Label in the phosphatidic acid pool was insufficient (<0.5%) and was poorly defined, making it impossible to follow ^{14}C-pulse chase kinetics. The label in the total polar lipid and phosphatidylcholine fractions remained constant over the chase period, as did the [^{14}C]acyl composition in these fractions. Thus there was no evidence to suggest that there was channeling of labeled MCFA through phosphatidylcholine or other polar lipid intermediates. In the light of evidence to the contrary it is likely that MCFA incorporation into triacylglycerols follows the classical Kennedy pathway.

Developmental studies. Figure 2 shows the developmental variation of several parameters associated with [^{14}C]acetate labeling experiments, while Table 5 gives total fatty acid compositions for four selected ages. The endogenous fatty acid composition of the embryo remains constant from day 8 onwards. Day 6 shows slightly higher percentages of C_{14}, C_{16} and C_{18} fatty acids. Maximum acetate incorporation occurs at day 7, when only 17% of the eventual lipid has been deposited. Labeled MCFA and triacylglycerol biosynthesis peak at this time.

TABLE 3

Composition of the [^{3}H]Acyl Pools in Acyl-ACP and Acyl-CoA Fractions from Developing Cuphea and Safflower Seeds Labeled with [^{3}H]Acetate

	Cuphea lutea				Safflower	
	Acyl-ACP		Acyl-CoA		Acyl-ACP	Acyl-CoA
	1 hr	1-hr chase	1 hr	1-hr chase	1 hr	1 hr
Total cpm ($\times 10^{-6}$)	0.015	0.003	0.3	0.36	0.1	1.86
Percentage ^{3}H distribution in acyl groups						
8:0	58	31	14	12	25	2
10:0	19	14	36	32	22	3
12:0	11	25	19	21	27	3
14:0	5	13	11	13	13	4
16:0 + 18:1	6	14	17	20	12	82
18:0	2	2	2	3	1	6

Incubation conditions as described in Materials and Methods, with assay and pulse chase times given above.

Later on the biosynthesis of C_{16} and C_{18} fatty acids becomes dominant, with appreciable labeling of long chain fatty acids too. The period between day 14 and day 20 represents the dehydration of the seed. Dry weight remains constant. During this dehydration period, acetate labeling showed a significant increase. A small amount of MCFA still is being synthesized. Presumably during this period the dehydrating seed is producing the enzymes needed for lipid synthesis during the very initial stages of germination while still containing the decaying systems of lipid biosynthesis found in the seed maturation phase. What we are observing in the assays is both systems.

TABLE 4

Labeling Patterns from the Pulse Chase Experiment

	Time course		Pulse chase
	2 Hr	6 Hr	2 + 4 Hr
Percentage ^{14}C distribution in acyl groups of total lipids			
8:0	17	14	18
10:0	37	27	40
12:0	12	12	14
14:0	4	4	4
16:0	7	8	6
18:0	4	5	2
18:1	7	17	6
18:2	1	3	1
20:0	8	7	5
22:0	4	2	3
Percentage lipid distribution in lipid classes			
Triacylglycerols	48.3	53.0	55.6
1,2-Diacylglycerols	6.8	6.3	2.1
Monoacylglycerols	1.4	0.9	0.5
Polar lipids	12.8	15.1	11.1
Phosphatidylcholine	4.4	6.5	4.7

Incubation conditions as described in Materials and Methods.

TABLE 5

Mass and [^{14}C] Fatty Acid Profiles of Total Lipids from [^{14}C]Acetate Labeling Experiments with *Cuphea lutea* Seed Embryos at Different Stages of Development

	Percentage ^{14}C and (percentage mass) distribution[a]			
Acyl group	Day 6	Day 8	Day 12	Day 18
8:0	1 (0.5)	5 (1.5)	0 (1.0)	0 (1.0)
10:0	26 (13.0)	33.5 (20.0)	7 (19.5)	4.5 (20.5)
12:0	40 (35.5)	24.5 (43.5)	9 (44.0)	5 (42.5)
14:0	12.5 (20.0)	6 (15.0)	5 (13.5)	5 (13.0)
16:0	9.5 (9.0)	11 (4.5)	17.5 (4.0)	23 (4.5)
18:0	1.5 (1.0)	3 (0.5)	6 (0.5)	5.5 (0.5)
18:1	4.5 (10.0)	8 (7.5)	25.5 (10.0)	43.5 (10.5)
18:2	3 (11.0)	2 (7.5)	3 (7.5)	2 (7.5)
20:0	1.5	5.5	19	6.5
22:0	0.5	1.5	8	5

Incubation conditions as described in Materials and Methods.
[a]Days after flowering.

Endogenous squalene and sterols. Because squalene is known to be a minor component of many vegetable oils, generally at 0.01–0.5% levels (22–24), the high level of [2-^{14}C]acetate incorporation into squalene was unexpected. An analysis of the levels of squalene and sterols in *Cuphea lutea* seed embryos therefore was performed. In a batch of mature seed that yielded 850 μg of total lipid per embryo, squalene was present at 0.1 μg/embryo, while free and total sterol concentrations were 3.6 and 4.8 μg/embryo, respectively. The identity of the sterols present was determined by retention times of the underivatized and derivatized (*tert*-butyldimethylsilyl-) sterols on the SE-30 capillary column when compared with standards and by GC-MS. The free sterol fraction was composed of 8% campesterol, 2% stigmasterol, 72% sitosterol and 16% isofucosterol, with less than 2% methyl sterols. This composition is typical of seed oils from plants (25). A similar composition was observed for the total sterol fraction. The observation that 7–22% of the lipid labeled from [2-^{14}C]acetate was incorporated into squalene, while the endogenous squalene and sterol pools are small relative to the triacylglycerol pool, indicates that a single acetate pool cannot be supplying single sites of fatty acid and isopentenoid biosynthesis. This conclusion is tenable because the labeled squalene pool does not appear to turn over, as described in the next section.

Mevalonic acid labeling. [2-^{14}C]R-mevalonic acid lactone labeling studies on developing *Cuphea lutea* embryos were

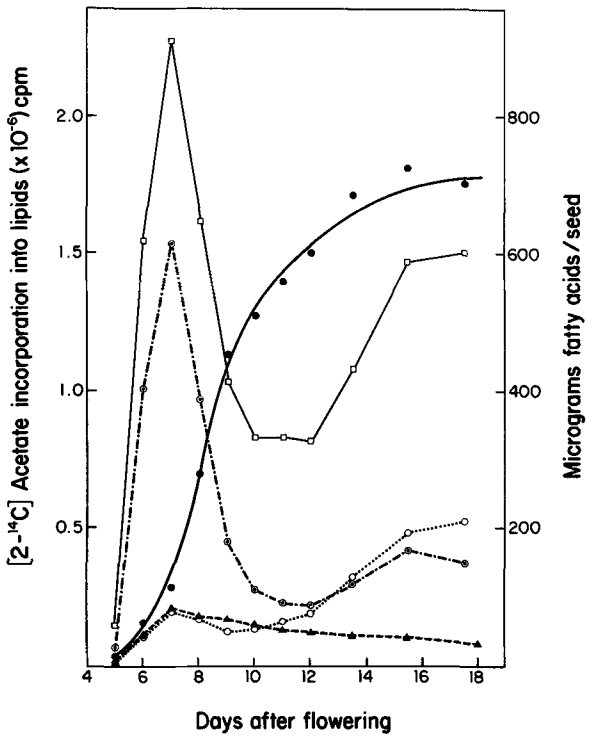

FIG. 2. Development profile for [2-^{14}C]acetate labeling of *Cuphea lutea* embryos. Day 8 was the time at which the seed capsule first showed signs of splitting. Incorporations of added label into the total lipid extract (□—□), triacylglycerols (⊙•—•—•⊙), polar lipids (○•••••○) and squalene (▲— — —▲) are shown, along with the mass of endogenous lipid (●—●) for this particular experiment. The incorporations are on a cpm per assay basis, with the standard assay as described in Materials and Methods. Figs. 2 and 3 describe experiments carried out with the same batch of maturing seeds.

attempted to confirm and extend the acetate labeling experiments. Squalene was always the dominant labeled lipid (Fig. 3). However, in later stage embryos a second major labeled species also was noted. Its R_f value on silica gel TLC plates developed with hexane/diethyl ether/acetic acid was very close to that of sitosterol, while on C_{18} reversed phase TLC plates developed in methanol it ran with phytol. This unknown did not contain a free hydroxyl or a free carboxylic acid group, as demonstrated by its TLC behavior after acetylation or diazomethane treatments, respectively. It was not further characterized. [2-^{14}C]R-mevalonic acid lactone also gave several minor labeled bands (2–5% each) in the sterol region of neutral lipid TLC plates. On acetylation, these bands migrated as alkyl acetates. Only a 4-desmethyl band was more fully characterized. It appeared to be sitosterol by radio-GLC of its silyl ether on a 10% SP-2330 column. The level of sitosterol labeling peaked with labeled triacylglycerol synthesis from [2-^{14}C]acetate (Figs. 2 and 3, which represent the same experiment).

The levels of endogenous squalene and sterols were assayed over seed development (Fig. 4). The rate of free sterol deposition is 0.4 μg/day/embryo, while for total sterols it is 0.55 μg/day/embryo. Squalene, however, reaches a maximum level of 0.3 μg/embryo before declining. The squalene and sterol levels in the developing seeds suggest a precursor-product relationship, as would be expected from the biosynthetic pathway. The numerical data suggest a turnover rate for a single squalene pool supplying sterol biosynthesis of about 12 hr. When a two-hr [2-^{14}C]R-mevalonic acid lactone labeling period was followed by a six-hr pulse chase in developing embryos at the earlier stages (mevalonate incorporation was approximately linear up to 8 hr in this experiment), depletion of the label from squalene was small (from 80% to 68% of the total label), while the increase in the label in 4-desmethyl sterols also was small (from 3% to 6%). A pulse chase of less than 5% of the label in the squalene pool to desmethyl sterols in six hr is not consistent with a single pool of squalene in the developing embryos. In the latter case our calculations would suggest a turnover of 50% in the six-hr chase period. We conclude that in developing *Cuphea lutea* embryos there well may be two sites of squalene biosynthesis. One is readily accessible to exogenous mevalonate and does not show developmental sensitivity. The second is less accessible to exogenous mevalonate and is the pool supplying endogenous sterol biosynthesis. Some labeled mevalonate may enter this second pool: note the coincident labeling of sterols from mevalonate and triacylglycerols from acetate (Figs. 2 and 3).

A specific interpretation of this two-pool phenomenon is difficult. The developing embryo is likely to be heterogenous in cell type, so the two-pool phenomenon could be inter- or intracellular in origin. This problem already has been noted for acetate labeling studies in developing nasturtium seeds, when it was necessary to postulate two separate sites of de novo fatty acid synthesis (15). An artifact of the incubation is also a possibility. An explanation that might be proposed is that the incubation is fairly anaerobic. Squalene biosynthesis from mevalonate is an anaerobic process, but the conversion of squalene to sterols is aerobic, so anaerobic conditions would favor a squalene build-up. However, in the incubations with acetate, oleate is formed preferentially over

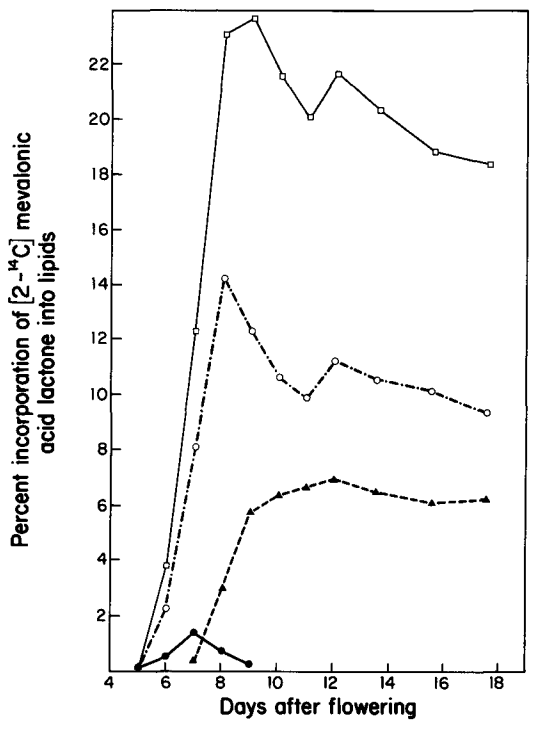

FIG. 3. Development profile for [2-^{14}C]mevalonate labeling of *Cuphea lutea* embryos. Incorporations of added label into the total lipid extract (□—□), squalene (○•—•—•○), 4-desmethyl sterols (●—●) and a major unknown (▲– – – –▲) are shown. Figs. 2 and 3 describe experiments carried out with the same batch of maturing seeds. The standard incubation conditions described in Materials and Methods were used.

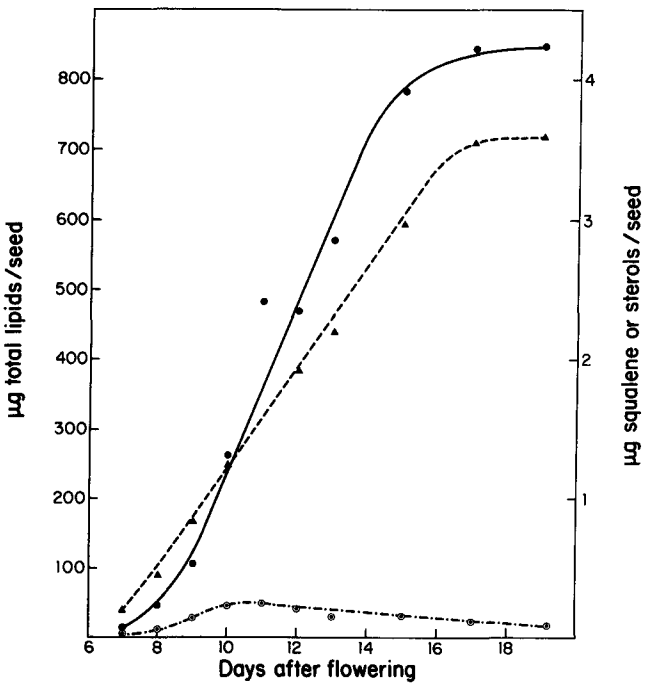

FIG. 4. Developmental profile for levels of squalene (⊙—•—•⊙) and free sterols (▲– – –▲) in *Cuphea lutea* embryos. The amount of total lipids (●—●) also is given, as this analysis was performed with a different batch of seeds than for the experiment shown in Figs. 2 and 3.

stearate. The Δ9-desaturation is aerobic, indicating an oxygen supply to the tissue. The high level of labeled squalene in *Cuphea lutea* embryos cannot be considered a general phenomenon for developing seeds. Baisted (26) observed high levels of triterpene alcohol and sterol labeling from mevalonate relative to squalene in developing peas. No other developing seed systems appear to have been investigated, though germinating seeds have received more attention. Germinating seeds are known to exhibit synthesis of squalene from the onset of imbibition, with sterol biosynthesis delayed for several days (27,28). Fang and Baisted (29) later defined this in peas as a phenomenon of the cotyledons, not the axis. In the developing embryos of cuphea seeds the cotyledons will be the dominant tissue. Perhaps the squalene biosynthesis we observe is a constitutive system not associated with the surge of lipid deposition but the same as observed in early germination.

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Distribution of ^{14}C after Oral Administration of (U-^{14}C)Labeled Methyl Linoleate Hydroperoxides and Their Secondary Oxidation Products in Rats

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To study the toxicity of low molecular weight (LMW) compounds formed during the autoxidation of oils, ^{14}C-labeled primary monomeric compounds (methyl linoleate hydroperoxides) and secondary oxidation products, i.e., polymer and LMW compounds prepared from autoxidized methyl [U-^{14}C]linoleate hydroperoxides (MLHPO) were orally administered to rats, and their radioactive distributions in tissues and organs were compared. The polymeric fraction consisted mainly of dimers of MLHPO. For the LMW fraction, 4-hydroxy-2-nonenal, 8-hydroxy methyl octanoate and 10-formyl methyl-9-decenoate were identified as major constituents by gas chromatography-mass spectrometry (GC-MS) after chemical reduction and derivatization. When LMW compounds were administered to rats, $^{14}CO_2$ expiration and the excreted radioactivity in urine in 12 hr were significantly higher than those from polymer or MLHPO administration. Maximum $^{14}CO_2$ expiration appeared 2–4 hr after the dose of LMW compounds. Radioactivity of the upper part of small intestines six hr after the dose of LMW compounds was higher than the values from administered polymer or MLHPO. The remaining radioactivity in the digestive contents and feces 12 hr after administration of LMW compounds was much lower than the values observed from administered polymer or MLHPO. Among internal organs, the liver contained the highest concentration of radioactivities from polymer, MLHPO and LMW fractions, and an especially higher level of radioactivity was found in liver six hr after the administration of LMW compounds. Six hours after the dose of LMW compounds, a relatively higher level of radioactivity also was detected in kidney, brain, heart and lung. These results show that the LMW compounds from MLHPO autoxidation are more easily absorbed in rat tissues than polymer and MLHPO.
***Lipids 21,* 150–154 (1986).**

Vegetable oils rich in unsaturated fatty acids undergo oxidative deterioration easily during processing and cooking. Clearly, this has considerable significance for the nutritional value of these oils. The primary oxidation products have less nutritional value. The substances produced in the autoxidation of oils are lipid hydroperoxides, which undergo polymerization and degradation reactions giving rise to several secondary oxidation products during the advanced oxidation process. The nutritional problems, cytotoxicities and metabolic fate of fatty acid hydroperoxides have been studied extensively (1–4). However, little is known about the absorption and distribution profiles resulting from ingestion of the secondary oxidation products of lipid hydroperoxides, especially LMW compounds. One of the authors has reported previously that LMW compounds formed in the autoxidation of methyl linoleate are more toxic than those of MLHPO in rats and mice, and among them hydroperoxyalkenals especially show the greatest cytotoxic effects (5,6).

In this study, we investigated and compared the absorption and distribution of [U-^{14}C]labeled secondary decomposition products, i.e., LMW compounds, of MLHPO with those of dimeric and monomeric (MLHPO) compounds in rat tissues and excreta to elucidate the toxicity. This is the first report showing higher absorption of dietary LMW compounds than of MLHPO and polymers in animal tissues.

MATERIALS AND METHODS

Preparation of MLHPO. Methyl linoleate (ML) was prepared from mixed methyl esters of safflower oil fatty acid by urea adduct formation (7). The purity of the ML prepared was 99% by gas liquid chromatography (GLC). The ML then was allowed to autoxidize in the dark at 12 C for 72 hr to a peroxide value of 1000 meq/kg. Isolation and purification of MLHPO from the autoxidized ML were carried out by dry column chromatography on Silica Woelm TSC (Woelm Pharma Co., Eschwege, Federal Republic of Germany) with hexane/ether (7:4, v/v) as the eluant. The final preparation had a peroxide value of 6080 meq/kg, determined by KI reduction, vs the theoretical value of 6125 meq/kg, indicating that the MLHPO prepared was 99% pure.

Fractionation of secondary MLHPO oxidation products. The purified MLHPO was autoxidized further at 40 C for 60 hr in the dark. The autoxidized products (1.2 g) were applied to a gel chromatography column on Bio-Beads S-X3 (190 × 2.8 cm; BioRad Laboratories, Richmond, California) with benzene as eluant, and divided into polymeric, monomeric (MLHPO) and LMW compound fractions which were monitored by UV absorption at 233 nm. The mean molecular weight of the chromatographic fraction was determined by the vapor pressure equilibrium method using benzene on a Hitachi Perkin-Elmer molecular weight measurement apparatus Model 115.

GC-MS of LMW compounds. Chemical reduction of the peroxide groups in the LMW compounds separated from oxidized MLHPO products was done with stannous chloride (8). The reduced products then were converted into their dimethylhydrazone (DMH) and trimethylsilyl ether (TMS) derivatives (9,10). The chemical structure of the DMH-TMS derivatives of the reduced LMW compounds was characterized by GLC and GC-MS. The GLC was performed with a Shimadzu gas chromatograph, Model JGC-20K, on a glass column (200 × 0.3 cm) packed with 3% SE-30 on Chromosorb W. The flow rate of N_2 carrier gas was 40 ml/min. The column temperature was programmed from 100 to 260 C at a rate of 4 C/min. The GC-MS was carried out with a Shimadzu LKB-900 apparatus on a glass column (200 × 0.3 cm) packed with

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3% SE-30 on Chromosorb W programmed from 100 to 260 C at 4 C/min. The molecular separator and ion source were maintained at 250 C and 270 C, respectively, and the carrier gas was helium. Ionizing voltage was 25 eV (13).

Preparation of [U-^{14}C]labeled compounds and their oral administration to rats. [U-^{14}C]Linoleic acid was purchased from New England Nuclear (Boston, Massachusetts) and the methyl ester was prepared by reaction with 5% methanolic HCl. The methyl [U-^{14}C]linoleate was autoxidized, and the resulting MLHPO were isolated by dry column chromatography. In the same manner as that described before, [U-^{14}C]labeled polymer, MLHPO (monomer) and LMW compounds were separated from the autoxidized products of [U-^{14}C]MLHPO by gel chromatography on Bio-Beads S-X3.

For the animal experiments, male Wistar rats purchased from Nihon Rat Co. (Urawa, Japan) were allowed to mature to a body weight of 200–250 g with ad libitum feeding of a basal diet (type F-2 pellet ration, Funabashi Farms Co., Chiba, Japan) containing all required minerals and vitamins. Rats were fasted for 12 hr prior to intubation. Oral administration of radioactive sample was performed by stomach tube after suspension in an appropriate amount of fresh ML. Each rat given the radioactive fraction was placed in a separate metabolic box. $^{14}CO_2$ was collected at 2 hr intervals by passing the expired gases through 1 N NaOH. Six and 12 hr after administration, rats were killed under ether anesthesia. Digestive tracts (stomach, small intestine, caecum and large intestine) and tissue organs (brain, lung, heart, liver and kidney) then were excised. Feces, urines and digestive contents also were collected. The sample tissues (100–450 mg) were solubilized with 1 ml of 1 N NaOH and neutralized by 0.98 N HCl. Their radioactivities were measured in a xylene scintillation cocktail with an Aloka liquid scintillation system LSC-903. The data were corrected to represent the radioactivity of whole tissue. All the animal experiments in this paper have been replicated at least twice to check the reproducibility.

RESULTS AND DISCUSSION

Gel chromatogram of autoxidized MLHPO products. The autoxidized MLHPO was fractionated tentatively into five fractions according to mean molecular weights—namely, polymer, polymer + MLHPO, MLHPO (monomer), MLHPO + LMW and LMW compounds—on Bio-Beads S-X3 gel chromatography as depicted in Figure 1. The non-UV active compounds, if they occurred, might be included in these fractions. In the present experiments, polymer, MLHPO (monomer) and LMW fractions were used. The polymer fraction was composed mainly of dimers of MLHPO. It has been determined that dimers with one or two hydroperoxy or hydroxy groups in a molecule linked through a single C—O—O—C or C—O—C bond are characteristic of those obtained on autoxidation of MLHPO (11). MLHPO was a major constituent of the combined MLHPO fraction in Figure 1. If endoperoxidic (cyclic) compounds with a carbon chain of 18 occurred, as has been observed in the case of autoxidized linolenate and photosensitized oxidized linoleate (12), these substances also were included as very minor constituents of the monomeric MLHPO fraction. Therefore, these three fractions contained monomer (MLHPO)

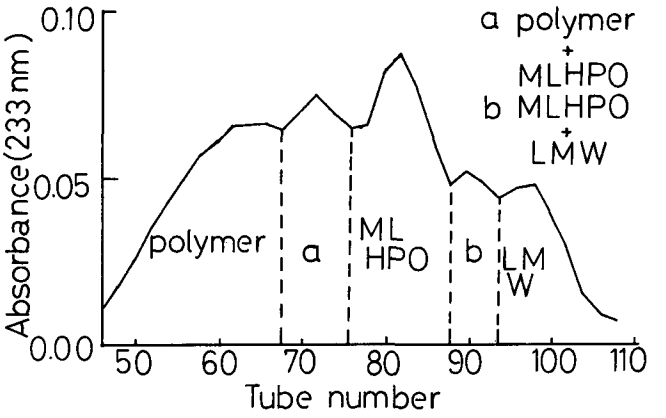

FIG. 1. Fractionation of autoxidized MLHPO products on a Bio-Beads S-X3 column. The autoxidized MLHPO products were eluted on Bio-Beads S-X3 and 7-ml fractions were collected. Absorbance of each fraction was measured at 233 nm.

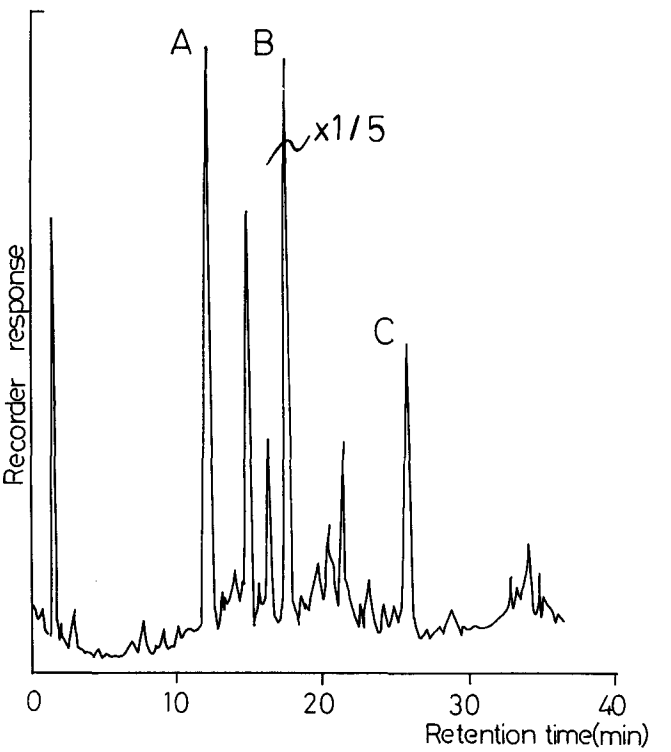

FIG. 2. Gas chromatogram of $SnCl_2$-reduced LMW compounds as DMH-TMS derivatives. For details, see text.

and typical secondary oxidation products of MLHPO, i.e., dimer and LMW degradation compounds, respectively.

Main constituents of the LMW fraction. A gas chromatogram of the DMH-TMS derivatives of the reduced LMW fraction is shown in Figure 2. The compounds isolated as peaks A, B and C on the chromatogram comprised 16.1%, 40.0% and 4.1% of the total LMW fraction, respectively, as determined by the peak areas. The chemical structures of the constituents of peaks A, B and C then were identified by their mass spectra. For the peak A compound, the molecular weight could be deduced from the fragment ion at m/e 231 $[M-15]^+$. The fragment ion at m/e 73 $[Si(CH_3)_3]^+$ indicated the presence

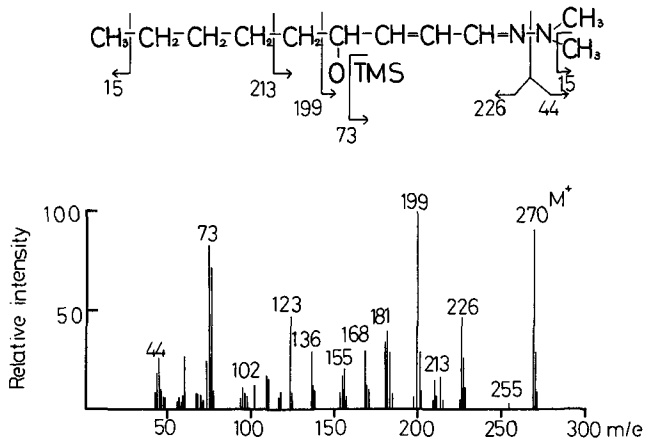

FIG. 3. Mass spectrum of DMH-TMS derivative of 4-hydroxy-2-nonenal identified as a peak B component in Fig. 2.

of a TMS group, and the fragments of m/e 215 $[M\text{-}OCH_3]^+$ and m/e 59 $[H_3COCO]^+$ demonstrated the presence of methyl ester in the molecule. The spectrum was identical to that observed for 8-hydroxy methyl octanoate (19). Thus, peak A was identified as a TMS derivative of 8-hydroxy methyl octanoate. For peak B, a main component of the LMW fraction, a molecular ion at m/e 270 was detected as shown in Figure 3. The fragment ions at m/e 73 $[Si(CH_3)_3]^+$ and m/e 181 $[M\text{-}(CH_3)_3SiO]^+$ showed the presence of the TMS group in the molecule. The peaks at m/e 226 $[M\text{-}N(CH_3)_2]^+$ and m/e 44 $[N(CH_3)_2]^+$ indicated the presence of the DMH group. The base peak at m/e 199 indicated that the double bond was located between C-7 and C-8. The spectrum was identical to that of 4-hydroxy-2-nonenal as reported by Nakamura et al. (20). The spectrum thus indicated that the peak B component was a DMH-TMS derivative of 4-hydroxy-2-nonenal. For the spectrum of peak C compound, a molecular ion appeared at m/e 254. The fragment ions at m/e 239 $[M\text{-}CH_3]^+$, m/e 223 $[M\text{-}OCH_3]^+$ and m/e 59 $[H_3COCO]^+$ showed the presence of methyl ester, those at m/e 210 $[M\text{-}N(CH_3)_2]^+$ and m/e 44 $[N(CH_3)_2]^+$ showed the presence of a DMH group in the molecule. The base peak at m/e 111 indicated that the double bond was located between C-9 and C-10. The spectrum was consistent with that of 10-formyl methyl-9-decenoate (19). Therefore, the peak C compound was identified as a DMH of 10-formyl methyl-9-decenoate.

Because we applied the LMW fraction after chemical reduction on GC-MS, the hydroperoxides reduced to the corresponding hydroxy derivatives. Before the reduction, several minor LMW compounds were revealed to have peroxy groups in the molecules, which could be deduced from thin layer chromatograms of the LMW fraction exhibiting several pink spots positive for peroxides on spraying with *N,N*-dimethyl-*p*-phenylenediamine reagent. Therefore, it is possible that the hydroxy alkenals identified originated in part from the hydroperoxy derivatives contained in the intact LMW fraction. Besides the three compounds characterized, several LMW and short chain compounds have been identified among the autoxidation products of ML (5,13-15). In this study, several minor peaks also were detected on GLC, as depicted in Figure 2. Therefore, it can be said that the LMW fraction included hydroxy (and/or hydroperoxy) alkenals as main components and that the several other components reported

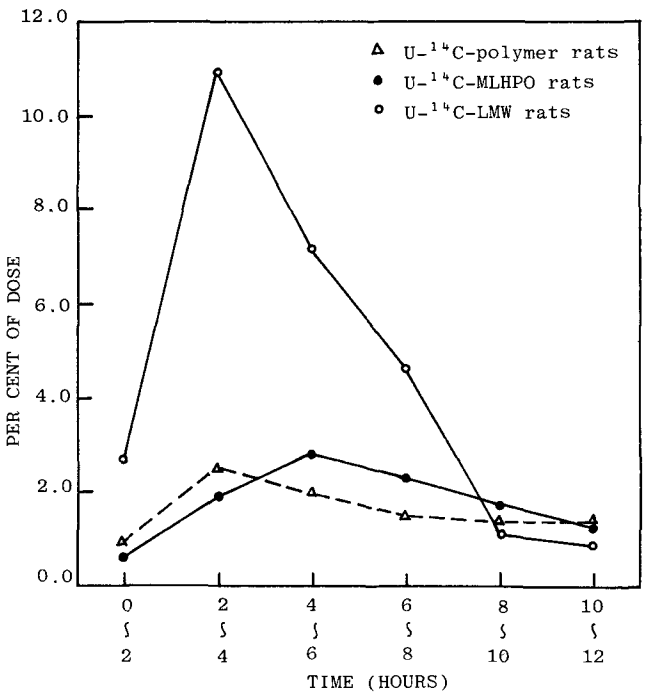

FIG. 4. $^{14}CO_2$ expiration during 12 hr following administration.

in the literature (5,13-15) may also be present as minor components of the LMW fraction. The presence of compounds A, B and C has been demonstrated, but the GLC information shows that the LMW is a very complex mixture. Although $SnCl_2$ was used for the reduction of LMW compounds in this study, reduction with triphenylphosphene is more suitable for these studies because the conditions of the reaction are milder using triphenylphosphene than when $SnCl_2$ is used.

$^{14}CO_2$ expiration and distribution of radioactivity after oral administration of ^{14}C-MLHPO and its secondary oxidation products. Table 1 compares total $^{14}CO_2$ expired and radioactivity in urine 12 hr after administration. $^{14}CO_2$ expiration and the radioactivity of urine collected from LMW compounds were evidently higher than those observed when the polymer and MLHPO fractions were administered. A typical $^{14}CO_2$ expiration curve indicates that maximum $^{14}CO_2$ production occurred about 2-4 hr after intubation of LMW compounds (Fig. 4).

The distribution ratios (percent to total dose) of radioactivity in the digestive tracts six hr after administration are shown in Table 2. For the polymer, MLHPO and LMW fractions, radioactive distributions in the stomach and small intestines were a little higher than those of the caecum and large intestine. The upper part of the small intestine contained higher concentrations of ^{14}C of the LMW fraction than polymer or MLHPO.

Twelve hr after intubation of LMW fraction, the distribution ratios in each digestive tract were lower than the values observed six hr after administration (Table 1). When the polymer or MLHPO fraction was administered, the radioactivities in the caecum and large intestine after 12 hr were higher than the values observed after six hr.

The distribution of radioactivities in the digestive contents and feces 12 hr after the doses is shown in Table 1. Total radioactivities of these digestive contents and feces amounted to 53% for polymer fraction, 41% for MLHPO

TABLE 1

Radioactivity in Digestive Contents, Sections of the Intestinal Tract, Organs and Excreta 12 Hr After Intubation

	Fractions administered					
	Polymer		MLHPO		LMW	
Distribution	DPM	%	DPM	%	DPM	%
Digestive contents						
Stomach	42,517	7.13	394,965	33.54	1,431	0.09
Small intestines (upper part)	5,130	0.86	17,381	1.48	5,954	0.37
Small intestines (lower part)	16,197	2.72	11,519	0.98	8,119	0.51
Caecum + large intestine	192,755	32.35	53,688	4.56	22,987	1.44
Feces	57,226	9.60	7,918	0.67	11,107	0.70
Digestive tracts						
Stomach	6,956	1.17	29,484	2.50	3,560	0.22
Small intestines (upper part)	972	0.16	4,528	0.38	2,785	0.17
Small intestines (lower part)	1,695	0.28	1,339	0.11	1,997	0.13
Caecum	4,352	0.73	1,093	0.09	2,101	0.13
Large intestine	3,028	0.51	1,411	0.12	1,911	0.12
Organs						
Brain	253	0.04	335	0.03	1,075	0.07
Lung	333	0.06	904	0.08	2,060	0.13
Heart	185	0.03	559	0.05	555	0.03
Liver	11,483	1.93	43,667	3.71	13,702	0.86
Kidney	1,115	0.19	2,838	0.24	4,240	0.27
$^{14}CO_2$	58,000	9.73	124,900	10.61	437,700	27.46
Urine	76,190	12.79	164,571	13.98	747,065	46.87
Total activity of labeled compounds administered (DPM)	595,914		1,177,579		1,593,796	

TABLE 2

Radioactivity in Digestive Tract and Other Organs 6 Hr After Intubation

	Fractions administered					
	Polymer		MLHPO		LMW	
Distribution	DPM	%	DPM	%	DPM	%
Digestive tracts						
Stomach	13,676	3.42	14,892	1.96	691	1.47
Small intestines (upper part)	1,131	0.28	3,884	0.51	765	1.62
Small intestines (lower part)	4,132	1.03	6,564	0.86	427	0.91
Caecum	808	0.20	1,930	0.25	193	0.41
Large intestine	297	0.07	1,518	0.20	345	0.73
Organs						
Brain	294	0.07	727	0.10	419	0.89
Lung	325	0.08	1,101	0.14	320	0.68
Heart	365	0.09	672	0.09	345	0.73
Liver	4,859	1.21	17,870	2.35	3,908	8.29
Kidney	1,073	0.27	3,750	0.49	687	1.46
Total activity of labeled compounds administered (DPM)	399,991		759,602		47,118	

fraction and only 3% for LMW compound fraction, respectively. The highest radioactivity remained in the caecum and large intestine for polymer fraction and in the stomach for MLHPO.

The radioactivities in the tissue organs of rats six hr after intubation is shown in Table 2. Among the organs examined, the liver contained the highest radioactivity—1.2%, 2.4% and 8.3% on intubating polymer, MLHPO and LMW compounds, respectively. When the ^{14}C-LMW compounds were administered, relatively higher levels of ^{14}C also were found in the brain, lung, heart and kidney, compared with polymer and MLHPO. Twelve hours after administration, the distribution in the liver was 1.9%, 3.7% and 0.9% for polymer, MLHPO and LMW compounds, respectively (Table 1). For LMW compounds, radioactivities in all organs examined after 12 hr decreased compared to the values after six hr.

Nakatsugawa and Kaneda (2) showed with rabbit that 0.23% of the administered MLHPO is found in the lymph as intact MLHPO. Their results indicated that some unchanged hydroperoxides are absorbed into the intestinal wall and transported to some organs through the lymph. In rats, it appeared that the MLHPO administered was absorbed directly from the intestinal wall and in part converted to derivatives such as methyl hydroxyoctadecadienoates and methyl oxooctadecadienoates, which also were absorbed from the intestinal wall (1). The LMW compounds may be more easily absorbed than MLHPO into the intestinal wall, because of their shorter carbon chain length. It is known that most of the medium and short chain fatty acids were carried into the liver directly through the hepatic portal vein in the free form without being derivatized to triacylglycerol (16-18). The short chain fatty acids are not good substrates for fatty acid thiokinase and are more hydrophilic than MLHPO and polymer (17). For this reason, it is thought that LMW compounds are carried into internal organs more easily and more rapidly than the MLHPO and polymer, resulting in the highest rate of expiration and excretion in CO_2 and urine at relatively early stages of the dose. These characteristics of LMW compounds may account for their high potency in cellular damage and toxicity. Esterbauer's group has extensively studied the biological effects of hydroxy alkenals (21). For example, 4-hydroxy octenal has been shown to react with functional sulphydryl groups of the enzymes, probably by linking to the 2,3-carbon-carbon double bond of the hydroxy alkenal (21). It also is known that hydroxy alkenals deactivate enzymes to different degrees because of their various reactivities against the thiol groups (21).

Additional work will be needed in the future on the purified oxidation compounds mentioned in this paper.

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Synthesis of the Dipalmitoyl Species of Diacyl Glycerophosphocholine by Rabbit Alveolar Macrophages

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The distribution of radioactivity among the molecular species of diacyl glycerophosphocholine of rabbit alveolar macrophages was determined after incubation with [^{3}H]glycerol and 1-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine. The highest percentage of radioactivity of [^{3}H]glycerol was found in the dipalmitoyl species (35% of the total) followed by the 1-palmitoyl-2-linoleoyl (23.6%) and 1-stearoyl-2-linoleoyl plus 1-palmitoyl-2-oleoyl species (19.7%) during the first 30 min incubation. The radioactivity of the dipalmitoyl species reached a maximum at 120 min incubation and decreased thereafter, although the radioactivities of other molecular species still increased. In contrast to the [^{3}H]glycerol labeling, only 4% of the total radioactivity in diacyl glycerophosphocholine derived from 1-[^{14}C]palmitoyl glycerophosphocholine was found in the dipalmitoyl species; 80% of the radioactivity was located in the 1-palmitoyl-2-arachidonoyl species at 10 min incubation. The present results indicate that the dipalmitoyl species of diacyl glycerophosphocholine are synthesized predominantly via a de novo pathway and not the deacylation-reacylation pathway in rabbit alveolar macrophages.
Lipids 21, 155–158 (1986).

The dipalmitoyl species of choline glycerophospholipid (CGP) is believed to be the major surface active component of the pulmonary surfactant (1,2). To investigate the pathway for its biosynthesis, it is necessary to separate this molecular species from other molecular species of glycerophospholipids. Although argentation thin layer chromatography (TLC) is a common technique to separate the molecular species of glycerophospholipids on the basis of the degree of unsaturation of fatty acids, it shows insufficient resolution for the separation of disaturated molecular species such as the dipalmitoyl and distearoyl species. Reverse-phase high performance liquid chromatography (HPLC) gives excellent separation of the molecular species of glycerophospholipids. Recently we developed a reliable HPLC procedure for the isolation of the individual molecular species of 1,2-diradyl-3-acetylglycerol derived from glycerophospholipids (3). We analyzed the compositions of the molecular species of glycerophospholipids of rabbit alveolar macrophages by HPLC and found a high amount of the dipalmitoyl species of diacyl glycerophosphocholine (GPC) (4).

The formation of CGP can occur in alveolar macrophages through de novo synthesis (5) and the deacylation-reacylation pathway (6). The incorporation of labeled glycerol (7), palmitic acid (7–9), choline (8,9) and 1-acyl glycerophosphocholine (10–12) has been demonstrated in intact alveolar macrophages or a homogenate of them. However, little is known about the rate of synthesis of the individual molecular species of CGP in alveolar macrophages via a de novo pathway and the deacylation-reacylation pathway.

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In the present work, we investigated the rates of incorporation of [^{3}H]glycerol and 1-[^{14}C]palmitoyl glycerophosphocholine into the individual molecular species of diacyl GPC and compared the patterns of the newly formed molecular species of diacyl GPC derived via a de novo pathway and the deacylation-reacylation pathway in rabbit alveolar macrophages.

MATERIALS AND METHODS

Materials. 1-[^{14}C]Palmitoyl-*sn*-glycerophosphocholine (60 mCi/mmol) and [1(3)-^{3}H]glycerol (2.5 Ci/mmol) were purchased from Amersham (Amersham, United Kingdom). HPLC grade organic solvents and distilled water were obtained from Wako Pure Chemical Ind. (Osaka, Japan).

Methods. Alveolar macrophages were harvested from rabbit lung according to the method of Myrvik et al. (13). Contaminating erythrocytes were removed by osmotic lysis and the cells were washed three times with isotonic saline. Examination of Giemsa stained smears showed that 95% of the lung washed cells were alveolar macrophages. From 3 to 8 $\times$ 10^7 cells were obtained from a single animal.

Alveolar macrophages (2 $\times$ 10^7 cells) were suspended in 20 ml minimum essential medium solution containing 5 mM N-2-hydroxyethylpiperazine ethanesulfonic acid buffer (pH 7.2). Incubation was started by the addition of 1 mCi of [^{3}H]glycerol or 0.4 μCi of 1-[^{14}C]palmitoyl glycerophosphocholine. An aliquot of the cell suspension was withdrawn at intervals and the reaction was terminated by adding the organic solvent mixture chloroform/methanol (1:2, v/v).

Cellular lipids were extracted by Bligh and Dyer's method (14). The labeled lipids were mixed with nonlabeled total lipids of bovine heart as the carrier and separated into individual glycerophospholipids by 2-dimensional TLC (15). The CGP were separated into diacyl, alkylacyl and alkenylacyl subclasses as described previously (16). CGP were hydrolyzed with phospholipase C *(Bacillus cereus)* and acetylated with acetic anhydride and pyridine. The resultant 1,2-diradyl-3-acetylglycerol derivatives were fractionated into the diacyl, alkylacyl and alkenylacyl subclasses by TLC according to Renkonen and Luukkonen (17). Diacyl GPC were extracted and further fractionated into the individual molecular species by reverse-phase HPLC. The analytical conditions were the same as those used in the previous study (3). 1,2-Diacyl-3-acetyl-glycerol was dissolved in 20 μl of methanol and injected into HPLC on the reverse-phase column (Zorbax ODS, 4.6 $\times$ 250 mm, Dupont, Wilmington, Delaware). The solvent system was acetonitrile/2-propanol/methyl-t-butyl ether/water (72:18:8:2, v/v/v/v). The flow rate was 1 ml/min and the column was controlled at 30 C. Each molecular species peak was monitored at 205 nm and fractions were collected from the column for measurement of radioactivities of individual molecular species.

TABLE 1

Incorporation of [^{3}H]Glycerol into Lipids of Alveolar Macrophages

	Incubation time (min)			
	30	60	120	240
	cpm ± SD/10^6 Cells			
CGP	12020 ± 670	57640 ± 4160	88520 ± 8640	118980 ± 2990
EGP	1453 ± 352	2897 ± 299	4626 ± 103	11050 ± 1034
IGP	2928 ± 12	2755 ± 467	6467 ± 1982	8018 ± 1566
Lyso-bis PA	1360 ± 189	2731 ± 305	3412 ± 159	2120 ± 142

Alveolar macrophages were incubated with [^{3}H]glycerol at 37 C for the indicated time. Lipids were extracted and separated into choline glycerophospholipid (CGP), ethanolamine glycerophospholipid (EGP), inositol glycerophospholipid (IGP) and lyso-bis-phosphatidic acid (lyso-bis-PA). Values are the averages for three separate experiments.

TABLE 2

Incorporation of [^{3}H]Glycerol into the Molecular Species of Diacyl Glycerophosphocholine

	Composition (%)[a]	Incubation time (min)			
		30	60	120	240
		cpm ± SD/10^6 Cells			
18:2-20:4	1.1	67 ± 20 (0.6)	298 ± 35 (0.6)	378 ± 19 (0.4)	514 ± 41 (0.5)
18:1-20:4	2.3	157 ± 11 (1.5)	601 ± 45 (1.2)	1308 ± 67 (1.5)	1880 ± 184 (1.7)
16:0-20:4	8.8	387 ± 21 (3.7)	2209 ± 123 (4.6)	4439 ± 357 (5.0)	6568 ± 510 (6.2)
18:0-20:4	2.4	130 ± 18 (1.2)	515 ± 35 (1.1)	1175 ± 81 (1.3)	1825 ± 201 (1.7)
18:1-18:2	6.7	546 ± 22 (5.2)	2306 ± 55 (4.8)	4349 ± 589 (4.9)	5751 ± 614 (5.4)
16:0-18:2	9.2	2463 ± 124 (23.6)	12192 ± 843 (25.5)	21093 ± 1805 (23.9)	30043 ± 975 (28.5)
18:0-18:2 16:0-18:1	48.2	2053 ± 103 (19.7)	12661 ± 1312 (26.4)	24300 ± 1789 (27.6)	31551 ± 933 (30.0)
18:1-18:1	2.7	322 ± 28 (3.1)	1632 ± 114 (3.4)	2985 ± 188 (3.4)	4003 ± 37 (3.8)
18:0-18:1	10.7	107 ± 3 (1.0)	411 ± 12 (0.9)	1111 ± 76 (1.2)	1624 ± 173 (1.5)
16:0-16:0	22.2	3962 ± 177 (37.9)	15047 ± 1001 (31.4)	23723 ± 2104 (26.9)	17782 ± 865 (16.9)
18:0-16:0	5.7	251 ± 14 (2.4)	1565 ± 121 (3.2)	3334 ± 213 (3.8)	3828 ± 164 (3.6)

The results for all molecular species are mean values ± SD (n = 3). Values in parentheses are the percentages of radioactivities of individual molecular species.

[a]Numbers are mean relative proportions (wt %) of each molecular species of CGP. These numbers were calculated from the results of previous paper (4).

RESULTS

The time-dependent incorporation of [^{3}H]glycerol into glycerophospholipids is shown in Table 1. [^{3}H]Glycerol was incorporated effectively into CGP, with about 65% of the total radioactivities being incorporated during the incubation period.

CGP were converted into 1,2-diradyl-3-acetylglycerol derivatives and separated into diacyl, alkylacyl and alkenylacyl subclasses. No significant radioactivities were found in the alkylacyl and alkenylacyl subclasses at any time (data not shown). The 1,2-diacyl-3-acetylglycerol derivatives from CGP were fractionated further into the molecular species by reverse-phase HPLC. Table 2 shows the relative distribution of radioactivities among the molecular species of diacyl GPC. The highest percentage of incorporated radioactivity was found in the dipalmitoyl species (38% of the total) followed by the 1-palmitoyl-2-linoleoyl (24%) and 1-stearoyl-2-linoleoyl plus 1-palmitoyl-2-oleoyl species during the first 30 min incubation. The radioactivity of the dipalmitoyl species reached a maximum at 120 min and decreased thereafter, whereas the radioactivities of other molecular species still increased up to 240 min.

To determine the contribution of the deacylation-reacylation pathway in the synthesis of the dipalmitoyl species of diacyl GPC of alveolar macrophages, macrophages were incubated with 1-[^{14}C]palmitoyl glycerophosphocholine as the direct precursor for the reacylation pathway and the distribution of radioactivity among the molecular species of diacyl GPC was determined. On incubation for 10 min, ca. 70% of the radioactivity of the total lipids was associated with CGP and other radioactivity was found predominantly in the lysophosphatidylcholine fraction (data not shown). The percentage distribution of the radioactivity derived from 1-palmitoyl-glycerophosphocholine among the molecular species of diacyl GPC is shown in Table 3. The distribution of radioactivity is dramatically different from that in the case of [^{3}H]glycerol labeling. The total radioactivity found in the 1-palmitoyl-2-arachidonoyl species amounted to 79%, which was the highest for all molecular species of diacyl GPC. In contrast to the [^{3}H]glycerol labeling,

TABLE 3

Distribution of Radioactivity Derived from 1-[^{14}C]Palmitoyl GPC among the Molecular Species of Diacyl GPC

	Radioactivity (cpm/10^6 cells)	Distribution (%)
16:0-16:0	254 ± 30	4.4 ± 0.2
16:0-18:1	141 ± 10	2.4 ± 0.3
16:0-18:2	845 ± 24	14.1 ± 0.6
16:0-20:4	4570 ± 95	79.0 ± 1.0

The results for all molecular species are the averages for three separate experiments.

the dipalmitoyl species were poorly labeled with 1-[^{14}C]palmitoyl glycerophosphocholine.

DISCUSSION

Previous analysis of the compositions of the molecular species of alveolar macrophage phospholipids showed the high level of the dipalmitoyl species of diacyl GPC (3). The dipalmitoyl species of CGP is not unique to alveolar macrophages; appreciable quantities of this molecular species have been found in erythrocytes (18), platelets (19), brain (20) and lung (20,21).

Several investigators have studied the synthesis of the dipalmitoyl species of CGP in alveolar macrophages using labeled precursors for a de novo pathway (7-9). Mason et al. (7) and Miles et al. (9) reported the incorporation of choline, palmitic acid and glycerol into the disaturated species of CGP. These investigators concluded that synthesis of the disaturated species of CGP occurs in alveolar macrophages and suggested that the majority of the synthesis of this molecular species occurs via a de novo pathway, although they did not examine the contribution of other pathways to the synthesis of the dipalmitoyl species of CGP. The present results showed that [^{3}H]glycerol was incorporated rapidly into the dipalmitoyl species of diacyl GPC as compared with other molecular species. The possible pathways for the synthesis of the dipalmitoyl species of CGP are a de novo synthesis pathway (Kennedy's pathway) (5), a deacylation-reacylation pathway (Land's pathway) (6) and methylation of ethanolamine glycerophospholipids (Bremer-Greenberg's pathway) (22). The methylation pathway seems to contribute little in the synthesis of the dipalmitoyl species of CGP, since Kikkawa et al. found that neither [^{14}C]ethanolamine nor [^{14}C]methionine were incorporated into CGP of rabbit alveolar macrophages (8). The reacylation-deacylation pathway also is not the major pathway for synthesis of the dipalmitoyl species, as the present studies showed that significant radioactivity was not found in the dipalmitoyl species of diacyl GPC fraction with 1-[^{14}C]palmitoyl glycerophosphocholine as the direct precursor for the reacylation reaction. Diacyl GPC formed through the acylation of 1-[^{14}C]palmitoyl glycerophosphocholine were exclusively of the 1-palmitoyl-2-arachidonoyl species (79% of the total). The result that the arachidonoyl molecular species of CGP were synthesized preferentially via the deacylation-reacylation pathway is in good agreement with the findings in liver (23) and platelets (24). These results are consistent with the view that alveolar macrophages synthesize the dipalmitoyl species of CGP via a de novo pathway.

Rapid degradation of the dipalmitoyl species of diacyl GPC occurred in alveolar macrophages. The radioactivity of the dipalmitoyl species decreased after 120 min incubation, although the radioactivities of other molecular species still increased. Miles et al. (9) found rapid degradation of the dipalmitoyl species of CGP prelabeled with [^{3}H]palmitic acid or [^{14}C]choline in rat alveolar macrophages, and determined that free [^{3}H]palmitic acid and water soluble [^{14}C]choline were the products of the catabolism of the labeled dipalmitoyl species of CGP but not a lyso compound. They concluded that the dipalmitoyl species of CGP were catabolized by both phospholipases A_1 and A_2. Several investigators have presented evidence for the existence of phospholipase A and lysophospholipase in alveolar macrophages (10,11,25,26). These data suggest that an active degradation pathway for the dipalmitoyl species of CGP may exist in alveolar macrophages. The degradation pathway for dipalmitoylglycerophosphocholine is interesting when the function of alveolar macrophages is considered, as alveolar macrophages may play an important role in the breakdown of the lung surfactant (9,27).

The present results indicate that the dipalmitoyl species of CGP were synthesized predominantly via a de novo pathway and not the acylation of 1-acylglycerophosphocholine and that they were rapidly turned over in rabbit alveolar macrophages.

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Effect of Clofibrate on Cholesterol Metabolism in Rats Treated with Polychlorinated Biphenyls

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Serum and hepatic cholesterol content in rats treated with polychlorinated biphenyls (PCBs, KC-400) were increased compared to those of control rats. This increase of cholesterol content was reduced to control level by simultaneous administration of ethyl p-chlorophenoxyisobutyrate (CPIB). Also, when lecithin-cholesterol acyltransferase (LCAT) (EC. 2.3.1.43) activity was expressed as the net cholesterol esterification, the acyltransferase activity in rats treated with PCBs was elevated, while the elevated acyltransferase activity was brought to control level by simultaneous administration of CPIB. On the other hand, the amount of bile of rats treated with CPIB, PCBs and PCBs-CPIB was increased, but free and total cholesterol content in bile of these treated rats was decreased to 40–60% of those of control rats. Moreover, cytochrome P-450 content in liver microsomes of rats treated with CPIB, PCBs and PCBs-CPIB was increased. At the same time, cholesterol-metabolizing activity in liver microsomes of rats treated with CPIB, PCBs and PCBs-CPIB also was elevated. Similar results were obtained for drug metabolizing (aniline hydroxylation and aminopyrine N-demethylation) activity. In addition, the amount of bile acids excreted from rats treated with CPIB, PCBs and PCBs-CPIB was increased compared to that of control rats.

These results suggest that hypercholesterolemia induced by oral ingestion of PCBs is recovered by CPIB treatment and that this hypocholesterolemic effect of CPIB may be related partly to the elevation of hepatic mixed function oxidase activity for cholesterol catabolism.
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PCBs are found as widespread contaminants in the environment. In particular, in 1968 poisoning (Yusho) caused by ingestion of rice oil contaminated with PCBs was found in more than 1,000 persons in northern Kyushu, Japan. In these clinical cases (1,2), serum triglyceride, cholesterol and phospholipid levels were increased, while serum LCAT, which catalyzes the formation of cholesterol ester and lysolecithin primarily from cholesterol and lecithin present in high density lipoproteins ($1.063 < d < 1.210$ g/cm^3) (3), was inhibited. In addition, in experimental PCBs poisoning in animals such as rats and rabbits, serum and liver cholesterol levels of these animals were similarly increased (4–6).

CPIB is a hypolipidemic agent commonly used in clinical practice. In a majority of hypertriglyceridemic and hypercholesterolemic human subjects, serum cholesterol and triglyceride levels were reduced moderately by the ingestion of CPIB (7–11). Similar phenomena have been observed in experimental hyperlipidemic animals (12–15).

In this study, we investigated the effect of CPIB on cholesterol content and cholesterol metabolism in serum and liver of rats treated with PCBs (KC-400).

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MATERIALS AND METHODS

Compounds. [4-^{14}C]-Cholesterol was purchased from New England Nuclear Corp. (Boston, Massachusetts). Glucose-6-phosphate (Na salt), NADP and glucose-6-phosphate dehydrogenase (EC. 1.1.1.49) were purchased from Sigma Chemical Co. (St. Louis, Missouri). CPIB was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). A kit (Free Cholesterol C-Test Wako) was purchased from Wako Pure Chemical Ind. (Osaka, Japan). Lecithin was prepared from egg yolk by the method of Faure (16) and purified by silicic acid column chromatography. PCBs (KC-400) were a gift from Dr. Uchiyama, National Institute of Hygienic Sciences, Tokyo, Japan. The other chemicals used were of reagent grade, purchased from Kanto Chemical Co. (Tokyo, Japan).

Treatment of animals. Male albino rats of Wistar strain weighing 180–220 g were used. The rats were fed a commercial diet obtained from Oriental Yeast Co. (Tokyo, Japan). Food was removed from the cages of all rats at 9 a.m. and returned at 6 p.m. To test the effect of CPIB, PCBs and PCBs-CPIB, rats were divided into four groups of three rats each and given orally 0.5 ml mineral oil, 0.5 ml mineral oil containing CPIB (300 mg/kg body), 0.5 ml mineral oil containing PCBs (100 mg/kg body) and 0.5 ml mineral oil containing CPIB-PCBs (300 and 100 mg/kg body, respectively) once a day (9 a.m.) for four days. Two hr after the final administration, serum, liver and bile samples were taken.

Preparation of serum and microsomes. Blood was drawn from the drug-treated rats by cardiac puncture into syringes and was centrifuged for 15 min at 3,500 rpm at 4 C or less. Rat serum was diluted with phosphate buffer (pH 7.4, ionic strength 0.1) to give a protein content of 60 mg/ml.

For preparation of liver microsomes, rats were killed by cervical fracture and livers were excised and homogenized with three vol of cold 0.01 M phosphate buffer (pH 7.4) containing 1.15% KCl. The homogenates were centrifuged at 9,000 × g for 30 min at 4 C or less. The resultant supernatant then was recentrifuged at 105,000 × g for 60 min at 4 C or less. The microsomal pellet was washed with 0.1 M phosphate buffer (pH 7.4) using the ultracentrifugal procedure as mentioned above. The washed microsomal pellets were suspended in the same buffer by gentle homogenation and used for the assays.

The protein content in serum and liver microsomal suspension was determined by the procedure described by Lowry et al. (17) using crystalline bovine serum albumin as a standard.

Collection of bile. For collection of bile from the drug-treated rats, rat abdomens were opened under urethane anesthesia and the common bile duct was exposed and cannulated with polyethylene tubing (PE-10) into the duct. After the incision was closed, bile sample was collected for five hr.

Determination of enzyme activity and cytochrome P-450 content. For determination of LCAT activity, the

incubation mixture contained 0.2 ml of rat serum (60 mg proteins/ml) and 0.3 ml of phosphate buffer (pH 7.4, ionic strength 0.1). The samples were placed in 15-ml screw-capped tubes, which then were flushed with N_2, sealed and incubated at 37 C for 2 hr with mechanical shaking. Free cholesterol in the incubation mixtures before and after incubation was determined by the use of the Free Cholesterol C-Test Wako kit based on an enzymatic reaction; serum-free cholesterol was oxidized by cholesterol oxidase to cholest-4-en-3-one with the simultaneous production of H_2O_2, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 505 nm.

For determination of aniline hydroxylation and aminopyrine N-demethylation activities, the incubation contained an NADPH-generating system (0.5 μmol NADP, 10 μmol glucose-6-phosphate, 25 μmol $MgCl_2$ and 1 unit of glucose-6-phosphate dehydrogenase), 1 ml (7–10 mg proteins) of microsomal suspension and 4 μmol substrate (aniline or aminopyrine). The final volume was adjusted to 4 ml with 0.1 M phosphate buffer (pH 7.4) (18). Activities of aniline hydroxylation and aminopyrine N-demethylation were measured by determining p-aminophenol (19) and formaldehyde (20), respectively. Under these experimental conditions, both enzyme activities increased linearly with time up to 20 min.

The determination of cholesterol-metabolizing activity was carried out essentially as described by Kwok et al. (21). The standard incubation medium contained potassium phosphate buffer pH 7.4 (80 mM), nicotinamide (3 mM), EDTA (1 mM), mercaptoethanol (5 mM), [4-^{14}C]cholesterol (0.025 μCi, 12.5 μM), microsomal fraction (4 mg proteins), 105,000 × g supernatant fraction (0.5 mg protein) and NADPH-generating system in a final volume of 0.5 ml. [4-^{14}C]Cholesterol as a substrate was added to the incubation medium as a lecithin-cholesterol liposome prepared by the procedure of Batzri and Korn (22), with a molar ratio of 1. After a 10 min preincubation, the reaction was commenced by adding an NADPH-generating system of glucose-6-phosphate (5 mM), NADP (2.5 mM), $MgCl_2$ (4.5 mM) and glucose-6-phosphate dehydrogenase (2 I.U.). All incubations were carried out in 10 ml screw-capped tubes with constant shaking for 10 min at 37 C. After incubation, extraction and separation of lipids and measurement of radioactivity were performed as described by Kwok et al. (21). Under these experimental conditions, 7α-hydroxylation of cholesterol increased linearly with time up to 10 min.

Cytochrome P-450 content in liver microsomes was measured by the method of Omura and Sato (23).

Determination of bile acids and cholesterol content. The determination of total bile acids in bile was performed by the spectrofluorometric procedure described by Levin et al. (24) using cholic acid as a standard.

The determination of free cholesterol in rat serum was performed by the use of the Free Cholesterol C-Test Wako kit as described above.

For determination of free cholesterol in rat bile and liver, 0.2 ml of bile or liver homogenates was placed in 15-ml screw-capped tubes and 5α-cholestane was added as an internal standard. The samples then were flushed with N_2 and extracted by chloroform/methanol (2:1, v/v) according to the procedure of Folch et al. (25). The chloroform extracts were evaporated to dryness and the amount of free cholesterol in bile and liver homogenates, as its trimethylsilyl ether derivative, was determined by gas liquid chromatography (GLC) as described by Marcel and Vezina (26).

For determination of total cholesterol in rat serum and liver, total lipid extracted from rat serum or liver homogenates by the procedure of Folch et al. (25) was hydrolyzed with 5% methanolic-KOH at 60 C for 1 hr, and total cholesterol was extracted with n-hexane as described previously (27). The hexane extracts were evaporated to dryness, and total cholesterol was determined by the colorimetric procedure of Muesing and Nishida (28).

RESULTS AND DISCUSSION

We first determined free and total cholesterol contents in serum and liver homogenates of rats treated with or without CPIB, PCBs and PCBs-CPIB. As shown in Table 1, serum free and total cholesterol contents were decreased to 70% and 54%, respectively, of those of control rats by CPIB treatment and were increased to 177% and 146%, respectively, of those of control rats by PCBs treatment. However, serum free and total cholesterol contents were unchanged by simultaneous administration of PCBs and CPIB compared to those of control rats. On the other hand, hepatic-free cholesterol content of rats treated with CPIB, PCBs and PCBs-CPIB was changed

TABLE 1

Effects of CPIB, PCBs and PCBs-CPIB on Free and Total Cholesterol Contents in Rat Serum and Liver

Treatment	Serum (μg/ml serum)		Liver (μg/mg protein)	
	Free cholesterol	Total cholesterol	Free cholesterol	Total cholesterol
None	263.6 ± 9.3 (100)	957.7 ± 78.4 (100)	5.69 ± 0.16 (100)	10.62 ± 2.20 (100)
CPIB	185.1 ± 13.5 (70)[a]	519.2 ± 16.5 (54)[b]	5.15 ± 0.30 (91)	8.88 ± 1.12 (84)
PCBs	465.7 ± 16.5 (177)[c]	1399.0 ± 51.1 (146)[a]	5.23 ± 0.30 (92)	15.83 ± 1.82 (149)[a]
PCBs-CPIB	291.1 ± 12.8 (110)	1080.3 ± 35.8 (113)	5.40 ± 0.25 (95)	11.85 ± 2.57 (112)

Three rats were used for each group in three separate experiments. Values are means ± S.D. of three separate experiments. Values in parentheses are percentages of the values obtained in control rats (taken as 100%).

[a]Significant at $p < 0.01$ by Student's t-test.

[b]Significant at $p < 0.02$ by Student's t-test.

[c]Significant at $p < 0.001$ by Student's t-test.

little compared to that of control rats. Also, although hepatic total cholesterol content of rats treated with CPIB and PCBs-CPIB was changed little, hepatic total cholesterol content of rats treated with PCBs was increased ca. 49% over that of control rats. Accordingly, the increase of free and total cholesterol contents in serum and liver induced by oral ingestion of PCBs may be reversed by CPIB treatment.

It has been reported in clinical cases of PCBs poisoning that serum lecithin-cholesterol acyltransferase activity is decreased (1). However, according to the data in Table 1, cholesterol ester content in serum of rats treated with PCBs was increased compared to that of control rats. This fact suggests that, besides increased secretion of cholesterol ester from liver to serum, the formation of cholesterol ester in serum also may be increased by PCB treatment. In addition, D'alessandro et al. (29) have reported that acyltransferase activity in hyperlipidemic subjects is decreased compared to that in normal subjects if expressed as the percentage of cholesterol esterification, but is slightly increased compared with normal subjects if expressed as the net cholesterol esterification. Similarly, we recently have reported that the apparent elevation of acyltransferase activity in serum of rats administered aspirin or salicylic acid orally is due mainly to the decrease of serum free cholesterol content (30). Therefore, to investigate the acyltransferase activity in serum of rats treated with or without CPIB, PCBs and PCBs-CPIB, we determined free cholesterol content in serum of rats treated with CPIB, PCBs, and PCBs-CPIB before and after incubation by the use of the kit based on enzymatic reaction. Under these experimental conditions, the esterification of endogenous cholesterol increased linearly with time up to 2 hr of incubation. As shown in Table 2, when acyltransferase activity was expressed on the basis of the percentage of cholesterol esterified, the acyltransferase activity in serum of rats treated with PCBs was decreased significantly compared to that of control rats, while the acyltransferase activity in serum of rats treated with CPIB was increased significantly. In addition, upon simultaneous administration of PCBs and CPIB, acyltransferase activity was the same level as that of control rats. However, if expressed as the net cholesterol esterification, the formation of cholesterol ester in serum of rats treated with PCBs was increased significantly compared to that of control rats, while the formation of cholesterol ester in serum of rats treated with CPIB and PCBs-CPIB was the same level as that of control rats. The same results were obtained by the use of exogenous lecithin-[^{3}H]-cholesterol liposomes as a substrate in vitro (data not shown). Accordingly, the increase of cholesterol ester content in serum of rats treated with PCBs may be due partly to the elevation of acyltransferase activity. In addition, the elevation of net cholesterol esterification by PCB treatments may be due to the increase of the enzyme or cofactor lipoprotein content in serum and/or the elevation of the enzyme activity itself.

On the other hand, the effect of CPIB on the increase of hepatic and serum cholesterol contents induced by PCB administrations may be related to the increase of cholesterol excretion from liver to bile and/or the increase of cholesterol degradation to bile acids in liver. In fact, CPIB is known to stimulate both the excretion of cholesterol into bile, without causing any compensatory increase in cholesterol synthesis, in hyperlipidemic patients (31) and the excretions of bile acid and cholesterol into bile in fasting dogs (32). Thus, we determined the amounts of excretion of bile and biliary cholesterol in rats treated with CPIB, PCBs and PCBs-CPIB. As shown in Table 3, the amount of bile excretion in rats treated with CPIB, PCBs and PCBs-CPIB was increased significantly compared to that of control rats, while free and total cholesterol contents in bile of rats treated with CPIB, PCBs and PCBs-CPIB were decreased to 63% and 70%, 60% and 73%, and 43% and 60%, respectively, of those of control rats. These results are incompatible with the observations obtained by Grundy et al. (31) and Horning et al. (32), who observed that CPIB stimulates cholesterol excretion into bile in hyperlipidemic patients and in fasting dogs. This discrepancy may be due to the difference of physiological and nutritional states of animals. In any event, the effect of CPIB on serum and hepatic cholesterol contents increased by PCB administration may be unrelated to the increase of excretion of hepatic cholesterol into bile. However, the results obtained here suggested that the enhancement of cholesterol catabolism in rats treated with CPIB, PCBs and PCBs-CPIB may cause the increase in bile and bile acids.

The first step in the sequence leading from cholesterol to bile acids is the introduction of hydroxyl group into 7α-position of cholesterol; it may be a rate-limiting step in bile acid formation (33–35). The terminal enzyme

TABLE 2

Cholesterol Esterification in Serum of Rats Treated with CPIB, PCBs and PCBs-CPIB

Treatment	Serum cholesterol content (μg/ml serum)		Esterification	
	Before 2-hr incubation	After 2-hr incubation	%	Net (μg/ml serum)
None	288.0 ± 17.0	220.5 ± 11.3	23.7 ± 0.6 (100)	68.5 ± 5.7 (100)
CPIB	186.6 ± 15.1	120.5 ± 11.1	35.4 ± 3.5 (149)[a]	66.1 ± 9.2 (96)
PCBs	484.5 ± 29.7	398.1 ± 38.2	18.0 ± 3.0 (76)[a]	86.4 ± 8.7 (126)[b]
PCBs-CPIB	289.1 ± 18.7	221.4 ± 22.9	23.6 ± 2.9 (99)	67.7 ± 4.6 (99)

Three rats were used for each group in three separate experiments. Values are means ± S.D. of three separate experiments. Values in parentheses are percentages of the values obtained in control rat serum (taken as 100%).

[a]Significant at $p < 0.05$ by Student's t-test.

[b]Significant at $p < 0.10$ by Student's t-test.

TABLE 3

Amounts of Bile and Biliary Cholesterol in Rats Treated with CPIB, PCBs and PCBs-CPIB

Treatment	Amount of bile (g/5 hr)	Cholesterol content (μg/bile of 5 hr)	
		Free cholesterol	Total cholesterol
None	3.96 ± 0.66 (100)	640.7 ± 25.8 (100)	679.3 ± 28.0 (100)
CPIB	5.36 ± 0.22 (135)[a]	410.0 ± 35.6 (63)[a]	477.0 ± 52.4 (70)[a]
PCBs	5.48 ± 0.40 (138)[b]	385.3 ± 53.3 (60)[a]	494.3 ± 44.6 (73)[a]
PCBs-CPIB	6.49 ± 1.01 (164)[b]	276.3 ± 28.6 (43)[c]	404.3 ± 25.2 (60)[c]

Values are means ± S.D. of three rats. Values in parentheses are percentages of the values obtained in control rats (taken as 100%).

[a]Significant at $p < 0.01$ by Student's t-test.

[b]Significant at $p < 0.05$ by Student's t-test.

[c]Significant at $p < 0.001$ by Student's t-test.

TABLE 4

Cytochrome P-450 Content and Drug- and Cholesterol-Metabolizing Enzyme Activities

Treatment	Cytochrome P-450 (nmole/mg protein)	Aniline hydroxylation (nmole p-aminophenol/ mg protein/20 min)	Aminopyrine demethylation (nmole HCHO/mg protein/20 min)	Cholesterol hydroxylation (p mole 7α-hydroxy-cholesterol/min/mg protein)
None	1.12 ± 0.14 (100	5.53 ± 0.66 (100)	31.35 ± 2.58 (100)	10.69 ± 0.55 (100)
CPIB	1.59 ± 0.10 (142)[a]	7.04 ± 0.32 (127)[a]	36.77 ± 1.98 (117)	13.35 ± 0.55 (125)[c]
PCBs	2.39 ± 0.08 (213)[b]	11.63 ± 0.50 (210)[b]	52.59 ± 2.76 (168)[b]	18.10 ± 1.31 (169)[c]
PCBs-CPIB	2.64 ± 0.07 (236)[b]	12.71 ± 1.44 (230)[c]	60.83 ± 4.91 (194)[c]	16.07 ± 2.00 (150)[a]

Three rats were used for each group in three separate experiments. Values are means ± S.D. of three separate experiments. Values in parentheses are percentages of the values obtained in control rats (taken as 100%).

[a]Significant at $p < 0.05$ by Student's t-test.

[b]Significant at $p < 0.001$ by Student's t-test.

[c]Significant at $p < 0.01$ by Student's t-test.

TABLE 5

Excretion of Bile Acids in Rats Treated with CPIB, PCBs and PCBs-CPIB

Treatment	Amount of bile (g/5 hr)	Bile acid content (cholic acid equivalent in mg/5 hr bile)
None	3.00 ± 0.22 (100)	6.20 ± 0.56 (100)
CPIB	5.29 ± 0.40 (176)[a]	11.05 ± 1.89 (178)[b]
PCBs	4.95 ± 1.04 (165)[a]	10.80 ± 1.15 (174)[a]
PCBs-CPIB	5.50 ± 0.55 (183)[a]	12.47 ± 1.15 (201)[a]

Values are means ± S.D. of three rats. Values in parentheses are percentages of the values obtained in control rats (taken as 100%).

[a]Significant at $p < 0.01$ by Student's t-test.

[b]Significant at $p < 0.05$ by Student's t-test.

catalyzing this step is cytochrome P-450 in liver microsomes. In general, CPIB and PCBs are known to induce the hepatic mixed function oxidase system which metabolizes many foreign chemicals and endogenous steroids (36,37). Therefore, we determined cytochrome P-450 content and drug- and cholesterol-metabolizing enzyme activities in liver microsomes of rats treated with or without CPIB, PCBs and PCBs-CPIB. As shown in Table 4, cytochrome P-450 content in liver microsomes of rats treated with CPIB, PCBs and PCBs-CPIB was increased significantly compared to that of control rats. Aminopyrine N-demethylase activity was also significantly elevated by PCBs and PCBs-CPIB, but not by CPIB, treatment. Furthermore, enzyme activity for the metabolism of cholesterol to 7α-hydroxycholesterol in liver microsomes of rats treated with CPIB, PCBs and PCBs-CPIB also was elevated significantly compared to that of control rats. To further confirm the effects of CPIB, PCBs and PCBs-CPIB treatment on cholesterol metabolism in rat liver, we determined the excretion of bile acids from liver to bile in rats treated with CPIB, PCBs and PCBs-CPIB. As shown in Table 5, bile acid ex-

cretion was increased significantly by CPIB, PCBs and PCBs-CPIB treatment. These results are in agreement with earlier observations: (i) that the increase in liver microsomal cytochrome P-450 after CPIB treatment is accompanied by the increase in the metabolism of testosterone to more polar metabolites but not by the increase in metabolism of model drug substrates such as aminopyrine, pentobarbitone or benzo(a)pyrene (38); (ii) that although the activities of hepatic microsomal hydroxylase and N-demethylase are increased by feeding PCB (Aroclor 1242) to rats, N-demethylase activity is much less sensitive than hydroxylase activity (39), and (iii) that steroid hydroxylase for the metabolism of oestradiol to more polar metabolites in avian species is induced by feeding PCB (Aroclor 1254 and 1262) (40). Accordingly, the phenomenon that the increase of serum and hepatic cholesterol contents in rats treated with PCBs is reversed by CPIB administration may be related partly to the elevations of hepatic mixed function oxidase activity for cholesterol catabolism and excretion of bile acids from liver to bile. However, in spite of the increase of cholesterol catabolism and excretion of bile acids in rats treated with PCBs alone, serum and hepatic cholesterol levels were elevated compared to those of control rats. Therefore, upon administration of PCBs, hepatic cholesterol synthesis and hepatic cholesterol secretion to serum may be elevated more than the elimination of cholesterol as bile acids. In addition, both hepatic cholesterol synthesis and hepatic cholesterol secretion to serum elevated by PCBs may be effectively depressed by CPIB. In fact, it has been reported so far that PCBs increase both the incorporation of [^{14}C]glucose or [^{14}C]acetate into sterols in rat liver (6) and hepatic lipoprotein secretion to serum (41) and that, on the contrary, CPIB decreases hepatic cholesterol synthesis (42-44) and hepatic cholesterol secretion to serum (45). Furthermore, since PCBs (unheated KC-400) are contaminated by polychlorinated quaterphenyl (PCQ) and polychlorinated dibenzofuran (PCDF) (46), there is a possibility that the results obtained here may be caused by PCQ or PCDF in PCBs (KC-400). These problems remain to be clarified.

In conclusion, our findings indicate that the increase of serum and hepatic cholesterol contents in rats treated with PCBs is reversed by CPIB treatment and also that the increase of net cholesterol ester formation in serum of rats treated with PCBs comes to normal level with CPIB treatment. In addition, the changes of hepatic, serum and biliary cholesterol concentrations by the drugs used here may depend partly on the degree of degradation, which is catalyzed by mixed function oxidase in liver microsomes, of cholesterol to bile acids.

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Subcellular Distribution of Dietary β-Carotene in Chick Liver

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Studies were conducted examining the subcellular distribution of β-carotene (BC), α-tocopherol (E) and retinol (A) in livers of control and BC-fed male White Leghorn chicks. Chicks were fed Cornell B chick starter diet with or without the addition of 0.5 g BC/kg diet. A first study involved liver fractionation by differential centrifugation in 0.25 M sucrose followed by high performance liquid chromatographic (HPLC) analyses of all fractions for quantitation of BC, E and A. A second study employed both intravenous injection of Triton WR-1339 four days prior to sacrifice and centrifugation in 1.0 M sucrose to separate mitochondria from lysosomes more efficiently. Fraction purity was assessed by marker enzyme analyses. Results showed that (i) chick liver accumulated BC; (ii) BC-fed chicks had higher concentrations of BC in all fractions relative to controls, and (iii) the mitochondrial fraction contained the highest concentration of BC, followed by lysosomes, microsomes and nuclei, respectively. Plasma BC increased more than fivefold in BC-fed chicks. Dietary BC increased A and E levels in liver and in the mitochondrial and lysosomal fractions while the plasma E level was decreased. Plasma A changed little with BC feeding. While dietary BC had no effect on fatty acid composition of subcellular fractions, the increase in E resulted in a large increase in the molar ratio of E to polyunsaturated fatty acids. The incorporation of BC and increased amounts of E into cellular membranes presumably would result in increased resistance to peroxidative damage.
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Human cancer risks are inversely correlated with dietary β-carotene (BC) (1). This apparent protective role may be related to BC's provitamin A activity. However, evidence also suggests that BC may have anticarcinogenic effects independent of its role as a vitamin A precursor. BC is known to function in plants as an effective electron acceptor/donor and singlet oxygen quencher, leading to the hypothesis that BC may perform similar activities in nonphotosynthetic tissues. Krinsky and Deneke (2) have found BC capable of inhibiting free radical and singlet oxygen-induced lipid peroxidation in liposomes. Recent evidence in vitro suggests that BC belongs to a previously unknown class of biological antioxidants particularly effective at low oxygen partial pressures such as those found in most tissues under physiological conditions (3). In vivo, BC has been shown to protect against skin tumors induced in mice by UV-B irradiation (290-320 nm) (4,5), dimethylbenz[a]anthracene/croton oil applications (4) and benzo[a]pyrene application followed by UV irradiation at 300-400 nm (6). The role free radicals may play in carcinogenesis recently has been reviewed (7-9).

The human diet contains numerous natural mutagens and carcinogens, many of which may act through the generation of oxygen radicals (10); therefore, dietary intake of natural antioxidants such as BC could be an important aspect of the body's defense mechanism against these agents. However, if the hypothesis that dietary BC protects against carcinogenesis or other diseases via the quenching of organic radicals or activated oxygen species is to be compelling, demonstration of BC in animal tissues in effective concentrations seems necessary. BC has been found in the spleen, liver, adrenals and ovaries of mice and the liver and adrenals of guinea pigs (11). A recent study examining BC content in 11 tissues from BC-fed rats showed that rat tissue exhibited a dose-response relationship between the amount of dietary BC ingested and the tissue content, with liver accumulating the most BC, followed by adrenals and ovaries (12). BC also has been found in human erythrocyte membranes (13) and in various subcellular fractions of bovine corpus luteum (14) and mammary gland (15). Both latter studies found BC to be associated with all subcellular fractions examined. In mammary gland, the highest BC concentration was found in the mitochondrial fraction; however, assessment of cross-contamination of subcellular fractions was not reported in either study. We know of no other studies concerning the subcellular distribution of BC in animals.

The use of carotenoids as colorants in poultry has resulted in much information demonstrating the ability of chicks to absorb and accumulate carotenoids (16). In the two experiments described below, BC, α-tocopherol (E) and retinol (A) levels were determined in subcellular fractions prepared from livers of BC-fed and control chicks. Fraction characterization was performed using a variety of marker enzymes. In addition, fatty acid analyses were conducted on all fractions. These data permit calculation of the concentration of BC in various organelles relative to other presumed antioxidants and peroxidizable substrates.

MATERIALS AND METHODS

Animals and diets. Male White Leghorn chicks, hatched from breeder hens maintained by the Department of Poultry Science, Cornell University, were fed from hatching Cornell B chick starter diet, which contains small amounts of BC, with or without the addition of crystalline BC (Sigma, St. Louis, Missouri; >94% purity by HPLC). Experimental diets were supplemented with BC (0.5 g/kg diet) in corn oil (20 g/kg diet); control diets were supplemented with corn oil (20 g/kg diet) only.

Subcellular fractionation—experiments 1 and 2. Experiment 1 was designed to examine the subcellular distribution of BC in chick liver following fractionation in 0.25 M sucrose. At six wk, plasma was obtained from heparinized chicks by cardiac puncture; chicks were killed by cervical dislocation, and livers were thoroughly perfused in situ with ice-cold isotonic saline. Each liver was weighed and three g were removed, minced over ice and placed in 15 ml of ice-cold 0.25 M STE buffer (0.25 M sucrose containing 5 mM Trizma base and 0.5 mM disodium EDTA, final pH 7.4). Each three g liver portion was homogenized by four complete passes in a loosely fitting Potter-Elvehjem homogenizer, then diluted with 0.25 M STE to a final volume of 30 ml (10% homogenate).

Cell nuclei, unruptured cells and debris were sedimented by centrifugation in a refrigerated international centrifuge at 700 × g for 10 min. Postnuclear supernatants

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were centrifuged at 15,000 × g for 5.5 min in a Sorvall RC2-B refrigerated centrifuge (SS-34 rotor), yielding the mitochondrial pellet which was washed by resuspension in 0.25 M STE and recentrifugation at 15,000 × g for 5.5 min. The combined postmitochondrial supernatants were centrifuged at 25,000 × g for 10 min, yielding the lysosomal pellet which was washed at 25,000 × g for 10 min. The postlysosomal supernatants were centrifuged at 105,000 × g for 60 min in a Beckman LC-65B ultracentrifuge (50.2 Ti rotor), yielding the microsomal pellet. Pellets were diluted to a final volume of 2 ml and resuspended thoroughly; 50 μl aliquots were removed for marker enzyme analyses. All manipulations were done at 0–4 C, and the fractions were stored immediately under N_2 in chloroform/methanol (2:1, v/v) containing BHT, BHA and propyl gallate at −70 C until analysis.

For experiment 2, 38-day-old control and BC-fed chicks were injected intravenously with 20% Triton WR-1339 in saline, 800 mg Triton/kg body weight. Four days later the chicks were killed by cervical dislocation after an overnight fast and livers were perfused and homogenized as described for experiment 1. Nuclei and cell debris were isolated as described previously, then discarded. Twenty-five ml of the postnuclear supernatant was added to 15 ml of ice-cold 2.25 M STE and mixed by gentle inversion to achieve a final concentration of 1.0 M STE. The mitochondrial pellet was isolated by centrifugation in the Sorvall RC2-B (SS-34 rotor) at 7,700 × g for 5.5 min and washed by resuspension in 1.0 M STE and centrifugation at 8,600 × g for 7.0 min. The postmitochondrial supernatant was centrifuged at 15,800 × g for 5.5 min, yielding a mixed pellet of light mitochondria and lysosomes. The resulting supernatant was centrifuged at 25,000 × g for 12 min, yielding the lysosomal pellet. Isolated pellets were treated as described for experiment 1.

Analysis of subcellular fractions. Lipids were extracted from each fraction according to the method of Folch et al. (17), then saponified in 5% ethanolic KOH at 65 C for 10 min in the presence of added BHT, BHA and propyl gallate. All steps were performed in dim light. Recovery studies indicated that both carotenoids and tocopherols are stable in this reaction. BC, A and E were extracted from the basic solution with hexane and analyzed on a Beckman 110A HPLC using reverse phase chromatography. Analysis of E and A involved a mobile phase of methanol/water (98:2, v/v), 1.2 ml/min, and detection at 300 nm with retention times of six and two min, respectively. BC analysis involved a mobile phase of acetonitrile/tetrahydrofuran/methanol/1% ammonium acetate (66:22:7.2:4.8, v/v/v/v), 1.8 ml/min, retention time of 11 min on a 25-cm Merck Lichrocart RP-18 cartridge (experiment 1) or acetonitrile/chloroform/water (80:18:2, v/v/v), 1.4 ml/min, retention time of four min on a 12.5-cm Merck RP-18 cartridge (experiment 2). Detection of carotenes was at 436 nm (experiment 1) or 460 nm (experiment 2). For both experiments, peaks were integrated by a Hewlett-Packard 3390A plotter-integrator. Recovery of known amounts of BC and E in ethanol exceeded 95% in the described saponification and HPLC procedures, with 90 ± 7% recovery of retinol (n = 8).

The postsaponification solution was acidified and free fatty acids were extracted with hexane and transesterified with 4% H_2SO_4 in dry methanol. The resulting fatty acid methyl esters were separated and quantitated by gas liquid chromatography (GLC). A 1 μl sample in hexane was injected onto a Varian 2100 gas chromatograph equipped with a flame ionization detector and a 6-ft column packed with 15% SP 2330 on 80/100 mesh Gas Chrom P (Supelco, Bellefonte, Pennsylvania) maintained at 190 C. Fatty acids were identified by comparison of retention times with those of standards. Peaks were integrated by a Hewlett-Packard 3390A plotter-integrator.

Phospholipid phosphorous was determined according to the method of Eng and Noble (18) using a perchloric acid digestion. Cytochrome C oxidase activity was determined according to the method of Wharton and Tzagoloff (19). Acid phosphatase activity was analyzed by Sigma Kit #104-AL using p-nitrophenyl phosphate as substrate; β-glucuronidase activity was analyzed by Sigma Kit #325A using phenolphthalein glucuronic acid as substrate, and cathepsin D activity was determined according to the method of Takayuki and Tang using bovine hemoglobin as substrate (20).

Results are expressed in terms of lipid phosphorous, as this parameter reflects yield and recovery of membrane lipids. Data were statistically analyzed using Student's t-test for samples with unequal variances.

RESULTS AND DISCUSSION

The distribution of BC in liver subcellular fractions is summarized in Table 1. The relatively large standard deviations are due to individual animal, not analytical, variation, as determined by duplicate analyses (not shown). Experiment 1 (fractionation in 0.25 M sucrose) showed that chick liver accumulated dietary BC, as BC-fed chicks had considerably higher concentrations of BC in all fractions. The mitochondrial fraction contained the highest concentration of BC, expressed either as ng/g liver or ng/mg phospholipid phosphorous (PL Pi). BC was not detected in the total liver sample in control chicks due to the small samples taken for this analysis. The lysosomal fraction contained nearly as much BC per mg PL Pi as the mitochondrial fraction. The finding of BC in lysosomes was not surprising, as BC is transported in human blood primarily by low density lipoproteins (21,22) which are taken up by the liver via LDL receptor-mediated endocytosis, followed by transport to lysosomes. Plasma BC was elevated more than fivefold in chicks fed BC.

To rule out the possibility that mitochondrial BC was due to mass transfer from lipid droplets during homogenization, liver was obtained from a male Sprague-Dawley rat fed a carotenoid-free diet, then exsanguinated by perfusion and homogenized briefly in 0.25 M STE. BC (6 μg in 50 μl corn oil) then was added in excess of any level previously observed in our lab for chick liver, and the mixture was rehomogenized. The mitochondrial fraction was prepared as described in experiment 1. No BC could be detected in this fraction, indicating that partitioning of BC from bulk lipid into membrane lipid during homogenization was not quantitatively important.

BC feeding resulted in a 51% decrease in plasma E level, while increasing total liver E level more than fourfold (Table 2; $p = 0.0085$ and $p = 0.0025$, respectively). Dietary BC also markedly increased the A level in all liver fractions (Table 3). However, the plasma A level remained essentially unchanged, consistent with Willett's results in humans (23).

TABLE 1

Subcellular Distribution of β-Carotene (BC) in Liver of Control and BC-Fed Chicks

	ng BC/g fractionated liver		ng BC/mg PL Pi[a]	
	Control	β-Carotene	Control	β-Carotene
Experiment 1				
Total liver	ND[b]	308 ± 221	ND	400 ± 250
Nuclei/debris	ND	5 ± 2	ND	70 ± 30
Mitochondria	9 ± 6	69 ± 43[c]	220 ± 160	840 ± 470[c]
Lysosomes	4 ± 2	14 ± 11	170 ± 85	720 ± 440[d]
Microsomes	3 ± 1	7 ± 4[d]	40 ± 20	110 ± 55[d]
Plasma (ng/ml)	2 ± 3	15 ± 13	—	—
Experiment 2				
Total liver	92 ± 61	678 ± 429[c]	100 ± 65	730 ± 445[c]
Mitochondria	2 ± 1	17 ± 15[d]	320 ± 90	1450 ± 1770
Mixed	ND	9 ± 5	ND	335 ± 140
Lysosomes	2 ± 3	5 ± 3	170 ± 105	335 ± 135

Values expressed as mean ± SD. Experiment 1, n = 3 for control, n = 6 for BC. Experiment 2, n = 2 for control, n = 6 for BC.

[a]PL Pi, phospholipid phosphorous.

[b]ND, not detected (<2 ng BC).

[c]Vs control, $p < 0.05$.

[d]Vs control, $p < 0.10$.

TABLE 2

Subcellular Distribution of α-Tocopherol (E) in Liver of Control and BC-Fed Chicks

	ng E/g fractionated liver		μg E/mg PL Pi[a]	
	Control	β-Carotene	Control	β-Carotene
Experiment 1				
Total liver	3850 ± 596	16020 ± 6110[b]	4.14 ± 1.44	21.06 ± 7.08[b]
Nuclei/debris	88 ± 53	107 ± 104	1.31 ± 0.27	1.01 ± 0.48
Mitochondria	309 ± 189	439 ± 202	6.50 ± 3.22	5.54 ± 2.46
Lysosomes	189 ± 24	275 ± 98	8.12 ± 0.95	13.78 ± 4.32[b]
Microsomes	194 ± 37	103 ± 23[c]	2.99 ± 0.39	1.79 ± 0.23[b]
Plasma (ng/ml)	1625 ± 194	794 ± 182[a]	—	—
Experiment 2				
Total liver	4210 ± 750	10370 ± 3453[b]	4.66 ± 0.77	11.49 ± 4.23[b]
Mitochondria	84 ± 64	950 ± 386[b]	22.80 ± 25.95	94.37 ± 85.92
Mixed	116 ± 59	507 ± 276[b]	5.48 ± 3.48	18.85 ± 6.15[b]
Lysosomes	110 ± 27	410 ± 247[b]	7.07 ± 1.79	28.05 ± 19.06[b]

Values expressed as mean ± SD. Experiment 1, n = 3 for control, n = 6 for BC. Experiment 2, n = 2 for control, n = 6 for BC.

[a]PL Pi, phospholipid phosphorous.

[b]Vs control, $p < 0.05$.

[c]Vs control, $p < 0.10$.

The distribution between fractions of cytochrome c oxidase, a mitochondrial marker enzyme, indicated that the lysosomal and microsomal fractions of experiment 1 were essentially free from mitochondrial contamination (Table 4). However, a high cytochrome c oxidase activity was observed in the nuclei/debris fraction, as reported by others (24). This most likely was due to contamination of this fraction by whole cells and heavy mitochondria. The distribution of acid phosphatase, a lysosomal marker enzyme, in experiment 1 showed more acid phosphatase activity in the mitochondrial fraction than the lysosomal fraction, indicating considerable cosedimentation of mitochondria and lysosomes in this mitochondrial fraction. Activities of β-glucuronidase and cathepsin D, also lysosomal marker enzymes, were determined on the same fractions and resulted in nearly identical enzyme distributions (data not shown).

Experiment 2 was designed to prepare liver mitochondrial fractions with minimal lysosomal contamination from chicks of the same sex, strain and age and on the same diets as used in experiment 1. Triton WR-1339, a nontoxic, nonmetabolizable detergent which accumulates

TABLE 3

Subcellular Distribution of Retinol (A) in Liver of Control and BC-Fed Chicks

	ng A/g fractionated liver		μg A/mg PL Pi[a]	
	Control	β-Carotene	Control	β-Carotene
Experiment 1				
Total liver	43830 ± 15800	314750 ± 154280[b]	14.95 ± 9.66	195.98 ± 116.48[b]
Nuclei/debris	336 ± 140	813 ± 980	5.80 ± 2.14	6.86 ± 4.13
Mitochondria	223 ± 181	845 ± 643[c]	4.64 ± 3.25	10.55 ± 7.71
Lysosomes	204 ± 70	492 ± 320	8.75 ± 2.91	22.80 ± 10.00[b]
Microsomes	128 ± 24	160 ± 68	2.00 ± 0.45	2.80 ± 1.16
Plasma (ng/ml)	706 ± 266	809 ± 237	—	—
Experiment 2				
Total liver	17760 ± 11650	97060 ± 27450[b]	19.77 ± 13.18	106.71 ± 31.37[b]
Mitochondria	240 ± 215	2870 ± 1440[b]	68.10 ± 81.73	271.75 ± 219.25
Mixed	400 ± 440	2280 ± 1427[b]	19.75 ± 22.77	83.05 ± 35.09[c]
Lysosomes	330 ± 49	1340 ± 941[b]	21.11 ± 2.92	85.85 ± 52.04[b]

Values expressed as mean ± SD. Experiment 1, n = 3 for control, n = 6 for BC. Experiment 2, n = 2 for control, n = 6 for BC.

[a]PL Pi, phospholipid phosphorous.

[b]Vs control, $p < 0.05$.

[c]Vs control, $p < 0.10$.

TABLE 4

Cytochrome C Oxidase and Acid Phosphatase Activities of Liver Subcellular Fractions from Control and BC-Fed Chicks

	Cytochrome C oxidase activity		Acid phosphatase activity	
	Units/sample[a]	Units/mg PL Pi[b]	Units/sample	Units/mg PL Pi
Experiment 1				
Nuclei/debris	81.4 ± 59.0	34.0 ± 6.8	7.3 ± 4.7	3.4 ± 1.4
Mitochondria	62.6 ± 30.9	29.6 ± 8.6	26.6 ± 9.5	13.5 ± 4.8
Lysosomes	1.6 ± 1.1	2.5 ± 1.3	7.5 ± 2.2	11.5 ± 2.3
Microsomes	2.0 ± 1.2	1.1 ± 0.6	11.4 ± 1.9	6.2 ± 0.8
Experiment 2				
Mitochondria	17.6 ± 14.2	35.4 ± 9.9	1.5 ± 0.7	3.7 ± 1.2
Mixed	19.7 ± 5.6	22.1 ± 5.6	6.9 ± 2.6	7.8 ± 2.5
Lysosomes	10.9 ± 5.4	18.2 ± 6.9	8.8 ± 3.8	15.7 ± 6.3

Values expressed as mean ± SD. Experiment 1, n = 9. Experiment 2, n = 8.

[a]Expressed as units in 50 μl of 2 ml total fraction volume.

[b]PL Pi, phospholipid phosphorous.

in liver lysosomes, was injected intravenously to decrease lysosomal density (25). In addition, 1.0 M STE buffer was used in the mitochondrial sedimentation, as previous fractionations had indicated that the denser sucrose resulted in less lysosomal cosedimentation and a higher cytochrome c oxidase:acid phosphatase activity ratio. Marker enzyme data (Table 4) confirmed the efficacy of this method, as the acid phosphatase activity of the mitochondrial fraction dropped from 26.6 units (experiment 1) to 1.5 units (experiment 2), an eighteenfold decrease. Concurrently, the mitochondrial yield (as determined by cytochrome c oxidase activity) decreased only 3.5-fold (from 62.2 units in experiment 1 to 17.6 units in experiment 2). The result was a net fivefold purification of the mitochondrial fraction in terms of lysosomal contamination. This procedure also increased the lysosomal yield by 17%, as determined by acid phosphatase activity.

The distribution of BC in subcellular fractions determined in experiment 2 was similar to that obtained in experiment 1 (Table 1). The absolute BC content found in the purified mitochondria was lower than that observed in the contaminated (experiment 1) mitochondria (69 vs 17 ng/g fractionated liver, respectively), as would be expected by the observed decrease in mitochondrial yield. However, mitochondrial BC content expressed per mg PL Pi increased 1.7-fold with mitochondrial purification, from 840 ng/mg PL Pi (experiment 1) to 1450 ng/mg PL Pi (experiment 2). This indicated that the bulk of the BC found in this fraction was not due to lysosomal contamination but rather was associated with mitochondrial lipid per se.

These results suggest that dietary BC is incorporated into chick liver mitochondria at levels exceeding those in other subcellular fractions. The importance and mechanism of the apparent high BC incorporation into this fraction is unclear. Burton and Ingold (3) predicted that BC and related compounds would tend to be concentrated in those membranes and organelles exposed to the lowest partial pressures of oxygen. While oxygen tensions may vary considerably between organs, it is unlikely that large differences in oxygen tension would exist between organelle microenvironments within a given tissue. Additionally, the similarities between mitochondria and microsomes with respect to membrane fatty acid composition and occurrence of oxidative reactions would suggest that these organelles would have more similar BC concentrations than those reported here or by Patton (15).

Also of interest are the observations from experiments 1 and 2 that BC feeding significantly increased the concentrations of E and A in chick liver. A fourfold increase in E and A concentrations, expressed as ng/mg PL Pi, was seen in mitochondrial, mixed and lysosomal fractions obtained from BC-fed animals. The mitochondrial fraction contained the most A and E. The finding of the highest subcellular E concentration in mitochondria is consistent with that of Taylor (26) in rat liver. Reports of the comparative subcellular concentration of A are lacking. In rat liver, the major portion has been found in association with lipid droplets within liver cells (27). Information on the distribution of A in membrane fractions is limited, but has shown A to be present in several crude membrane preparations (24,28).

The mechanism for the significant increase in hepatic E in BC-fed chicks is unclear. It is unlikely that BC enhanced E absorption in light of the decreased E concentration seen in plasma of BC-fed chicks. BC may have exerted a sparing effect on membrane E by quenching active oxygen species before their interaction with E. While such an interaction may seem unlikely because the mitochondrial molar ratio of E to BC (in both control and BC-fed chicks) calculated from experiment 2 was ca. 80:1, studies of the stability of BC during singlet oxygen attack lend support for such a concept. It has been observed that approximately one molecule of E is irreversibly oxidized for every 120 molecules of singlet oxygen quenched (29), whereas one molecule of BC is irreversibly oxidized for every 2000–2500 molecules of singlet oxygen quenched (30). Similar comparisons using other active oxygen species have not been reported. We recently have observed a decreased loss of E during NADPH/Fe^{3+}/ADP induced peroxidation of liver mitochondria isolated from BC-fed rats relative to control preparations (31). Coprotection of BC and δ-tocopherol during singlet oxygen-initiated oxidation of methyl linoleate also has been reported (32). Therefore, although BC concentration in membranes may be low relative to that of E, BC may retard oxidative destruction of E.

Dietary BC had no significant effect on mitochondrial fatty acid composition. Linoleate, arachidonate and docosahexaenoate represented 18.8, 11.5 and 9.5% of mitochondrial fatty acids, respectively, in the purified mitochondrial fraction of experiment 2. Respective values for these fatty acids in the mitochondrial fraction of experiment 1 were 20.4, 20.2 and 2.3%. Differences between these two mitochondrial preparations point out the necessity of fraction characterization and the limitations of mitochondrial preparation in 0.25 M sucrose. Molar ratios of these selected polyunsaturated fatty acids to E in mitochondrial fractions from experiments 1 and 2 are presented in Table 5. Results show that in the lysosome-containing mitochondrial fraction (experiment 1), no effect of BC feeding on the calculated molar ratios was observed, whereas the decreases in molar ratios were substantial in purified mitochondria (experiment 2). These decreases reflect the increase in E content of purified mitochondria from BC-fed chicks. It is unlikely that the increase in mitochondrial E content from experiment 1 to experiment 2 resulted from increased hepatic uptake or intracellular redistribution of E caused by Triton, since total liver and lysosomal E levels were not significantly altered from experiment 1 to experiment 2 (Table 2).

TABLE 5

Fatty Acid/E Molar Ratios in Liver Mitochondria from Control and β-Carotene-Fed Chicks

	18:2/E	20:4/E	22:6/E
Experiment 1			
Control	826	759	93
β-Carotene	841	756	75
Experiment 2			
Control	714	527	294
β-Carotene	149	103	81

Values expressed as mean of the following number of observations: Experiment 1, n = 3 for control, n = 6 for BC; Experiment 2, n = 2 for control, n = 6 for BC.

In summary, dietary BC was shown to be incorporated into several subcellular fractions of chick liver, with the concentration in mitochondria exceeding that in other fractions. Dietary BC also was found to increase the concentrations of E and A in both total liver and subcellular fractions, although plasma E was reduced. As membrane concentrations of carotenoids, retinoids, tocopherols and fatty acids are likely to influence membrane characteristics collectively, simultaneous quantitation of these components such as reported here is important. Both the occurrence of BC in cellular membranes and the concomitant increase in E concentration in BC-fed chicks should afford an increase in the resistance of such membranes to oxidative damage. We currently are testing this hypothesis in subcellular fractions similar to those obtained in this study. While the relationship of membrane peroxidation to specific disease states remains obscure, these results lend credence to the suggestion that BC and other carotenoids may influence carcinogenesis by altering membrane stability.

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METHODS

A Method for Isolation of Milk Fat Globules

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The traditional procedure for isolating milk fat globules involves repeated cycles of centrifuging to obtain globules and redispersion of them in fresh buffer to eliminate other milk components. We have evaluated a simpler, less manipulative method whereby globules are centrifuged out of the milk and through an overlying buffer layer. Human milk samples ranging from 0.1 to 35 ml were centrifuged at 1500 × g for 20 min after deposition under a suitable quantity of buffer. This yielded purified globules, in less time, which could be dispersed more satisfactorily than those by the traditional procedure. Protein, phospholipid and cholesterol contents of globules by the two methods were quite similar. A lower protein content (10.4 vs 13.2 mg/g of lipid) was characteristic of globules prepared by the multiple wash method. However, large differences could not be seen in gel electrophoresis patterns of the proteins. By using plastic centrifuge tubes, tube freezing and cleavage just below the globule layer enables clean separation of globule and nonglobule phases for analysis of milk component distributions. Macro (5 to 35 ml of sample) and micro (200 μl or less) versions of the method are described. Limited trials showed that the method can be applied satisfactorily to cow's and goat's milks, but for highly pure globules a deeper buffer column than that used with human milk is required because of their much higher casein content.
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In lactating mammary cells, triacylglycerols accumulate as droplets which at secretion range from 1 to 10 μm and average 3 to 4 μm in diameter. Secretion of these droplets from the cell into the alveolar lumen is accomplished by their envelopment in plasma membrane at the cell apex (1; for reviews, 2-4). The resulting milk fat globules are important as nutrients, as a record of synthetic and secretory activity of the lactating cell and as a remarkable source of plasma membrane components. In recent years certain proteins of the human milk fat globule membrane have gained special significance in breast cancer research. They are used to raise antibodies that are capable of detecting this malignancy (5-9). Further progress in understanding the milk fat globule is hampered by a number of complications. Microscopists have stated that the surface structure (membrane) of the globule is unstable and appears to undergo progressive rearrangement, with shedding of membranous vesicles following secretion (see ref. 4, p. 48 for review). Some species secrete milk fat globules with attached crescents of cytoplasm (10-14), notably the human (13,14) and goat (10,12), but not the cow (11). Because these crescents can represent a substantial volume of material as compared to globule membrane, they can influence analyses of interest, such as proteins, lipids and enzymatic activities. The problems of crescent contamination and postsecretion deterioration of globule structure may be resolvable provided that a satisfactory procedure for isolating globules from the other milk components can be devised.

The classical method with bovine globules involves washing them by repeated collections and redispersions with the aid of a cream separator (15). Because of the limited quantities involved, human milk fat globules usually are isolated by spinning them up in a centrifuge tube and then repeating several times a cycle of dispersing the cream layer in buffer and recentrifuging to recover the globules. These extensive and time-consuming manipulations hasten changes in globule membrane structure, including partial churning of the globules (butter granule formation) and some loss of their surface components. It has been shown that 85% of the xanthine oxidase and alkaline phosphatase activities are removed from bovine milk fat globules by four successive water washes (16). We present here a relatively simple and rapid way of isolating milk fat globules, based on centrifuging them out of the milk through an overlying buffer layer.

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MATERIALS AND METHODS

Human milk samples were obtained from area mothers as complete expressions of one breast, by hand or pump, following a lapse of at least four hr since the breast was last suckled. Bovine and caprine samples were aliquots of milkings from single animals in commercial herds. All samples were transported immediately to the laboratory, uncooled, for analysis.

Globule isolation. In the old procedure, a 40- or 50-ml plastic centrifuge tube was filled with fresh milk and centrifuged at 2000 × g and ambient temperature for 15 min. The resulting skim milk was decanted or siphoned from under the globule layer. This layer was resuspended in approximately 10 ml of appropriate medium (buffer, saline or water at 37 C). Gentle vortexing is satisfactory for this step. The tube contents then were made to original volume with medium and centrifuged as before. This washing process was repeated two more times and the final globule layer was dispersed in the desired kind and volume of medium.

In the macro version of our new procedure, sucrose was dissolved in the fresh milk sample at the rate of 5 g/100 ml, and 35 ml of the treated milk was delivered under 15 ml of buffer, saline or water, as appropriate, in a 50-ml plastic centrifuge tube (with screw cap) held in a support stand. This delivery was accomplished with a 50-ml bulb-type pipette connected to a rubber bulb flow controller. The loaded pipette was inserted through the buffer to the bottom of the tube and then slowly drained completely. Alternative methods were used successfully to deliver the milk; for example, see adaption to 15-ml tube, following. The drained pipette was removed, avoiding agitation so far as possible. The tube was capped and centrifuged in a swinging bucket-type rotor at 1500 × g for 20 min. The resulting globule layer was transferred with a spoon-shaped spatula (14 mm diam) to an appropriate container filled with dispersing medium.

Gentle hand mixing, in a vial or flask, for a minute or so readily accomplished resuspension of the globules. This version of the new procedure can be carried out with milk sample sizes down to 5 or 10 ml. A compensating increase in overlayer volume is made in order to fill the tube.

Depending on the analytical or research need, various media may be employed for the (washing) overlayer. Ordinarily we use phosphate-buffered salt solution (PBS), i.e., 0.14 M NaCl in 0.01 M phosphate buffer (pH 7.3). In constructing the two layers in the tube, it is imperative to avoid mixing. Air bubbles, which can cause turbulence, must be eliminated from the sample and avoided in creating the layers. In the case of the 50-ml pipette, this is simply a matter of allowing time for any air bubbles to rise out of the delivery stem. In the case of the long Pasteur pipette, once milk has entered, its level must be maintained above the capillary constriction. The long capillary is absolutely essential. It slows the flow of milk and prevents mixing. Releasing trace amounts of milk into the buffer layer either when inserting or removing the pipette causes no problem. The milk sinks out of the layer readily because of its greater density enhanced by the added sucrose. Other devices for producing the layers are available, but the method as described uses simple equipment common to most laboratories. Plastic tubes can be frozen and then sawed just below the globule layer to enable independent analyses of globule and nonglobule phases of milk (see also micro version of the procedure). The centrifugation selected, 1500 × g for 20 min, is very nearly quantitative for lipids, as shown subsequently, and yields a globule layer which is sufficiently compacted for efficient removal with a spatula, yet not so firm that it will not disperse readily. Under these conditions the globule layer has a slight clot-like quality. However, it also can be picked up with a syringe.

It is important to fill tubes close to the top so that a spatula can be inserted easily under the globule layer. Because of the wider mouth, globule layers are more easily manipulated with a spatula from a 50-ml than from a 15-ml tube. The globule layer first should be released from the tube wall by inserting a thin spatula. Then the tube should be inclined slightly to expose the underlying medium and a spatula inserted under the layer at that point. We ordinarily obtain 80–90% yields of globules (as lipid) based on total milk lipids. Removing substantial quantities of medium (buffer) layer to improve yield is not recommended if obtaining highly pure globules is important.

In the micro version of our new procedure, buffer (250 μl) is placed in the bottom of a 0.4 ml plastic centrifuge tube with attached cap (West Coast Scientific Inc., Berkeley, California, Cat. No. 2070). Sucrose-treated milk (100 μl) is loaded in a microliter syringe, the needle is wiped clean, and the syringe contents are slowly delivered under the buffer at the bottom of the tube. After withdrawing the syringe, the tube is capped, put into a suitable adapter and centrifuged (swinging bucket) as for the macro procedure. Following the spin the tube is inspected for preservation of the buffer layer and formation of a globule layer. It then is placed upright in a freezing compartment. The frozen tube is sectioned on a <0 C surface with a single edge razor blade or scalpel just below the globule layer. The two segments can be submitted to analysis or further experimentation.

Globule analysis. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis of milk fat globules was carried out by established methodology precisely as applied previously at our laboratory (17). Human and bovine skim milks were used as a reference to evaluate the relative contamination of globule preparations as revealed by SDS-polyacrylamide gel electrophoresis. The major human milk proteins lactoferrin, lysozyme, α-lactalbumin (all from Sigma Chemical Co., St. Louis, Missouri) and β-casein were used to establish the positions of skim milk proteins on gels. β-Casein was isolated as casein micelles by centrifugation of human skim milk at 50,000 × g and 2 C for one hr.

Protein content of globule suspensions was determined by the procedure of Lowry et al. (18) using bovine serum albumin (Sigma) as standard. Before carrying out these analyses lipids were removed by Roese-Gottlieb solvent extraction (19) with addition of 1.5% NaCl to maximize yield of phospholipid (20). Protein then was determined on the aqueous phase. Following solvent removal from these extracts, total lipids were quantified gravimetrically at constant weight. The extracted lipids were used to isolate and analyze the globule total phospholipids as follows: The total lipids were dissolved in chloroform/methanol (19:1, v/v). A quantity of this solution equivalent to 1 to 5 μg of phosphorus was spotted at the origin of a silica gel thin layer chromatography plate (Merck, Darmstadt, West Germany) together with a solvent extract control. The plate was developed with hexane/ethyl ether/acetic acid (50:50:1, v/v/v). Along with control areas, sample phospholipids, which remain at the origin, were scraped from the plate into ignition tubes and then assayed as by Rouser et al. (21). Samples were analyzed in duplicate and corrected for the controls. Total cholesterol of globule suspensions was analyzed as for bovine milk by the colorimetric method of Bachman et al. (22).

RESULTS

Figure 1 shows a 15-ml centrifuge tube and contents resulting from our globule isolation procedure. Above the sucrose-treated milk phase the buffer column can be seen, and on top of it the compacted layer of fat globules produced by the centrifuging. The appearance is essentially the same with a 50-ml centrifuge tube when 35 ml of milk and 15 ml of buffer are used. The relative clarity of the buffer layer following centrifugation varies, and we presume that the proportion of small globules in the milk may influence this condition. However, we have checked the amount of lipid left in the skim milk phase following centrifugation of 19 random samples of human milk (using no buffer layer) and found it to average less than 4% of the total (23).

To detect contamination of isolated globules with nonglobule components of milk, we have used SDS-polyacrylamide gel electrophoresis because it is a sensitive means of detecting proteins of the skim milk phase. The principal skim milk proteins in mature human milk and their approximate concentration ranges are lactoferrin, 0.15 to 0.4%; β-casein, 0.15 to 0.3%; and α-lactalbumin, 0.15 to 0.4% (24–27). In cow's milk the principal skim milk proteins and their approximate concentrations are α- and β-caseins plus small amounts of related

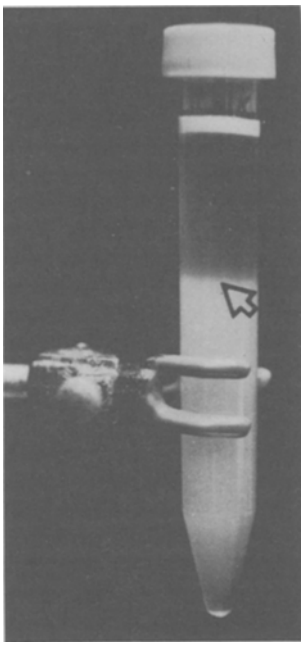

FIG. 1. Photograph of a tube containing sucrose-treated human milk which has been centrifuged (1500 × g for 20 min) to isolate the milk fat globules by passing them out of the milk through overlying buffer. Note interface (arrow) between milk and buffer with white layer of globules atop buffer.

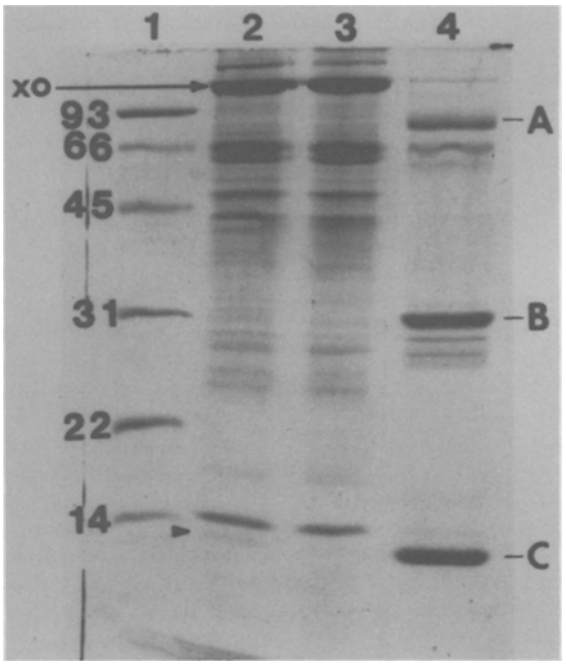

FIG. 2. SDS-polyacrylamide gel showing electrophoretic separation of proteins in human milk (lane 1), fat globules isolated from that milk (lane 2), bovine milk fat globules (lane 3), the milk from which those globules were isolated (lane 4) and reference proteins (lane 5) with Mr × 10^{-3} as indicated. The samples contained 20, 50, 50, 30 and 6 μg of protein respectively. Human globules were isolated by the single spin procedure using 10 ml of sucrose-treated milk under 5 ml of PBS in a 15-ml tube; bovine globules were obtained by the same procedure employing 30 ml of treated milk under 15 ml of PBS in a 50-ml tube. Bands corresponding to the principal human milk proteins are A, lactoferrin; B, β-casein; and C, α-lactalbumin. The gel was 12.5% acrylamide, 1.5 mm thick and stained with Coomassie blue.

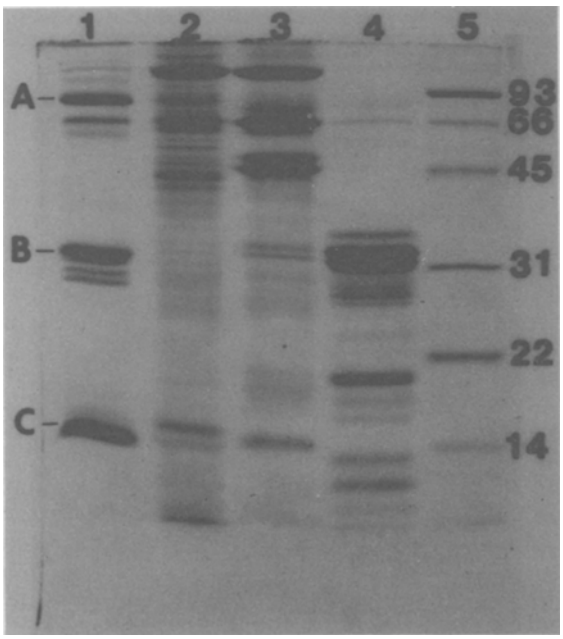

FIG. 3. SDS-polyacrylamide gel showing electrophoretic separation of proteins in human milk fat globules. Samples were reference proteins with Mr × 10^{-3} as indicated (lane 1), human milk fat globules isolated by the single spin procedure as in Fig. 2 (lane 2), human globules prepared by multiple washing and centrifuging (lane 3) and milk from which the globules (lanes 2 and 3) were prepared (lane 4). The samples contained 6, 30, 30 and 20 μg respectively. Putative xanthine oxidase band (XO arrow) and band corresponding to α-lactalbumin in globules (arrowhead) are indicated. Major milk proteins (A, B, C) and gel conditions are as in Fig. 2.

caseins (2.6%) and β-lactoglobulin (0.3%) (28). These proteins can be seen in the gel reproduced in Figure 2. The human milk proteins are in lane 1 (A, B and C) and those for the cow in lane 4 with caseins as a double band at Mr 31,000 and the β-lactoglobulin at Mr 20,000. Also shown on this gel are the protein patterns of human (lane 2) and bovine globules (lane 3) isolated by the single spin procedure from the corresponding milks resolved in lanes 1 and 4. It can be seen that the bovine globules are contaminated slightly with casein as shown by the two bands corresponding to the major doublet in bovine milk (Mr 31,000). Contamination of the cow globules with β-lactoglobulin is almost imperceptible. The human globule protein pattern (lane 2) shows no definite band corresponding to β-casein (B in Fig. 2) but weak bands can be seen in the pattern adjacent to lactoferrin (A) and α-lactalbumin (B), suggesting the possibility of minor contamination with those proteins.

α-Lactalbumin and galactosyltransferase constitute the enzyme complex required for lactose synthesis (29). It is known that galactosyl transferase occurs in the milk fat globule membrane (30). Thus α-lactalbumin may have a normal structural-functional association with milk fat globules. For this reason and as discussed following, we do not feel that the band in human globule isolates corresponding to α-lactalbumin is useful as a criterion of globule contamination.

In Figure 3, a representative gel is shown comparing protein patterns of globules isolated by the single spin

TABLE 1

Protein, Phospholipid and Cholesterol Contents of Milk Fat Globules Isolated from Human Milk Samples by Two Procedures[a]

	mg/g of Lipid	
	3× Washed	Single spin
Protein	10.4 ± 3.7	13.2 ± 2.8[b]
Phospholipid	5.1 ± 1.7	5.2 ± 0.8
Cholesterol	2.0 ± 1.5	2.1 ± 1.3

[a]For details of procedures, see text. Data are for seven samples (means ± S.D.).

[b]In a paired t-test, difference in protein by the two procedures was significant ($p < 0.01$); differences for phospholipid and cholesterol were not.

procedure (lane 2) with those obtained by multiple washing (lane 3). The two patterns are almost identical. The only consistent difference we have noted is that the band corresponding to α-lactalbumin (arrowhead) is invariably stronger in the patterns for single spin globules. This band is almost always faintly evident in protein patterns of multiply washed globules. In consequence of this tendency of human globules to bind what appears to be α-lactalbumin, the bands for lactoferrin and β-casein seem most useful in judging globule contamination.

The bands for reference proteins in Figure 3 each correspond to approximately 1 μg. If one such band were to appear in the sample patterns (lanes 2 and 3), it would be detected easily and would represent a sample contamination of 1 in 30 μg or about 3.3%. In our experience, none of the skim milk proteins approached this level of contamination in globules properly prepared by the single spin procedure using either the 15- or 50-ml tube. Protein patterns of globules isolated by the microversion of our method (not shown, but essentially the same as for globules in Fig. 2, lane 2) closely resembled those by the macro procedure. However, minor contaminating bands of lactoferrin, β-casein and α-lactalbumin were consistently present in patterns via the micro version. We estimated that, in the aggregate, these protein contaminants represented 10 to 20% of the total sample protein.

To enable further comparison, we analyzed globules isolated by the two methods from the same sample of human milk for total protein, phospholipid and cholesterol. Milk samples from seven donors at various stages of lactation were so analyzed. The results (Table 1) provide additional evidence that globules isolated by the two methods are quite similar. It is not unexpected that repeated washing tends to produce globules with somewhat lower protein content. Our gel electrophoresis data (for example, Fig. 3) demonstrate evidence that a portion of this loss is due, at least in part, to removal of α-lactalbumin. Removal of xanthine oxidase from multiply washed globules, as shown for bovine globules by Zittle et al. (16), may be a further contributor. However, the major bands, XO, presumed to be xanthine oxidase on the gel in Figure 3 and corresponding to the xanthine oxidase band in bovine globules (Fig. 2, lanes 2 and 3) appear equally intense. Of course, slight contamination of single spin globules with skim milk proteins occurs in some instances (see Discussion) and this would raise their protein content.

DISCUSSION

The new method described here for isolating fat globules from human milk accomplishes the task in less time and with fewer manipulations than the conventional multiple washing procedure. Using protein, phospholipid and cholesterol contents (Table 1) and protein patterns of globules by gel electrophoresis (Fig. 3), it is established that products by the two procedures are quite comparable, the principal variant being a somewhat greater recovery of protein by the new method. An interesting issue regarding this difference concerns the presence and stability of cytoplasmic crescents on human milk fat globules (see Introduction). Their quantity and stability to isolation procedures are not known. Secretory vesicles containing casein micelles are seen occasionally in such crescents (10). Thus, some human milk fat globules may have skim milk proteins (lactoferrin, β-casein, α-lactalbumin) as natural associates by cellular occlusions in distinction to contamination. Amounts of these proteins in globule populations isolated by either the multiple wash or single spin methods must be low.

It is reasonable to assume that the single spin method does less than multiple spinning and washing to alter structure of the milk fat globules. Not infrequently, minute butter granules are seen in globule preparations by the multiple wash method. These are evidence of globule surface disruption such that triacylglycerols from the core are exposed. In our experience, globules by the new method disperse easily and without churning. Bovine milk contains about 10 times as much casein as human milk (27,28), which makes it easier to contaminate bovine globules with casein (Fig. 2). While we have not explored this problem in depth, using 10 ml of buffer and 5 ml of sucrose-treated goat milk in place of 5 ml of buffer and 10 ml of treated milk in a 15-ml tube overcame the problem of casein contamination in the fat globules (data not shown). This change doubles the washing path length through the overlying buffer and halves the number of globules to be washed. The greater globule contamination problem we have encountered with the micro version of the method also suggests the importance of this path length. The 0.4-ml tubes used can provide a buffer layer depth of only 1 cm or so. However, we calculate that with a 10–20% contamination of globule protein with skim milk protein, this is ca. 0.5 to 1% of the protein in the skim milk phase. In analyzing partitioning of milk components between globules and skim milk, this would be within the error of most analyses. In any such analyses a longer centrifuging time also should help to remove casein micelles and to collect the very small globules into the globule layer.

Such factors as size of milk sample, species of donor, degree of globule purity required and the particular analytical or experimental need will dictate conditions of the single spin procedure for obtaining best results. These factors should be accommodated satisfactorily as the principle of the procedure is effective under varied conditions of its application. The clean separations of globule and skim milk phases which can be achieved should make

the tube freezing and cleaving aspect valuable. By this means one should be able to determine milk component distribution between the two phases with an accuracy heretofore difficult to obtain. A useful application of the micro version with tube cleavage would be in the modified Lowry procedure (31) for milk protein, in which it is essential to remove the fat globules in order to avoid turbidity in the assay.

A progress report on the development of this methodology has been published (32).

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Localization of Fatty Acid Double Bonds by Gas Chromatography of Intermediate Aldehydes as 1,3-Dioxane

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A semi-micro quantitative method has been developed for the determination of double bond positions in unsaturated fatty acid methyl esters differing in position, number of double bonds and chain length. This method involves ozonolysis in methylene chloride in the presence of 1,3-propanediol, reduction of the hydroperoxides with dimethyl sulfide and conversion of aldehyde and aldester fragments to stable dioxanes, followed by gas chromatographic analysis under programmed temperature. Propanediol acts as a proton donor for the generation of hydroperoxides as well as a reactant for the formation of dioxanes. All the steps are carried out in a culture tube without any transfers. The absence of unoxidized ester and unreduced hydroperoxide or ozonide has been shown by thin layer chromatography. The method has been tested with pure esters and their binary mixtures. Experimental values for mole percentage composition in the mixtures agreed with the actual values. This method enabled us to isolate and estimate malondialdehyde for the first time as a stable bis-dioxane derivative.
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Double bond positions in unsaturated fatty acids often are determined either by oxidation or mass spectrometry (1,2). For determination of positionally isomeric unsaturated acids in mixtures, oxidation is the method of choice (3,4). Periodate-permanganate and ozone are the most frequently used oxidizing agents (4). However, the β-dicarbonyl compounds which are of value in structural analysis are destroyed during the periodate oxidation (5). Since periodate-permanganate oxidation as well as oxidative ozonolysis lead to short chain acidic fragments which are difficult to recover and quantitate, reductive ozonolysis to aldehydic fragments often is used. The common reducing agents of ozonides obtained by ozonolysis in a nonparticipating solvent are triphenylphosphine (6) and Lindlar catalyst (7). Dimethylsulfide, a mild homogeneous reagent, has been found to quantitatively reduce hydroperoxides obtained by ozonolysis of olefins in methanol to aldehydes (8). This reagent has been used for the reduction of products obtained by ozonolysis in pentane and the subsequent addition of methanol as a means of determining the double bond position in monoenoic fatty acid methyl esters (9). The dimethylsulfide method has not been extended to polyunsaturated fatty acids. Other reductive ozonolysis procedures using triphenylphosphine or sodium borohydride do not give satisfactory yields of the C_3 fragment (10).

Most methods employing reductive ozonolysis followed by gas liquid chromatography (GLC) analyze the aldehyde fragments, which are volatile and unstable. Analysis of more stable and less volatile derivatives therefore is desirable. Dimethyl acetals are not suitable as they decompose during GLC (11). 1,3-Dioxanes have been prepared from aldehydes and shown to be superior to aldehydes and dimethyl acetals for quantitative analysis by GLC (12). In this communication a semi-micro quantitative method is reported for the determination of double bond positions in unsaturated fatty acid methyl esters differing in position and number of double bonds and chain length. This method is based on ozonolysis in methylene chloride in the presence of a protic solvent, followed by reduction of hydroperoxides with dimethyl sulfide, conversion of aldehyde and aldester fragments to 1,3-dioxanes and GLC. This method also provides for the isolation and determination of malondialdehyde as a stable bis-1,3-dioxane.

MATERIALS AND METHODS

Materials. Hexane (95%, Phillips, Bartlesville, Oklahoma) was washed with sulfuric acid, freed of acid, dried and distilled. Dimethyl sulfide (Superior grade; Matheson, Coleman and Bell, Norwood, Ohio) was dried by shaking with neutral alumina (grade I, Woelm, Eschwege, Germany) and the alumina was settled. Methylene chloride and methanol were GLC spectral grade. Other solvents and reagents were reagent grade.

The following fatty acids were purchased as methyl esters from Applied Science Laboratories (State College, Pennsylvania): 9-16:1; 18:0; 9-18:1; 11-18:1; 6-18:1; 9,12-18:2; 9,12,15-18:3; 5-20:1; 11-20:1; 11,14-20:2; 5,8,11,14-20:4; 13-22:1; 5,13-22:2 and 15-24:1. All the double bonds had the *cis* configuration.

Malondialdehyde-bis-1,3-dioxane was synthesized in the following manner. Ten ml of malondialdehyde-bis-dimethyl acetal (Aldrich, Milwaukee, Wisconsin), 30 ml of 1,3-propanediol and 1 g of p-toluene sulfonic acid (PTS) were dissolved in 3.8 l of thiophene-free benzene. The mixture was refluxed in a Dean-Stark apparatus for six hr and the benzene-water azeotrope was removed periodically. The benzene solution was concentrated to ca. 200 ml by distillation, neutralized with calcium carbonate, filtered and diluted with 500 ml of pentane (permanganate-purified, Mallinckrodt). This solution was washed six times with 100 ml portions of ethanol/water (1:6, v/v), dried over sodium sulfate and distilled. Malondialdehyde-bis-1,3-dioxane was dissolved in pentane and crystallized at −20 C. The crystals were dried in a vacuum desiccator containing phosphorus pentoxide and a paraffin block, m.p. 48–48.5 C. The product gave one peak of GLC and a single spot when silica gel H plates were developed either with xylene, petroleum ether (30–60 C)/diethyl ether (90:10, v/v) or petroleum ether/diethyl ether (50:50, v/v). The elemental percentage analysis (C 57.5, H 8.5, O 34.0) agreed with the calculated values (C 57.4, H 8.6, O 34.0).

Reductive ozonolysis and synthesis of 1,3-dioxanes. One ml of methylene chloride and 2 drops (ca. 0.04 ml) of 1,3-propanediol were placed in a Kimax culture tube (2 ×

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15 cm) with a Teflon-faced and rubber-lined screw cap, cooled to −70 C and saturated with ozone generated in a Welsbach laboratory ozonator. A methylene chloride solution (0.2 ml) containing 1-2 mg of the methyl ester of an unsaturated fatty acid and 1 mg of methyl stearate was cooled to 2 C and pipetted into the blue ozone solution. After 1 min, 1 ml of dried dimethyl sulfide, cooled to −70 C, was added along the walls of the tube to reduce hydroperoxides and excess ozone. A small capillary (ca. 6 cm) was partially filled to a length of ca. 7 mm with ca. 4 mg of PTS and dropped into the tube so that the empty end fell into the solution. The tube was kept capped tightly except during the addition of reagents. The tube was warmed to room temperature and allowed to stand for two hr with occasional gentle mixing. The tube was tilted to dissolve the PTS, heated slowly to 75 C in one hr to avoid polymerization of aldehydes and maintained at this temperature for three hr. The contents were cooled to room temperature and neutralized with solid sodium bicarbonate. The solution was evaporated with a stream of nitrogen at room temperature. One ml of hexane was added and the mixture shaken. The hexane supernatant was analyzed by GLC.

Thin layer chromatography (TLC). TLC was carried out on 0.25-mm layers of Silica gel H. The products of ozonolysis and subsequent reduction with dimethyl sulfide were separated using mixtures of hexane/diethyl ether (90:10 and 70:30, v/v). Fuchsin sulfurous acid (13), which gave magenta spots with aldehydes and aldesters, iodine vapor and 50% sulfuric acid were used for locating the separated components. Dioxanes of aldehydes and aldesters were separated using hexane/diethyl ether (30:70, v/v). The separated bands were scraped off, extracted with chloroform and analyzed by GLC.

Gas liquid chromatography. A Varian Aerograph 1200 was used. The temperature of the column was programmed from 100-180 C (6 C/min). The stationary phase was 15% EGSS-X on Gas Chrom P (100-120 mesh) in a 10′ × 1/8″ column. The injection chamber was maintained at 210 C and the flame ionization detector at 270 C. The flow rates for helium, air and hydrogen were 30, 200 and 30 ml/min. Peak areas were obtained by multiplying peak height with peak width at half the height of the peak. Peak areas were divided by the respective molecular weights of the compounds to get mole fractions. Peaks were identified from relative retention times with respect to methyl stearate. Recoveries of 1,3-dioxane derivatives of aldehydes (ADO) and 1,3-dioxane derivatives of aldesters (AEDO) were calculated with an internal standard generating appropriate ADO and AEDO derivatives. The recovery of the bis-1,3-dioxane derivative of malondialdehyde (bis-ADO) was calculated with methyl stearate as an internal standard after correcting the peak area of the bis-ADO for the flame ionization response (55.5%) due to ionizable carbons (14).

RESULTS AND DISCUSSION

Determination of double bond positions in unsaturated fatty acid esters by reductive ozonolysis followed by GLC suffers from high volatility and instability of aldehyde fragments. It would be better to analyze aldehydes as stable derivatives such as dioxanes if they could be obtained without the isolation of aldehydes. This objective has been achieved by carrying out ozonolysis in low boiling methylene chloride in the presence of 1,3-propanediol which acts as a proton donor for the generation of hydroperoxides as well as a reactant for the formation of 1,3-dioxanes of aldehydes. The reagent used for reducing hydroperoxides was dimethylsulfide, which is low boiling and easily removed as only a small amount of the dimethylsulfide is converted to the high boiling dimethyl sulfoxide. That ozonolysis and reduction were complete was demonstrated with all methyl esters (see Materials) by the absence of unoxidized ester (R_f: 0.88, 0.95) and the exclusive presence of aldehydes (R_f: 0.64, 0.84) and aldesters (R_f: 0.14, 0.34) in the products as shown by TLC using hexane/diethyl ether (90:10 or 70:30, v/v) and fuchsin sulfurous acid to detect aldehydes and iodine vapor or 50% sulfuric acid to detect other compounds. 1,3-Propanediol and dimethyl sulfoxide remained at the origin. That conversion of aldehydes and aldesters to 1,3-dioxanes was complete was demonstrated by the exclusive presence of dioxanes and the absence of aldehydes and aldesters on thin layer chromatograms. GLC confirmed the absence of unoxidized methyl esters as well as unconverted aldehydes and aldesters. The R_f values of the dioxanes obtained from fatty acid esters 9-18:1, 9-12-18:2, 9-12,15-18:3, 5-20:1 and 5,13-22:2 are given in Table 1. There was a slight increase in R_f value with increase in chain length of dialdehyde and aldester dioxanes.

Pure dioxanes were isolated from ozonolysis products of pure esters by preparative TLC on 0.5-mm layers of silica gel H and were analyzed by GLC. The bis-dioxane of malondialdehyde, prepared from the bis-dimethyl acetal of malondialdehyde, also was used as a standard for GLC. The retention times, relative to methyl stearate, of various dioxanes are given in Table 2. The dioxanes of the C_{12} aldehyde and the C_5 aldester, and of the C_6 aldester and malondialdehyde, were not separated by GLC. Also, the dioxane of the C_{15} aldehyde had the same retention time as methyl stearate. As these derivative mixtures are unlikely to arise from natural fatty acids, the utility of the method will not be affected.

The dioxane method was tested for its applicability in quantitative determinations. A number of monounsaturated fatty acid esters including those made up of positionally isomeric acids were mixed with 18:1 as an internal standard and subjected to the procedure. The

TABLE 1

R_f Values of 1,3-Dioxanes of Aldehydes and Aldesters[a]

Aldehyde dioxanes		Dialdehyde bis-dioxanes		Aldester dioxanes	
Carbon number	R_f	Carbon number	R_f	Carbon number	R_f
3	0.97 (2)[b]	3	0.55 (10)	5	0.63 (2)
6	0.97 (7)	8	0.63 (2)	9	0.79 (8)
9	0.95 (9)				

[a]Silica gel H, 0.25 mm; hexane/dimethyl ether (30:70, v/v); development, 40 min; indicator, 50% sulfuric acid.

[b]Mean value is given; number of determinations is in parentheses.

TABLE 2

Relative Retention Times (RRT) in GLC[a] of 1,3-Dioxanes

Aldehyde dioxane		Aldester dioxane		Dialdehyde bis-dioxane	
Carbon number	RRT[b]	Carbon number	RRT	Carbon number	RRT
6	0.22 (11)[c]	5	0.68 (13)	3	0.77 (19)
7	0.28 (6)	6	0.77 (3)	8	1.95 (2)
9	0.43 (10)	9	1.22 (11)		
12	0.67 (3)	11	1.82 (10)		
15	1.00 (4)	13	2.83 (4)		
		15	4.64 (4)		

[a]15% EGSS-X on Gas Chrom P (100–120 mesh); 10′ × 1/8″ column; 100–180 C (6 C/min); helium flow rate, 30 ml/min.

[b]Relative to 18:0. Retention time for 18:0, 19 min and 10 sec.

[c]Mean value is given. Variability in RRT data did not exceed ± 0.01. Number of determinations is given in parentheses.

TABLE 3

Recovery of 1,3-Dioxane Derivatives of Aldehydes (ADO), Aldesters (AEDO) and Malondialdehyde (bis-ADO) Obtained by Reductive Ozonolysis of Unsaturated Fatty Acid Methyl Esters

	Recovery in %		
Ester	ADO	AEDO	bis-ADO
9-16:1	102.5[a]		
6-18:1	112.3	89.1[b]	
6-18:1	110.4	85.8	
11-18:1	96.1	97.3	
11-18:1	88.1	104.7	
5-20:1	110.5	110.6	
11-20:1		110.5	
9,12-18:2			88.8[c]
9,12,15-18:3			91.5
11,14-20:2			94.4
5,8,11,14-20:4			91.2
Mean ± SEM	103 ± 3.9	100 ± 4.6	91.5 ± 1.1

[a]Recovery is calculated using the 1.3-dioxane of the C_9 aldehyde synthesized from 9-18:1 as an internal standard. Each value is the average of two or more determinations.

[b]Recovery is calculated using the 1,3-dioxane of the C_9 aldester synthesized from 9-18:1 as an internal standard. Each value is the average of two or more determinations.

[c]Recovery is calculated using methyl stearate as an internal standard and the theoretical yield of malondialdehyde (1, 2 and 3 for diene, triene and tetraene esters). The peak area of the bis-1,3-dioxane is corrected for the flame ionization detector response (55.5%) due to ionizable carbons. Each value is the average of two or more determinations.

results are given in Table 3. Recoveries calculated either on the basis of ADO or AEDO agreed with the actual fatty acid ester content of the mixture (Table 3).

The determination of malondialdehyde arising from methylene-interrupted unsaturated acids was examined by using polyunsaturated acids yielding 1, 2 or 3 mol malondialdehyde per mol fatty acid ester. Methyl stearate was used as an internal standard and the peak area of the bis-ADO was corrected for ionizable carbons (14). The recovery of the bis-ADO was 101.2 ± 0.7 before and 91.5 ± 1.1 after extraction with hexane (Table 3). Hexane extraction is preferred as the C_9 aldehyde dioxane is masked by propanediol. Furthermore, propanediol and dimethyl sulfoxide give large peaks and skew the methyl stearate peak. Neither propanediol nor dimethyl sulfoxide is extracted in hexane.

The dioxane method has been applied successfully to estimate the content of petroselinic acid in the presence of oleic acid in maturing coriander seeds (15). The salient feature of the method is that stable derivatives of aldehydic fragments are prepared in a single culture tube without resorting to any isolation or transfer. The method has potential for analysis of a wide variety of mixtures of unsaturated fatty acid esters. The method is particularly useful for the estimation of malondialdehyde as a stable bis-dioxane. It may be possible to use the dioxane derivatives of various aldehydic products including malondialdehyde in studies on the decomposition of lipid peroxides in biological systems.

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Two Geometrical Isomers of Linoleic Acid: Improved Total Syntheses

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The total syntheses of 9(Z),12(E)- and 9(E),12(Z)-octadecadienoic acids have been carried out. A useful intermediate in both syntheses, 8-bromo-octanoic acid, recently has become available from commercial sources. This compound has been used to expedite the preparation of these isomers. The remaining carbon atoms were derived from propargyl alcohol along with either 1-heptyne or acetylene and 1-bromopentane. Because the overall yield for each sequence was roughly 15% and there were no extraordinary reaction conditions in any of the synthetic steps, the compounds could be prepared readily in multiple gram quantities. The syntheses of the two compounds were supported by data from a variety of spectroscopic techniques.
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Interest has been raised in recent years over the nutritional influence of the geometrical isomers of naturally occurring unsaturated fatty acids in the human diet. This has occurred to a large extent over concern about the increased dietary intake of highly processed foods. The isomerization of the natural *cis* olefinic isomer in polyunsaturated fatty acids, for example, is known to occur in the hydrogenation step commonly used in the processing of seed oils (1). While the nutritional impact of these so-called unnatural fatty acids has been considered in a number of experimental studies (2,3), the importance of identifying specific effects of individual compounds in such research has been emphasized recently (4). Experiments of this kind can be hampered by the lack of availability of sufficient quantities of the isomeric materials. The use of partially hydrogenated fats has inherent problems in this regard because the complex mixture of compounds present makes the interpretation of observed effects difficult. For studies on the effects of geometrical isomerism in individual monoene fatty acids, the availability of the isomeric materials generally is good. This is not the case, however, for polyunsaturated fatty acids as exemplified by linoleic acid. Because the availability of the two isomers (9[E],12[Z]- and 9[Z],12[E]-octadecadienoic acids) is not good, a more easily obtained mixture of the two compounds or the commercially available all-*trans* linoelaidic acid commonly have been used (5,6).

Two published approaches to the synthesis of the geometrical isomers of linoleic acid currently are available. A total synthesis was reported in 1962 by DeGaudmaris and Agnaud (7). This paper provided a general outline for the preparation of the appropriate compounds but was lacking in yield data and experimental detail at critical stages of the procedure. A complete description of the synthesis of the geometrical isomers of linoleic acid was reported by Gunstone and Jacobsberg in 1972 (8). This approach, however, started with the relatively rare natural starting materials of crepenynic acid and vernolic acid. Porter and Wujek recently have updated the Gunstone approach to the syntheses by using linoleic acid as the starting material (9). In that study, the methyl ester of the fatty acid was monoepoxidized and hydrolyzed to a pair of *vic*-diols that required high performance liquid chromatography (HPLC) separation before conversion to dienes. The necessity of this chromatographic step makes this approach effective for the preparation of small amounts of the desired compounds, but tedious for the acquisition of the larger quantities required for biochemical or nutritional studies (Porter, N.A., personal communication). Faced with these available options and a requirement for gram quantities of the isomeric materials, we chose to modernize the total syntheses of the geometrical isomers of linoleic acid. By taking advantage of the availability of 8-bromooctanoic acid as a starting material, the efficiency of the syntheses has been substantially increased.

MATERIALS AND METHODS

8-Bromooctanoic acid, 1-bromopentane, propargyl alcohol, triphenylphosphine, carbon tetrabromide, lithium acetylide-ethylenediamine complex, lithium amide, 1-heptyne and palladium on barium sulfate were obtained from Aldrich (Milwaukee, Wisconsin). Diethyl ether and tetrahydrofuran (THF) were distilled from sodium/benzophenone. Methylene chloride was distilled from barium oxide and stored under a nitrogen atmosphere. Copper(I) cyanide from MCB (Norwood, Ohio) was used without further purification. Chromatography was carried out by the method described by Still et al. (10). Large quantities were purified as 10-g batches using a 75-mm i.d. column.

11-Hydroxy-9-undecynoic acid. This material was prepared by the method of Ames et al. (11): 75.8 g (85.3%); mp 57.0 C (white plates from ethyl acetate/pentane), lit. (11) mp 56.5–57.0 C. ^{1}H NMR (90 MHz, $CDCl_3$): 1.11–1.77 (m, 10 H), 2.09–2.41 (m, 4 H), 4.21 (t, 2 H, 2.2 Hz), 7.93 (br s, 2 H); ^{13}C NMR (22.5 MHz, $CDCl_3$): 179.11, 86.36, 78.47, 51.26, 33.92, 28.81, 28.46, 24.56, 18.66; IR (KBr) 3500–2400, 2920, 2850, 1695, 1010 cm^{-1}.

11-Hydroxy-9(E)-undecenoic acid. The alkynoic acid was *trans*-hydrogenated by the method of Dear and Pattison (12). To a cooled solution of 11-hydroxy-9-undecynoic acid (1.00 g, 5.05 mmol) in anhydrous THF (15 ml) was added liquid ammonia (ca. 20 ml). Lithium metal (0.80 g, 115 mmol) was added portionwise to the above prepared solution under vigorous stirring. The solution was stirred and refluxed for three hr in a pressure bottle. The ammonia was evaporated and the residue was decomposed with water. The mixture was adjusted to pH 3 with 6 N HCl and extracted with diethyl ether (5×, 50 ml). The extracts were dried ($MgSO_4$) and evaporated. The product was purified by chromatography on silica using hexane/ethyl acetate/acetic acid (89.9:10:0.1, v/v/v): 0.83 g (82.2%), white solid; mp 40–41 C; ^{1}H NMR (90 MHz, $CDCl_3$): 1.22 (m, 10 H), 1.98 (m, 2 H) 2.23 (t, 2 H, 7.0 Hz), 3.98 (d, 2 H, 4.2 Hz), 5.55 (m, 2 H), 7.23 (br s, 2 H); ^{13}C NMR (22.5 MHz, $CDCl_3$): 178.85, 133.17, 128.40, 63.22,

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33.92, 31.93, 28.81, 24.56; IR (KBr) 3600–2500, 2920, 2850, 1690, 1090, 1000, 970 cm^{-1}.

11-Bromo-9(E)-undecenoic acid. The alcohol was converted to the bromide by the method of Carvalho and Prestwich (13). To a stirred solution of 11-hydroxy-9(E)-undecenoic acid (1.22 g, 6.10 mmol) and carbon tetrabromide (2.52 g, 7.59 mmol) in methylene chloride (50 ml), solid triphenyl phosphine (2.40 g, 9.16 mmol) was added gradually. After stirring for a further four hr, the solvent was evaporated in vacuo. A mixture of ethyl acetate (50 ml) and hexane (50 ml) was added to the residue. The solution was isolated by filtration. The combined filtrate was evaporated and the residue was purified by chromatography on silica using hexane/ethyl acetate (90:10, v/v): 1.09 g (68.1%), colorless oil; ^1H NMR (90 MHz, $CDCl_3$): 1.23 (m, 10 H), 1.97 (m, 2 H), 2.25 (t, 2 H, 7.1 Hz), 3.85 (m, 2 H), 5.62 (m, 2 H), 8.11 (br s, 1 H); ^{13}C NMR (22.5 MHz, $CDCl_3$): 180.06, 136.20, 126.23, 33.83, 33.23, 31.75, 28.72, 28.46, 24.39; IR (KBr) 3300–2500, 3020, 2920, 2850, 1700, 1460–1400, 1280, 1205, 955 cm^{-1}.

9(E)-Octadecen-12-ynoic acid. A solution of 1-heptyne (3.50 g, 36.5 mmol) in THF (20 ml) was added by drops to a 2.0 M THF solution (18.3 ml) of ethylmagnesium bromide (36.6 mmol) under a nitrogen atmosphere. The mixture was warmed to reflux for two hr, then cooled to room temperature. To the solution was added solid copper(I) cyanide (300 mg, 3.35 mmol). After 30 min, a solution of 11-bromo-9(E)-undecenoic acid (4.00 g, 15.2 mmol) in THF (25 ml) was added by drops. The resulting suspension was stirred under reflux for 16 hr. The reaction mixture was cooled to room temperature, decomposed with water, acidified to pH 3 with 6 N HCl, saturated with NaCl and extracted with ether (5×, 50 ml). The ether extracts were extracted with saturated aqueous ammonium chloride solution (3×, 50 ml). The ether layer was dried ($MgSO_4$) and concentrated in vacuo. The product was obtained as a colorless oil (2.75 g, 65.0%) following chromatography on silica using hexane/ethyl acetate/acetic acid (89.9:10:0.1, v/v/v); ^1H NMR (90 MHz, $CDCl_3$): 0.85 (t, 3 H), 1.17–1.69 (m, 16 H), 2.03 (m, 4 H), 2.30 (t, 2 H, 7.3 Hz), 2.80 (m, 2 H), 5.14–5.83 (m, 2 H), 9.56 (br s 1 H); ^{13}C NMR (22.5 MHz, $CDCl_3$): 180.2, 131.6, 124.8, 82.03, 77.52, 34.01, 32.19, 31.06, 28.98, 28.72, 24.65, 22.22, 21.96, 18.75, 13.99; IR (neat) 3300–2500, 3030, 2930, 2860, 1700, 1465–1400, 1320–1190, 970 cm^{-1}; CI MS (methyl ester, CH_4) M + 1, 293.

9(E),12(Z)-Octadecadienoic acid. A solution of 9(E)-octadecen-12-ynoic acid (5.0 g, 18.0 mmol) in pyridine (50 ml) was treated with palladium on barium sulfate (0.5 g, 5% palladium) and hydrogenated at atmospheric pressure for six hr at room temperature. The reaction mixture was poured into deionized water (500 ml). This mixture was acidified to pH 3 and extracted with ether (3×, 400 ml). The extracts were washed with water, dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by chromatography on silica using hexane/acetone/acetic acid (92.25:7.5:0.25, v/v/v). The product was obtained as a colorless oil (3.52 g, 69.6%). ^1H NMR (90 MHz, $CDCl_3$): 0.86 (br t, 3 H), 1.26 (m, 14 H), 1.60 (m, 2 H), 1.94 (m, 4 H), 2.31 (t, 2 H), 2.66 (m, 2 H), 5.29 (m, 4 H), 9.17 (br s, 1 H); ^{13}C NMR (22.5 MHz, $CDCl_3$): 180.29, 130.71, 130.54, 128.46, 127.68, 34.06, 32.50, 31.55, 30.42, 29.47, 29.04, 27.13, 24.70, 22.62, 14.04; IR (neat) 3400–2500, 3020, 2925, 2850, 1710, 1470–1405, 1320–1210, 970 cm^{-1}; CI MS (methyl ester, CH_4) M + 1, 295.

2(E)-Octen-1-ol. To a mechanically stirred suspension of lithium amide (8.60 g, 375 mmol) in liquid ammonia (250 ml), propargyl alcohol (10.80 g, 193 mmol) was added gradually and the mixture was stirred for 30 min under reflux. 1-Bromopentane (29.00 g, 193 mmol) was added by drops and the reaction mixture was stirred for three hr. Anhydrous THF (150 ml) was added to the reaction mixture. Lithium then was added to the reaction mixture in small pieces until the blue color persisted (4.20 g). The reaction mixture was stirred for a further 4.5 hr. The ammonia was evaporated and the residue was decomposed with water and acidified with 6 N HCl to pH 3. The mixture was saturated with NaCl and extracted with ether (5×, 500 ml). The extracts were dried ($MgSO_4$) and evaporated. The product was obtained as a colorless oil (12.95 g, 50.0%) from distillation at 53–55 C (0.6 mm Hg), lit. (14) 96–97 C (18 mm Hg): ^1H NMR (90 MHz, $CDCl_3$): 0.82 (br t, 3 H, 6.3 Hz), 1.22 (m, 6 H), 1.93 (m, 2 H), 3.13 (br s, 1 H) 4.00 (d, 2 H, 4.2 Hz), 5.58 (m, 2 H); ^{13}C NMR (22.5 MHz, $CDCl_3$): 133.25, 128.83, 63.48, 32.10, 31.32, 28.81, 22.48, 13.90; IR (neat) 3300, 2960, 2935, 2865, 1672, 965 cm^{-1}.

1-Bromo-2(E)-octene. A stirred solution of 2(E)-octen-1-ol (5.00 g, 39.1 mmol) and carbon tetrabromide (16.2 g, 48.9 mmol) in methylene chloride (125 ml) was treated gradually at 0 C with solid triphenyl phosphine (15.3 g, 58.4 mmol). The mixture was stirred at 0 C for one hr, then warmed to room temperature and stirred for another hour. The solvent was evaporated in vacuo. Pentane (300 ml) was added to the residue and the solution was isolated by filtration on silica gel. The product was obtained as a colorless oil (5.13 g, 68.8%) by distillation at 54 C (2.2 mm Hg), lit. (15) 58–59 C (1.5 mm Hg): ^1H NMR (90 MHz, $CDCl_3$): 0.85 (br t, 3 H 6.2 Hz), 1.25 (m, 6 H), 2.01 (m, 2 H), 3.90 (m, 2 H), 5.69 (m, 2 H); ^{13}C NMR (22.5 MHz, $CDCl_3$): 136.64, 126.23, 33.49, 32.01, 31.23, 28.46, 22.39, 13.99; IR (neat) 2960, 2920, 2865, 1665, 1468, 1442, 1205, 975 cm^{-1}.

9-Decynoic acid. This material was prepared by a slight modification of the procedure of DeJarlais and Emken (16). A solution of 8-bromooctanoic acid (100.0 g, 0.448 mol) in anhydrous DMSO (200 ml) was added by drops to a stirred suspension of lithium acetylide ethylene diamine complex (100.0 g, 1.09 mol) in anhydrous DMSO at 20 C. The mixture was stirred for three hr and combined with ice cold aqueous HCl (6 N, 600 ml, to pH 3). The solution was extracted with ether (6×, 130 ml). The combined extracts were washed with water, dried ($MgSO_4$) and concentrated in vacuo. The product was obtained as a pale yellow oil (58.0 g, 77.0%) by distillation (without cooling water in the condensor) at 129 C (1.5 mm Hg), lit. (17): 88 C (0.1 mm Hg): ^1H NMR (90 MHz, $CDCl_3$): 1.31 (m, 10 H), 1.86 (t, 1 H, 2.5 Hz), 2.09 (m, 2 H), 2.27 (t, 2 H, 7.0 Hz), 9.21 (br s, 1 H); ^{13}C NMR (22.5 MHz, $CDCl_3$): 180.32, 84.28, 68.07, 33.92, 28.72, 28.55, 28.20, 24.39, 18.15; IR (neat) 3400–2500, 3290, 2930, 2860, 2120, 1705, 1460–1410, 1290–1220, 935 cm^{-1}.

12(E)-Octadecen-9-ynoic acid. A solution of 9-decynoic acid (16.90 g, 100.6 mmol) in THF (100 ml) was added by drops to the 2.0 M THF solution (100 ml) of ethylmagnesium bromide (200 mmol) under nitrogen atmosphere. The mixture was warmed to reflux for one hr, then cooled to room temperature. Solid copper(I) cyanide (250 mg,

2.8 mmol) was added to the solution. After 15 min a solution of 1-bromo-2(E)-octene (9.60 g, 50.3 mmol) in THF (100 ml) was added by drops. The resulting suspension was stirred at room temperature for four hr and refluxed for 20 min. The reaction mixture was decomposed with ice water, acidified to pH 3 with 6 N HCl, saturated with NaCl and extracted with ether (5×, 120 ml). The combined extracts were extracted with saturated aqueous ammonium chloride solution (3×, 500 ml). The ether layer was dried ($MgSO_4$) and purified by chromatography on silica using pentane/ethyl acetate/acetic acid (89.9:10:0.1, v/v/v). The product was obtained as a pale yellow oil (which solidifies in the refrigerator) (7.81 g, 55.9%). ^1H NMR (90 MHz, $CDCl_3$): 0.85 (br t, 3 H, 7.0 Hz), 1.31 (m, 14 H) 2.06 (m, 4 H), 2.31 (t, 2 H, 7.3 Hz), 2.82 (m, 2 H), 5.49 (m, 2 H), 9.10 (br s, 1 H); ^{13}C NMR (22.5 MHz, $CDCl_3$): 179.37. 131.78, 124.85, 81.77, 77.87, 33.92, 32.19, 31.41, 28.98, 28.63, 24.65, 22.48, 21.96, 18.75, 13.91; IR (neat) 3300-2500, 3020, 2940, 2860, 2360, 1710, 1470-1400, 1330-1190, 970 cm^{-1}. CI MS (methyl ester, CH_4) M + 1, 293.

9(Z),12(E)-Octadecadienoic acid. A sample of 12(E)-octadecen-9-ynoic acid (5.0 g, 18.0 mmol) was hydrogenated in pyridine using palladium on barium sulfate, *vide supra.* The product was obtained as a colorless oil (3.25 g, 64.5%). ^1H NMR (90 MHz, $CDCl_3$): 0.87 (br t, 3 H), 1.29 (m, 14 H), 1.61 (m, 2 H), 1.96 (m, 4 H), 2.31 (t, 2 H) 2.67 (m, 2 H), 5.29 (m, 4 H), 9.18 (br s, 1 H); ^{13}C NMR (22.5 MHz, $CDCl_3$): 179.95, 130.89, 130.36, 128.29, 127.85, 34.06, 32.59, 31.46, 30.51, 29.64, 29.27, 29.04, 27.13, 24.70, 22.54, 14.04; IR (neat) 3400-2500, 3010, 2910, 2830, 1705, 1470-1400, 1320-1190, 1095, 970 cm^{-1}. CI MS (methyl ester, CH_4) M + 1, 295.

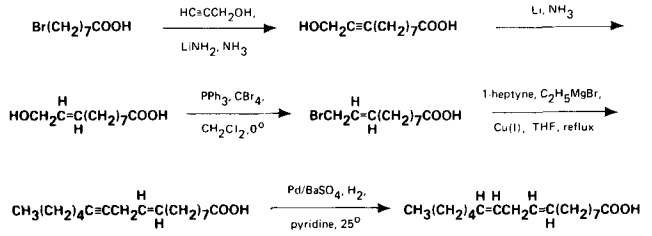

FIG. 1. Synthetic outline for 9(E),12(Z)-octadecadienoic acid.

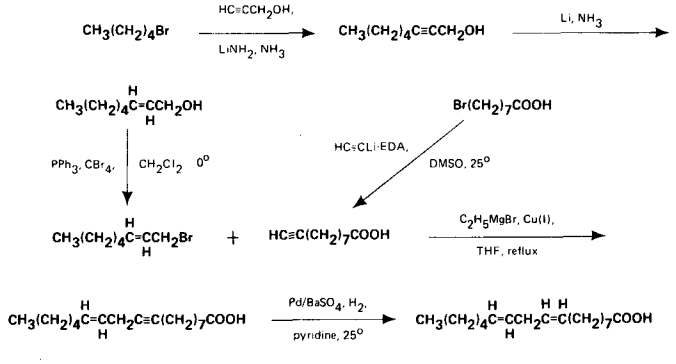

FIG. 2. Synthetic outline for 9(Z),12(E)-octadecadienoic acid.

RESULTS AND DISCUSSION

We have found that 8-bromooctanoic acid is a useful starting material in the syntheses of 9,12-octadecadienoic acids. The use of this compound circumvents many of the time-consuming steps in the previous total syntheses in which carbons 1-8 were derived from 1,6-hexanediol and malonic acid. The structures of the two polyunsaturated fatty acids were elaborated in one of two pathways (Figs. 1 and 2) in which the position of the *trans* unsaturation was controlled.

9(E),12(Z)-Octadecadienoic acid. The insipient Δ^9 unsaturation was incorporated into the molecule by condensation of propargyl alcohol with 8-bromooctanoic acid using lithium amide as the base. The resulting alkynol was then *trans*-hydrogenated and converted into the corresponding bromide. This compound was condensed with 1-heptyne using ethylmagnesium bromide as the base and copper(I) cyanide as the catalyst. Catalytic hydrogenation afforded the final product.

9(Z),12(E)-Octadecadienoic acid. In this case, 9-bromooctanoic acid first was condensed with acetylene using the lithium acetylide-ethylene diamine complex. This alkyne was condensed with 1-bromo-2(E)-octene using ethylmagnesium bromide and copper(I) cyanide. The bromoalkene was obtained from 1-bromopentane and propargyl alcohol by condensation, *trans* hydrogenation and bromination. The final product was prepared from the en-ynoic acid by catalytic hydrogenation.

The two isomers were obtained in roughly 15% isolated yield starting from 8-bromooctanoic acid. Multiple gram quantities of these compounds could be prepared readily in this way.

The two isomers are not distinguished easily on the basis of their spectroscopic properties. Small differences in the ^{13}C NMR resonances for the olefinic carbons in the two compounds have been reported (18). These differences are illustrated in Figure 3 for the compounds produced

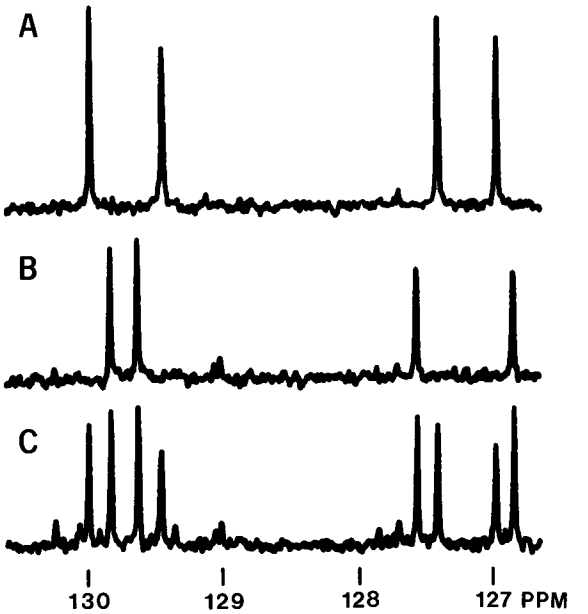

FIG. 3. ^{13}C NMR spectroscopy (22.5 MHz) for the olefinic region of the two synthetic isomers of linoleic acid. A, 9(Z),12(E)-octadecadienoic acid; B, 9(E),12(Z)-octadecadienoic acid; C, both.

by our new synthetic methods. This confirmed that these two isomers can be distinguished if the olefinic region of the ^{13}C NMR spectrum is scrutinized carefully. It is hoped that the publication of straightforward syntheses of the two *cis,trans* geometrical isomers of linoleic acid will stimulate further useful experiments in the evaluation of the biochemical and nutritional effects of these compounds.

ACKNOWLEDGMENTS

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ERRATA

In "Moderate Changes in Linoleate Intake do not Influence the Systemic Production of E Prostaglandins," by A. Ferretti, J.T. Judd, M.W. Marshall, V.P. Flanagan, J.M. Roman and E.J. Matusik Jr. in the May 1985 issue of *Lipids* an error was made in Table 3 of page 271. The PGE-M values are given in μg/g creatinine, not in ng/g creatinine as indicated in the heading (far right column) and in footnote c.

Comparative Study of the Molecular Species of Chloropropanediol Diesters and Triacylglycerols in Milk Fat

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In an effort to establish the origin of the fatty acid esters of 3-chloropropanediol, which recently have been isolated in small amounts from goat milk, we compared the molecular species composition of the chlorohydrin diesters and of goat milk triacylglycerols. The chloropropanediol diesters were found to be composed of molecular species containing C_{10}–C_{18} fatty acids and corresponded closely in carbon number to those calculated for the long chain sn-1,2-diacylglycerol moieties of goat milk triacylglycerols. The molecular species of goat milk total triacylglycerols contained C_4–C_{18} fatty acids. It is suggested that triacylglycerols and chloropropanediol diesters are derived from the same pool of long chain fatty acids. A molecular distillate of bovine milk fat did not contain chloropropanediol diesters, while the available samples of human milk fat were shown to contain alkyldiacylglycerols as the major components of a neutral lipid fraction corresponding in polarity to the chloropropanediol diesters.
Lipids 21, 183–190 (1986).

During a detailed investigation of the lipids of goat milk, Cerbulis et al. (1,2) observed a neutral lipid fraction that migrated on thin layer chromatography (TLC) plates slightly ahead of the major triacylglycerols. Isolation and preliminary characterization of the fraction showed that it contained 3-chloro-1, 2-propanediol diacyl esters as major components (3). Similar compounds have been demonstrated independently by Gardner et al. (4) in adulterated Spanish cooking oils. In both instances the small amounts of the chloropropanediol diesters were isolated by preparative TLC from a large excess of triacylglycerols. The chemical nature of these substances was demonstrated by mass spectrometric identification of the chloropropanediol (3) or the [M-RCO_2] fragment ions of chloropropanediol diesters (4) by high resolution mass spectrometry.

Since Davidek et al. (5) previously had shown that monoesters and diesters of chloropropanediol may be produced by hydrolysis of triacylglycerols with HCl at 110 C, Gardner et al. (4) suggested that the chloropropanediol esters found in the adulterated Spanish cooking oil may have also been generated by exposure to HCl. These workers speculate that HCl may have been used to remove the aniline contained as a denaturant in the rapeseed oils used to adulterate the cooking oils. The goat milk, however, was fresh and had not been exposed to HCl at any time. The identification of the chloropropanediol diesters in fresh goat milk, therefore, excluded exposure to HCl as an explanation of their origin. The results of the present study are consistent with an esterification of either chloropropanediol or its monoester in the mamary gland.

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MATERIALS AND METHODS

The triacylglycerol and chloropropanediol diester fractions of goat milk were prepared as described by Cerbulis et al. (3). The analyzed sample represents material (Fig. 1) pooled from many TLC plates. Similar methods of isolation were used to obtain the corresponding fractions of triacylglycerols and chloropropanediol diesters from fresh samples of milk from six Philadelphia, Pennsylvania, mothers and from an Ontario cow. A large sample of the most volatile 2.5% of a molecular distillate of butterfat prepared in 1960 (6) was available in the laboratory and was also worked

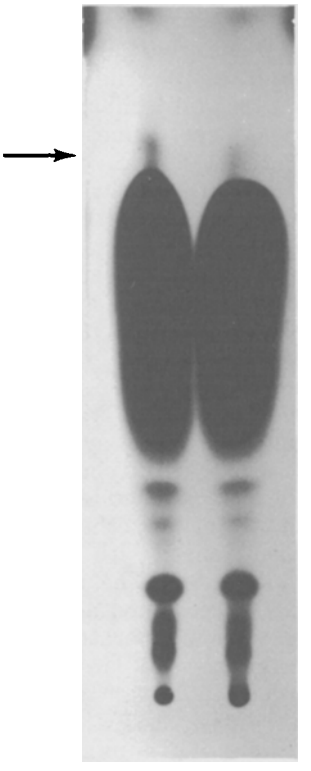

FIG. 1. Thin layer chromatogram of the neutral lipid fractions of goat milk. The plate was silica gel G and the solvent was freshly prepared petroleum ether/diethyl ether/acetic acid (90:10:1, v/v/v). The arrow indicates the position of the fraction studied in this paper. Each track represents ca. 0.50 mg of lipid applied to the plate. The plates were charred with sulfuric acid/acetic acid/$FeCl_3$.

up as described by Cerbulis et al. (3). Rac-1-chloro-2,3-dipalmitoyl-, rac-1-chloro-2,3-dioleoyl- and rac-1-chloro-2-palmitoyl-3-butyroylpropanediols were synthesized from rac-1-chloro-2,3-propanediol as described by Myher et al. (7). 1-Alkyl-2-palmitoyl 3-chloropropanediol was made from chimyl alcohol. 1-Alkyl-2,3-dipalmitoylglycerol was obtained by acylation of chimyl alcohol with palmitic anhydride in the presence of dimethylaminopyridine in benzene. The purified product then was digested with pancreatic lipase and the resulting 1-alkyl-2-palmitoyl glycerol was isolated by TLC. Conversion to the 3-chloro derivative was carried out by reacting this material with pyridine-phosphorus oxychloride (3:1) for one hr at 40 C.

Analysis of intact acylglycerols. Capillary gas liquid chromatography (GLC) of the acylglycerols was performed as previously described (8) on a Hewlett-Packard Model 5880A gas chromatograph (Hewlett-Packard Co., Palo Alto, California) equipped with an on-column injector and a fused silica capillary column (8 m × 0.32 mm I.D.) coated with SE-54 (Hewlett-Packard) using hydrogen as the carrier gas. The column temperature was programmed as indicated in the appropriate figures. Column bleed was minimal and was automatically subtracted via the single column compensation mode of the microprocessor terminal.

The high performance liquid chromatography (HPLC) analyses were performed with a Hewlett-Packard Model 1084B liquid chromatograph equipped with a Supelcosil LC-18 reversed phase column (Supelco, Bellefonte, Pennsylvania) using a gradient of 30-90% propionitrile in acetonitrile. The columns were operated at a flow rate of 1.5 ml/min and 30 C oven temperature. The mass spectrometry was done on a Hewlett-Packard Model 5985B quadrupole mass spectrometer equipped with a Hewlett-Packard direct liquid inlet interface and positive and negative chemical ionization detectors. The general layout of the liquid chromatography/mass spectroscopy (LC/MS) system and the methods of data analysis have been described previously (9,10). The mass spectrometer scans were taken every seven sec in the 200-900 mass range over the entire elution profile.

Analysis of alcohol moieties. Transmethylation of TLC purified natural lipids and of synthetic diesters of chloropropanediol was carried out as previously described (3); 50 μl of water was added and the mixture was extracted with petroleum ether. The remaining aqueous phase was neutralized with Dowex 1X8 in the hydroxyl form and decanted. The resin was washed with methanol, and the combined aqueous phase and methanol washings were evaporated. The resulting methanol/water mixture was analyzed by GLC; separation was by a modification of the method of Snyder and Franko-Filipasic (11). In this work a 1 m × 2 mm I.D. column of Tennax GC, previously conditioned under He at 300 C for 20 hr, was used on a Perkin Elmer Sigma 3B Chromatograph (Perkin Elmer Corp., Norwalk, Connecticut) with glass liner parts. The injector temperature was 240 C and the detector temperature was 300 C. Helium was the carrier gas at 45 ml/min, and the samples were eluted isothermally at 150 C. Detection was by flame ionization. Standard diols, triols and chloropropanediol were injected in a solvent made up of water/methanol (90:10, v/v). Under these conditions glycerol and chloropropanediol had retention times of 6.4 and 7.9 min, respectively.

The alkylglycerol moieties of alkyldiacylglycerols were determined by capillary GLC following acetylation of the transmethylation products, as previously described (12). The identities of the alkylglycerols were confirmed by chemical ionization MS using methane as reagent gas.

Analysis of fatty acid moieties. The fatty acid composition of the milk lipids was determined using either packed columns in combination with the n-butyl esters (6) or capillary columns in combination with the methyl esters (12).

RESULTS

Goat milk fat. Table 1 compares the fatty acid composition of the goat milk triacylglycerol and the chloropropanediol diesters. While the triacylglycerols contain significant amounts of both short and long chain acids (C_4–C_{18}), the chloropropanediol diesters possess largely long chain fatty acids (C_{10}–C_{18}). There is a great similarity in the relative content of the different unsaturated long chain fatty acids in the two acylglycerol fractions. In addition, both ester classes appear to possess comparable relative amounts of the odd carbon number fatty acids. The short chain fatty acids have either been excluded during the acylation of the chloropropanediol or the chloropropanediol esters of short and medium chain length fatty acids have not been resolved from the triacylglycerol bulk during the TLC separation. Interesting is the high content of stearic acid in the chloropropanediol diester fraction.

Capillary GLC on nonpolar columns was used to obtain detailed carbon number profiles for the triacylglycerol and the chloropropanediol diester fractions of goat milk. The carbon numbers of the

TABLE 1

Fatty Acid Composition of Goat Milk Triacylglycerols and Diacylchloropropanediols

Fatty acids	Triacylglycerols[a] (mol %)	Diacylchloropropanediols[b] (mol %)
4:0	3.5	
6:0	3.1	
8:0	3.2	
10:0	11.2	1.5
12:0	5.7	3.0
14:0	11.2	12.9
15:0	1.9	1.7
16:0	31.2	39.7
16:1 (n−7)	1.6	1.1
17:0	1.5	3.1
18:0	6.2	20.4
18:1 (n−9)	17.6	16.0
18:2 (n−6)	1.7	0.1
18:3 (n−3)	0.5	
20:0		0.5

[a]Goat milk fraction 5.

[b]Goat milk fraction 3.

triacylglycerols ranged from C_{28}-C_{54} and included small but significant amounts of species with odd carbon numbers. This pattern was similar to that reported by Cerbulis et al. (1) and Marai et al. (13), except that the odd carbon number species were not resolved by the columns used in the earlier studies. The carbon numbers of the chloropropanediols ranged from C_{26}-C_{38} and also included small amounts of species with odd carbon numbers. An accurate peak matching revealed that the latter esters were eluted together with the triacylglycerol of corresponding carbon numbers. For example, the synthetic dioleoyl chloropropanediol was eluted with an equivalent chain length of 37.9 relative to the set of triacylglycerols. Similarly, the butyroyl-palmitoylchloropropanediol was eluted slightly ahead of the retention time anticipated for a synthetic triacylglycerol with 24 acyl carbons. The goat milk fats examined in this study did not show significant peaks in the range of C_{16}-C_{28} triacylclycerols, which suggests that short chain fatty acid esters of chloropropanediol also were absent, although the very small peaks detected in this region were not examined by GC/MS. Table 2 gives the carbon number distributions for the two classes of esters of goat milk fat, along with that of another goat milk fat sample analyzed earlier (13). The earlier sample of goat milk triacylglycerols contains significantly more of the longer chain species (C_{50}-C_{54}) than the present sample. Table 3 compares the carbon number distribution of the chloropropanediol diesters with those of the sn-1,2- and the sn-2,3-diacylglycerol moieties as calculated from the known stereospecific fatty acid distribution of the earlier analyzed goat milk long chain triacylglycerol fraction. There is a close match between the carbon numbers of the chloropropanediol diesters and those of the sn-1,2- but not the sn-2,3-diacylglycerol moieties of the goat milk triacylglycerols.

Figure 2 compares the HPLC elution profiles of the goat milk triacylglycerols (lower panel) and of the

TABLE 2

Carbon Number Distribution of Goat Milk Triacylglycerols and Diacylchloropropanediols

Carbon numbers	Triacylglycerols (mol %)		Diacylchloro-propanediols[c] (mol %)
	Fraction 5[a]	Control[b]	
26			1.8
27			0.6
28			6.1
29			1.0
30	0.8	0.7	14.0
31			3.0
32			25.4
33	0.2	0.1	4.1
34	3.9	3.9	28.1
35	0.6	0.5	2.7
36	7.7	8.5	13.3
37	0.9	0.7	
38	10.8	12.4	
39	1.2	trace	
40	13.0	10.4	
41	0.4		
42	14.1	8.1	
43	1.6		
44	11.9	7.4	
45	1.5		
46	8.8	5.9	
47	0.9		
48	6.6	7.6	
49	0.9		
50	6.1	12.3	
51	0.5		
52	4.1	13.1	
53	0.3		
54	1.6	6.2	

[a]Goat milk fraction 5.

[b]Literature value (13).

[c]Goat milk fraction 3.

TABLE 3

Carbon Number Distribution of Diacylchloropropanediols and Diacylglycerol Moieties of Goat Milk Triacylglycerols

Carbon number	Diacylchloro-propanediols[a]	Diacylglycerol moieties (mol %)	
		sn-1,2-[b]	sn-2,3-[c]
26	1.8	3.3	6.8
27	0.6	0.2	0.3
28	6.1	5.6	7.7
29	1.0	0.7	0.7
30	14.0	14.1	6.3
31	3.0	2.4	1.8
32	25.4	28.2	16.1
33	4.0	2.4	3.5
34	28.1	27.8	27.9
35	2.7	0.6	1.4
36	13.3	11.2	19.0

[a]Goat milk fraction 3.

[b]1-random 2-random distribution calculated from literature data (13).

[c]2-random 3-random distribution calculated from literature data (13).

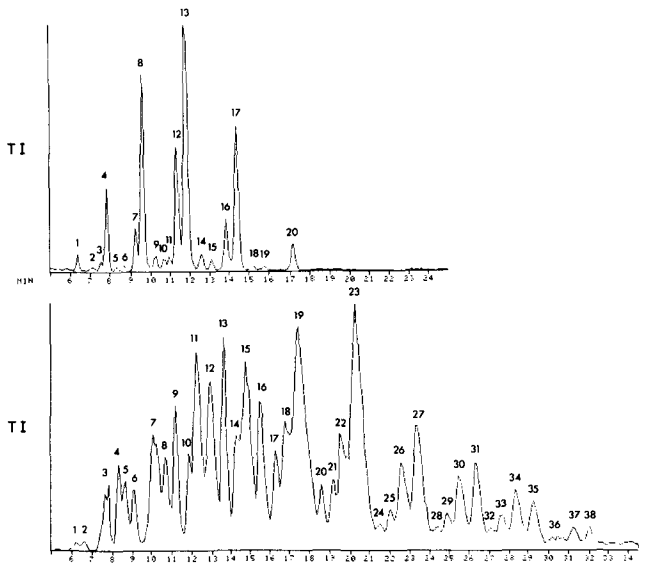

FIG. 2. HPLC elution profiles of the chloropropanediol diester (upper panel) and triacylglycerol (lower panel) fractions of goat milk fat. Chloropropanediol diester peaks are identified as in Table 4. LC/MS conditions as given in text.

chloropropanediol diesters (upper panel) as detected in the total positive ion current in the mass spectrometer. There is an extensive overlap between the two types of esters of corresponding partition number. Since the synthetic butyroylpalmitoylchloropropanediol was eluted well ahead of the short chain triacylglycerols, and in an area free of any other components in detectable amounts, it must be concluded that short chain esters of chloropropanediol were absent from the goat milk. This conclusion was confirmed by a search for characteristic positive and negative molecular and fragment ions over the anticipated range of elution times.

The chloropropanediol diester spectra obtained in the positive CI had $[MH\text{-}RCOOH]^+$ ions as the base peaks. The molecular weights of the esters were confirmed by the weak $[MH]^+$ ions. Thus, similar patterns were seen for the complete positive chemical ionization spectra of the synthetic dioleoylchloropropanediol and a major component (peak 12) from the HPLC elution profile (Fig. 2, upper panel) of the goat milk chlorohydrin esters. Because the synthetic compound had two identical fatty acids, only one mass (m/z 357) was seen for the $[MH\text{-}RCOOH]^+$ ion. The HPLC peak from the natural mixture apparently contained two different fatty acids in the same ester molecule, as indicated by the two different masses (m/z 357 and m/z 331) for the $[MH\text{-}RCOOH]^+$ ion. This was confirmed by the small pseudomolecular ion at m/z 613. These masses and fragment intensities were consistent with the presence of palmitoyloleoyl chloropropanediol as the sole component in this HPLC peak. The other chlorohydrin peaks showed the presence of two or more chloropropanediol diesters per peak. Table 4 lists the major ions detected in each of the chloropropanediol diester peaks. Table 5 gives the relative composition of the molecular species of the chloropropanediol diesters identified in the pooled goat milk sample. The major components are the dipalmitoyl, myristoylpalmitoyl, palmitoyloleoyl and palmitoylstearoyl species.

Figure 3 shows mass chromatograms corresponding to the m/z values of the MH^+ ions for the odd carbon-number diacylchloropropanediols. Because the major peaks elute with equivalent chain lengths (relative to the saturated diacylchloropropanediols) of 32.8, 34.8 and 36.8 instead of 29, 31 and 33, respectively, as expected for the odd carbon-numbered components, they must represent a new set of compounds. They were identified as 1-alkyl 2-acyl 3-chloropropanediols. Figure 4 shows the spectrum corresponding to one of the molecular species found in the natural sample along with the spectrum of a synthetic standard. Both the mass spectra and the chromatographic retention times are consistent with the proposed identity of these compounds.

The positive chemical ionization spectra of the goat milk fat triacylglycerols were similar to those recorded for other natural mixed-acid triacylglycerols (14). Prominent intensities were recorded for the protonated molecular ions $[MH]^+$, along with low abundance ions corresponding to adducts of acetonitrile and propionitrile. The $[MH\text{-}RCOOH]^+$ ions resulting from a random loss of an acid moiety from the protonated molecular ion were responsible for the base peaks in most of

TABLE 4

Major Ions[a] in the Chemical Ionization Spectra of Diacylchloropropanediols as obtained by LC/MS with a Gradient of Propionitrile in Acetonitrile

HPLC Peak	$(MH\text{-}RCOOH)^+$ (m/z)	$(MH)^+$ (m/z)	Major species[b] of diacylchloropropanediols
1	247, 275, 303, 331	503	10:0 16:0, 12:0 14:0
2	247, 331	503	10:0 16:0
3	275, 357	557	12:0 18:1, 14:0 16:1
4	275, 303, 331, 359	531	12:0 16:0, 14:0 14:0
			10:0 18:0
5	275, 289, 303, 317	545	14:0 15:0, 12:0 17:0
6	275, 289, 303, 317	545	14:0 15:0, 12:0 17:0
7	303, 357	585	14:0 18:1
8	275, 303, 331, 359	559	14:0 16:0, 12:0 18:0
9	303, 317, 331, 345	573	14:0 17:0, 15:0 16:0
10	303, 317, 331, 345	573	14:0 17:0, 15:0 16:0
11	357	639	18:1 18:1
12	331, 357	613	16:0 18:1
13	303, 331, 359	587	16:0 16:0, 14:0 18:0
13a	345, 357	627	17:0 18:1
14	317, 331, 345, 359	601	16:0 17:0, 15:0 18:0
	345, 357	627	17:0 18:1
15	317. 331, 345, 359	601	16:0 17:0, 15:0 18:0
16	357, 359	641	18:0 18:1
18	331, 359	615	16:0 18:0
17	331, 345, 359, 373	629	17:0 18:0, 19:0 16:0
19	345, 359	629	17:0 18:0
20	359	643	18:0 18:0
21	331, 387, 415	671	18:0 20:0, 16:0 22:0

[a]The identity of the ions is described in text.

[b]No distinction is being made among the sn-2 and sn-3 positions of the diacylchloropropanediol molecule.

TABLE 5

Relative Proportions of Molecular Species of Diacylchloropropanediols in a Pooled Sample of Goat Milk Fat[a]

HPLC peak	Molecular species	Area %	HPLC peak	Molecular species	Area %
1	10:0 16:0	0.8	10	14:0 17:0	0.7
	12:0 14:0	0.2		15:0 16:0	0.6
2	10:0 16:0	0.6	11	18:1 18:1	1.4
3	12:0 18:1	0.8	12	16:0 18:1	12.6
4	12:0 16:0	3.3	13	16:0 16:0	23.5
	14:0 14:0	1.7		14:0 18:0	3.2
	10:0 18:0	1.6	14	16:0 17:0	2.0
5	14:0 15:0	0.2	15	16:0 17:0	0.6
	12:0 17:0	0.2		15:0 18:0	0.5
6	14:0 15:0	0.2	16	18:0 18:1	5.1
	12:0 17:0	0.2	17	16:0 18:0	14.4
7	14:0 18:1	3.7	18	17:0 18:0	0.4
8	14:0 16:0	14.6		19:0 16:0	0.1
	12:0 18:0	1.6	19	17:0 18:0	0.5
9	14:0 17:0	0.8	20	18:0 18:0	2.8
	15:0 16:0	0.8	21	18:0 20:0	0.1

[a]Molecular species making up less than 10% of the HPLC peak have been ignored, with the peak area being divided among the identified species.

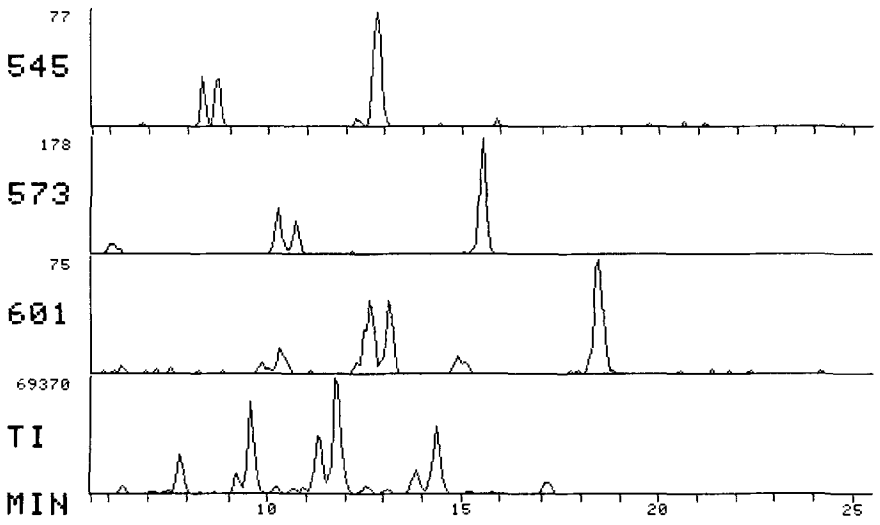

FIG. 3. Mass chromatograms of diacylchloropropanediols showing m/z values corresponding to MH^+ ions for odd carbon number species. The major peaks at m/z 545, 573 and 601 constitute a separate series of homologous species identified as alkylacylchloropropanediols.

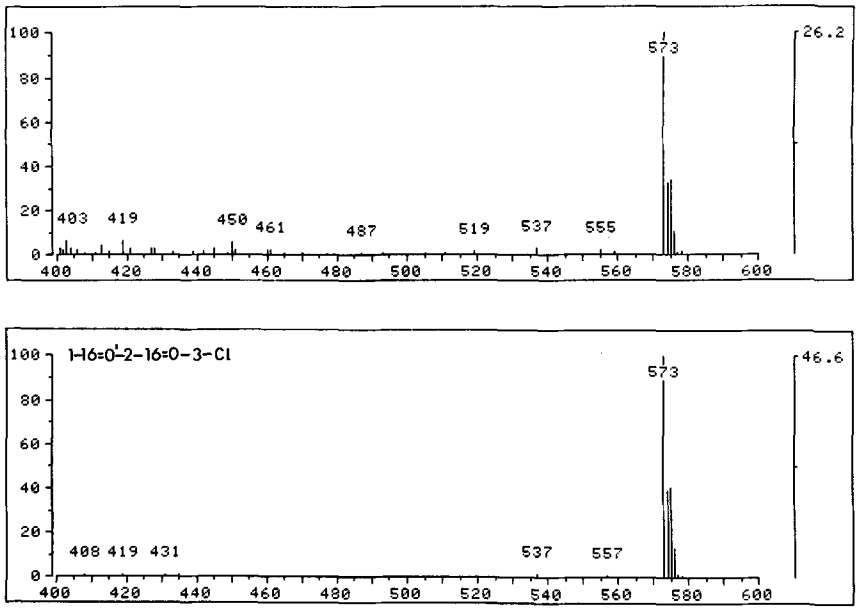

FIG. 4. Chemical ionization spectra of synthetic 1-palmityl 2-palmitoyl 3-chloropropanediol (lower panel) and one of the unknown series of species found in the natural sample of goat milk fat (upper panel).

these spectra. Using the LC/MS positive chemical ionization approach, it was possible to estimate that the short chain esters of chloropropanediol, if present, could not have exceeded 10% of the long chain chloropropanediol diesters in the goat milk.

Bovine milk fat. Figure 5 shows the total ion current profile of an LC/MS separation of a molecular distillate of bovine milk fat along with the pattern of the chloropropanediol diesters isolated from goat milk. Although there is an extensive overlap among the various components, a positive identification of any chloropropanediol diesters in the milk fat distillate cannot be made on the basis of the positive chemical ionization spectra of the components. A positive identification of these esters also could not be obtained by capillary GLC of the distillate or of the TLC fractions of the molecular distillate corresponding in migration rate to synthetic butyroylpalmitoyl and butyroyloleoylchloropropanediols.

To increase the specificity of detection of the chloropropanediol diesters, we attempted LC/MS with negative ion detection. Figure 6 shows the negative ion

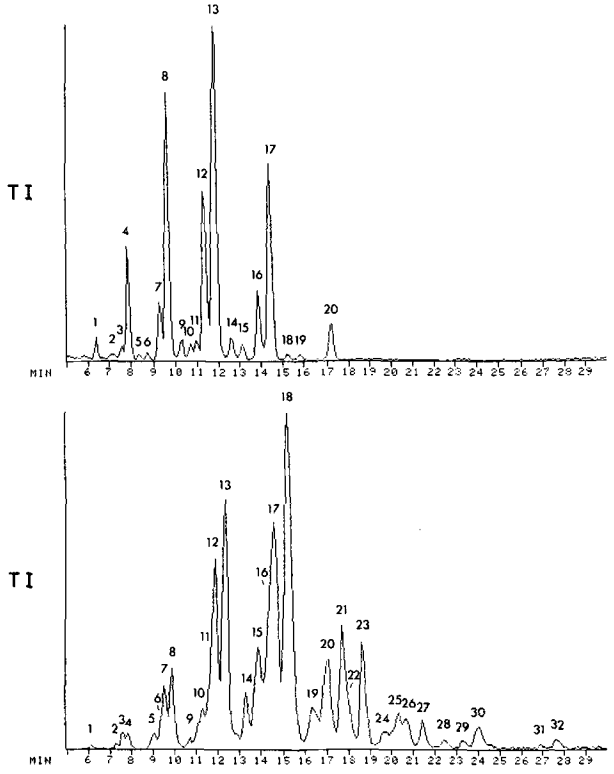

FIG. 5. HPLC elution profiles of the chloropropanediol diesters of goat milk (upper panel) and the 2.5% most volatile molecular distillate of bovine milk fat (lower panel). The chloropropanediol diester peaks are identified as in Table 4. The identity of the major peaks in the molecular distillate is as follows: 7—12:0, 18:1, 4:0; 8—14:0,14:0,4:0; 12—14:0, 18:1, 4:0 + 14:0, 14:0, 6:0; 13—14:0, 16:0, 4:0; 15—14:0, 18:1, 6:0 + 10:0, 12:0, 14:0; 17—16:0,18:1, 4:0 + 16:0, 14:0, 6:0; 18—16:0, 16:0, 4:0; 19—16:0, 17:0, 4:0; 20—16:0, 18:1, 12:0, 8:0; 21—16:0, 16:0, 6:0; 23—18:0, 16:0, 4:0; 25—16:0, 14:0, 10:0,; 27—18:0, 16:0, 6:0.

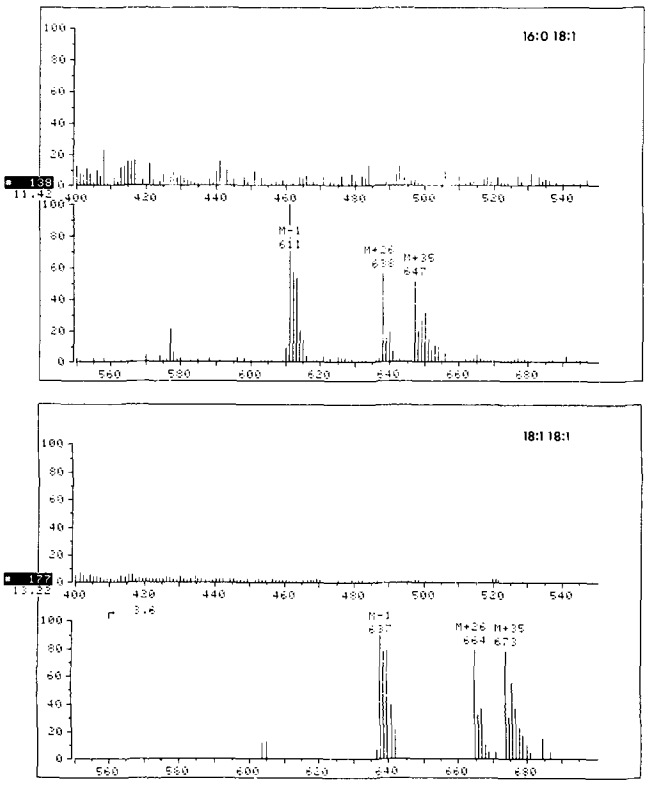

FIG. 6. Negative chemical ionization spectra of the natural palmitoyloleoylchloropropanediol (upper panel) and synthetic dioleoylchloropropanediol (lower panel) as obtained under LC/MS conditions using a gradient of propionitrile in acetonitrile. LC/MS conditions as given in text.

chemical ionization spectra for the dipalmitoylchloropropanediol (upper panel) recovered from the goat milk sample (peak 12, Fig. 2, upper panel) and the synthetic dioleoylchloropropanediol (lower panel). The spectra obtained in the negative CI mode exhibited ions at m/z $[M-1]^+$, $[M+26]^+$ and $[M+35]^+$. The latter two ions represent the addition of CN^- and Cl^- ions, respectively. The isotope clusters of the ions at m/z $[M+26]^+$ and $[M+35]^+$ are consistent with the presence of one and two chlorine atoms, respectively. The negative chemical ionization detection provided characteristic fragment ions not found in the ordinary triacylglycerol spectra. An examination of the distillate fraction by this method, however, gave negative results for chloropropanediol diesters in the 2.5% distillate of the butterfat. Likewise, neither the original triacylglycerol mixture nor the unknown fatty esters recovered from the front of the triacylglycerol spot on the TLC plate gave ions characteristic of the chloropropanediol diesters in either positive or negative chemical ionization mass spectra.

Human milk fat. Figure 7 compares the capillary GLC elution patterns of human milk triacylglycerols (lower panel) and of the fatty acid esters recovered from a TLC spot corresponding to the goat milk chloropropanediol diesters (upper panel). The unknown esters of the human milk are of higher molecular weight than the chloropropanediol diesters. These esters give an elution pattern similar to that obtained for human milk triacylglycerols under the same GLC conditions, but the major peaks are eluted somewhat earlier than the anticipated major triacylglycerol peaks. Such a behavior is characteristic of the alkyldiacylglycerols compared to triacylglycerols of corresponding carbon number. The presence of the alkyldiacylglycerols in the minor neutral lipid fraction of human milk was confirmed by capillary GLC of the acetylated transmethylation products, which showed the presence of both fatty acid methyl esters and of the diacetates of palmityl, stearyl and oleylglycerols as major components. The composition is shown in Table 6. Furthermore, the unknown fatty ester fraction of human milk failed to produce any of the positive or negative chemical ionization products seen in the LC/MS spectra of the goat milk chloropropanediol diesters.

Ten other human milk fat samples were examined by TLC. Each milk contained a TLC spot corresponding to the chloropropanediol diesters. Enough material was isolated from five sources to attempt to determine if the chloropropanediol diesters were present. The TLC spots were transmethylated as previously described (3), and the aqueous phase was recovered and analyzed

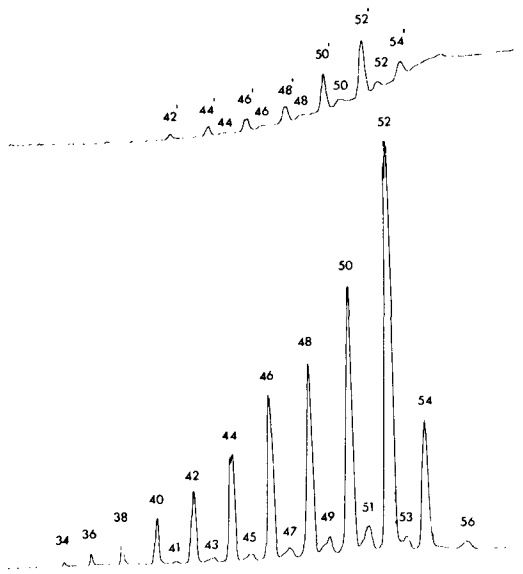

FIG. 7. Capillary GLC elution patterns of the alkyldiacylglycerol (upper panel) and the triacylglycerol (lower panel) of human milk fat. The triacyglycerol peaks are identified by the number of total acyl carbons and the alkyldiacylglycerols by the number of total fatty chain carbons per acylglycerol molecule. GLC conditions as given in text.

TABLE 6

Composition of Alkyldiacylglycerols, Triacylglycerols and Monoalkylglycerols from Human Milk

	Fatty acid methyl esters (mol %)		
Chain length	Alkyldiacyl-glycerols	Triacyl-glycerols	Monoalkylgly-cerols (mol %)
10:0 & shorter		3.9	
12:0	3.3	8.2	
14:0	6.6	8.3	
15:0	0.4	0.5	
16:0	34.0	24.2	23.9
16:1	3.2	3.1	
17:0	0.5	0.4	1.7
18:0	8.8	10.3	43.3
18:1	35.6	34.7	31.1
18:2	5.0	5.6	
20:0	1.2	0.6	
20:1	1.4	0.4	

Ca. 7% of the ether fraction contained alkenyl groups having either 16 or 18 carbon atoms.

by GLC for the presence of 3-chloropropanediol. Only three samples showed the presence of 3-chloropropanediol as judged by retention times, and in these cases it accounted for less than 5% of the total recovered alcohols. The major component in each case was glycerol, and a number of other minor components with unidentified retention times were observed. Thus, chloropropanediol diesters are not a major component of the corresponding TLC spot in human milk.

DISCUSSION

Our results confirm the presence of fatty acid esters of chloropropanediol in goat milk fat, from which they have been isolated as a minor TLC fraction migrating just ahead of the triacylglycerols in a neutral lipid solvent system. Although similar neutral lipid spots have been noted for milk fat samples from other herds of goats, it is not possible to conclude that the chloropropanediol diesters are characteristic components of goat milk fat. Detailed analyses of the corresponding fractions from human and bovine milk fat revealed the alkyldiacylglycerols as the major or sole components. It is therefore essential to confirm in each instance the presence of the chloropropanediol diesters by chromatographic and mass spectrometric means before the origin of these compounds is considered.

LC/MS of milk fats. The present study constitutes the first practical application of LC/MS to the study of the acylglycerol composition of the highly complex ruminant milk fats, although the suitability of the method has been demonstrated previously (14,15). Of special interest is the resolution of the short and medium chain triacylglycerols within a carbon number. Both specific molecular association and positional distribution of the fatty acids apparently contribute to their partition on the reversed-phase HPLC column as well as to the relative retention time on a polar GLC column (16). Carbon or partition numbers cannot be used to characterize and identify these molecules by HPLC. Accurate peak collection and analysis of fatty acid composition might help, but the necessary confidence in the correctness of the results can be obtained only by MS.

The LC/MS also allows the detection and identification of nonglyceride components that may contaminate the triacylglycerol fraction. We have previously described the overlaps between triacylglycerols and the fatty acid esters of cholesterol and plant sterols (14,15), which can be effectively demonstrated by mass chromatography. The present study shows that mass chromatography can also be used to demonstrate the presence of other minor components in the milk fat, provided the masses of the potential contaminants are accurately known and do not coincide with the masses of the major triacylglycerol ions or their P+1 and P+2 companions. In view of the extreme complexity of the milk fat triacylglycerols, however, the levels of contaminants that can be detected without prior enrichment by TLC or molecular distillation are rather high, except in those instances where the contaminants are clearly resolved from the bulk of the triacylglycerols by the reversed-phase HPLC column. The long chain diesters of the chloropropanediol and the long chain alkyldiacylglycerols require a preliminary isolation. The long chain alkyldiacylglycerols have been previously detected in both human and bovine milk fats (17). The present study shows that on TLC these migrate to the same spot as the long chain fatty acid esters of chloropropanediol.

Identification of chloropropanediol diesters. Electron impact spectra of chloropropanediol diesters have been obtained previously by Velisek et al. (18), Davidek et al. (5) and Gardner et al. (4), who identified fragments

characteristic of the fatty acid components, $[RCO]^+$, and of the loss of fatty acids from the parent molecule, $[M\text{-}RCOO]^+$. The presence of chlorine in the $[M\text{-}RCOO]^+$ fragment ions was confirmed by high resolution EIMS (4).

The present study confirms the presence of the molecular species of carbon numbers C_{26}–C_{38} identified in the chloropropanediol diesters of goat milk by Cerbulis et al. (3) on the basis of the fatty acid composition and the molecular weight estimates derived by ammonia/direct chemical ionization analyses. The isolated chloropropanediol diester fraction indicated the presence of nine major components with molecular weights of 502, 528, 530, 556, 558, 584, 586, 612 and 614. Compounds with molecular weights of 638, 640 and 642 also were observed in the ammonia/direct chemical ionization analyses, although intensities were low. The quasi-molecular ions of these compunds showed characteristic isotopic patterns of one chlorine atom. The LC/MS analysis allowed a complete identification of the fatty acid pairing within each carbon number, as well as a matching of the obtained pattern to that derived for the long chain sn-1,2-diacylglycerol moieties of the goat milk triacylglycerols. No evidence was obtained for the presence of short chain diesters of chloropropanediol in the goat milk fat.

Origin of fatty esters of chloropropanediol. In view of the close similarity in composition of the molecular species of the chloropropanediol diesters and the sn-1,2-diacylglycerol moieties of goat milk triacylglycerols, it would appear that a chlorination of the hydroxyl group had taken place after the formation of the sn-1,2-diacylglycerol intermediates of triacylglycerol biosynthesis. This also is consistent with the finding of the 3-chloro-3-deoxyderivative of 1-alkyl 2-acylglycerol known to be synthesized by a stereochemically specific mechanism involving dihydroxyacetone phosphate. Furthermore, this possibility is likely in view of the absence of the short chain esters of chloropropanediol. The short chain fatty acids are believed to be introduced into the 3-position of the sn-glycerol molecule as the final acylation step in the biosynthesis of milk fat triacylglycerols (19). Although a coincidence cannot be ruled out, the present results would appear to justify a further comparative stereospecific study of the chloropropanediol diesters and of the triacylglycerols collected from the same goat milk sample (7).

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Phospholipid Requirement of Alkylglycerol Monooxygenase from Rat Liver Microsomes

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The alkylglycerol monooxygenase catalyzing the cleavage of the ether bond in alkylglycerol resides in rat liver microsomes. The enzyme preparation was freed of phospholipids by sodium deoxycholate treatment followed by gel filtration in the presence of deoxycholate. The removal of phospholipids markedly decreased the alkylglycerol monooxygenase activity. The activity of the delipidated enzyme, however, could be completely restored by the addition of phospholipid vesicles without detergent. When individual phospholipids were added, anionic phospholipids such as phosphatidylglycerol and diphosphatidylglycerol were the most effective. These findings, along with our previous observation of a similar effect of liposomes on the purified enzyme, indicate that the amphipathic nature of the protein is responsible for the lipid dependence of enzymatic activity.
Lipids 21, 191-194 (1986).

Glycerophospholipids with ether linkages are found in nearly all animals. Although the distinctive functions of ether lipids generally are not known, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, which acts as a platelet-activating factor (PAF), has been shown to be of great biological importance (1-4). The biological activity of PAF is highly structure-dependent, i.e. the 1-alkyl ether group, the 2-acetyl group and the choline moiety in the polar head group are required (1,2).

The alkyl group has been known to be metabolized through oxidative cleavage by alkylglycerol monooxygenase, which requires tetrahydropteridine as a cofactor (5,6). Cleavage of the alkyl chain also requires that at least one of the glycerol carbons is unsubstituted (7,8). This cleavage enzyme may regulate the total ether lipid content of cells, as tissues rich in the enzyme are low in alkylglycerol and vice versa (9,10). Furthermore, the biosynthesis of the alkyl ethers and their conversion to plasmalogens also may play a role in regulating the ether lipid content (11).

Recently we succeeded in purifying and characterizing alkylglycerol monooxygenase from rat liver microsomes (12,13) and observed that some phospholipid vesicles stimulated the purified enzyme (12). However, the phospholipid dependence of the enzyme has not been examined in detail. In this communication, we present more detailed data on the phospholipid requirements of alkylglycerol monooxygenase. In our experiments, the microsomal membrane was separated into phospholipids and protein fractions and then reconstituted.

EXPERIMENTAL PROCEDURES

Materials. The following chemicals were obtained from commercial sources: 1-*O*-hexadecyl-*rac*-glycerol, fatty acid-poor albumin and phospholipase A_2 (naja naja) from Sigma (St. Louis, Missouri); 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride from Aldrich (Milwaukee, Wisconsin); reduced glutathione from Yamanouchi (Tokyo, Japan); sodium deoxycholate from Difco (Detroit, Michigan); Sephadex G-25 and G-50 from Pharmacia (Uppsala, Sweden); precoated Silica Gel F_{254} plates from E. Merck (Darmstadt, West Germany); dye reagent for protein determination from Bio-Rad (Richmond, Virginia); asolectin (a soybean phospholipid preparation) from Iwai Kagaku (Tokyo, Japan), and various phospholipids from Serdary (London, England). 1-*O*-[1-^{14}C]Hexadecyl-*rac*-glycerol (1.1 Ci/mol) was donated by H. K. Mangold (14).

Phospholipase A_2-treatment of microsomes. Rat liver microsomes were prepared as described previously (15) using a homogenization medium containing 8 mM $CaCl_2$ (16). Digestion with phospholipase A_2 was performed as follows with a minor modification of the method given in a previous report (15): a mixture containing microsomes (33 mg of protein), $CaCl_2$ (3 mM), fatty acid-poor albumin (10 mg) and phospholipase A_2 (30 units) was incubated at 37 C for 15 min. The reaction was stopped by addition of 10 mM EDTA, followed by centrifugation at 25,000 × g for 15 min. The resulting precipitate was washed twice and suspended in 0.1 M Tris-HCl, pH 7.5 (11.3 mg of protein/ml).

Preparation of phospholipid-depleted microsomal protein. To microsomes (33 mg of protein/ml) suspended in 20 mM Tris-acetate buffer (pH 8.1) containing 0.2 mM EDTA-0.1 M NaCl, as described by Rogers and Strittmatter (17), solid sodium deoxycholate was added to yield a final detergent concentration of 5% and then was stirred gently for 20 min. The suspension was centrifuged at 105,000 × g for 90 min, and the resulting supernatant (4 ml) was applied to a Sephadex G-50 column (1.5 × 100 cm) equilibrated with the buffer described above in the presence of 1% deoxycholate. The column was developed with the same buffer. In this column chromatography, microsomal protein was separated from membrane phospholipids. Lipid-free microsomal protein appears to be a heterogenous mixture of high molecular weight aggregates, even in the presence of deoxycholate. The solubilized protein was concentrated by ultrafiltration using an Amicon macrosolute concentrator (B15), and then sodium deoxycholate was removed by gel filtration on a Sephadex G-25 column (1.5 × 52 cm) equilibrated with the same buffer without deoxycholate. It has been shown that at least 99.99% of deoxycholate is removed from microsomal protein by this gel filtration procedure (17).

Analytical methods. The alkylglycerol monooxygenase activity was determined by method I using 1-*O*-[1-^{14}C]-hexadecylglycerol as described previously (13) with the following slight modification. The incubation mixture consisted of the following: 1-*O*-[1-^{14}C]hexadecylglycerol (dissolved in ethanol), 23 nmol; 0.1 M Tris/HCl, pH 8.8; 5 mM $(NH_4)_2SO_4$; 5 mM GSH; 1 mM tetrahydropteridine, and enzyme in a final volume of 0.1 ml. After incubation and shaking at 37 C for 15 min, the reaction was stopped by adding 0.5 ml of chloroform/methanol (2:1, v/v). Following vigorous mixing and centrifugation, the lower

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solvent phase was evaporated under nitrogen, dissolved in a minimum of chloroform and applied to a precoated Silica Gel F_{254} plate, which was developed with light petroleum/ethyl ether/acetic acid (80:30:1, v/v/v) for 50 min. The band corresponding to authentic hexadecanal was scraped off and counted in a liquid scintillation counter. One unit of enzyme activity was defined as 1 nmol hexadecanal formed/15 min under the above conditions.

Protein was measured by the method of Lowry et al. (18), or using the Bio-Rad protein assay kit; bovine serum albumin used as standard. Total microsomal lipid was extracted from microsomes by the method of Folch et al. (19), and the phospholipid fraction was obtained by silicic acid column chromatography (20). To prepare the liposomes, solvent was evaporated under a stream of nitrogen, and the dry phospholipid was suspended in 20 mM Tris-HCl buffer (pH 7.5). The suspension was sonicated (100 W, 30 min) in a Branson sonifier until no further decrease in turbidity was observed. Phospholipid phosphorus was determined by the method of Ames (21) after ashing in the presence of $Mg(NO_3)_2$ (22).

RESULTS

Separation of phospholipids from microsomal protein. Figure 1 shows the elution profile of the deoxycholate-treated microsomes on a Sephadex G-50 column equilibrated with 1% deoxycholate. The separation of protein from phospholipids is virtually complete. The phospholipid-depleted microsomal protein was recovered from the void volume and designated as microsomal protein.

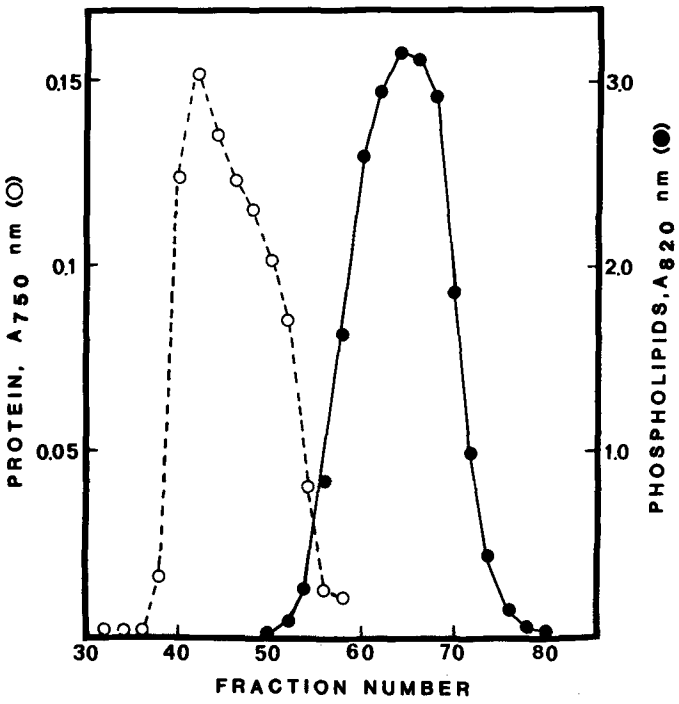

FIG. 1. Elution profile for deoxycholate-treated microsomes from a Sephadex G-50 column. Microsomes were treated with 5% sodium deoxycholate and chromatography was performed as described in Experimental Procedures. Fractions of 2.5 ml were collected at a flow rate of 20 ml/hr. Protein and phospholipid were measured in 10 μl and 100 μl of an aliquot, respectively.

Effect of phospholipid separation and reconstitution with phospholipid vesicles. Phospholipase A_2 treatment of microsomes resulted in ca. 60% decrease of the enzyme activity (Table 1). The activity was restored by the addition of asolectin depending on its concentrations. On the other hand, removal of phospholipids by gel filtration in the presence of deoxycholate resulted in about 70% decrease of the alkylglycerol monooxygenase activity. The enzyme activity of the microsomal protein also was completely restored with asolectin or total phospholipids extracted from liver microsomes. When individual phospholipids were added to the microsomal protein, the largest stimulation was observed with phosphatidylglycerol or diphosphatidylglycerol at concentrations of 0.26 mM. Asolectin has been shown to contain phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (23). Individual addition of these phospholipids showed little stimulation; nevertheless, asolectin was fully effective.

Kinetics of phosphatidylglycerol effect on delipidated-microsomal protein. The effect of phosphatidylglycerol, which was found to be the most effective individual phospholipid, was kinetically examined. Phosphatidylglycerol stimulated the enzyme activity in a concentration-dependent manner and its effect saturated at about 0.26 mM (Fig. 2). Furthermore, the phospholipid vesicles particularly increased the initial velocity (Fig. 3).

DISCUSSION

Use of reconstitution experiments to elucidate the effect of membrane components on the biological activity of membrane-bound proteins has been reviewed by Radin (24). It has become apparent that phospholipids are an integral part of membrane-bound enzymes and are necessary for the maintenance and expression of enzyme activity (25,26).

In the present work, we compared the catalytic activity of the lipid-free aggregates of microsomal proteins with the reconstituted system to describe the lipid requirements of the system. Use of asolectin and other phospholipids is becoming a very common method to stabilize and stimulate membrane-bound enzyme (23,27). The method of phospholipid depletion described herein offers several advantages over the more widely used technique of extraction with 90% acetone (24). Similar procedures have been reported by Helenius and Simons (28). Deoxycholate, although ionic, resembles the nonionic detergents in its minimal denaturating effect on proteins (29). Thus, alkylglycerol monooxygenase was not affected by treatment with deoxycholate (data not shown). Our detergent extraction procedure removes at least 95% of phospholipids, whereas extraction with acetone removes only about 80% (30). Finally, deoxycholate was easily removed by gel filtration, a vital step in the reconstitution procedure. The very low level of detergent that might have escaped detection was not expected to affect our data.

The fact that all the purified, microsomal proteins and phospholipase A_2-treated microsomes required phospholipids to express full enzyme activity indicates that the amphipathic properties of the alkylglycerol monooxygenase are primarily responsible for the lipid-dependence of this enzyme. Jones and Hajra (31) showed that the

TABLE 1

Effect of Delipidation and Reconstitution with Phospholipid Vesicles on Alkylglycerol Monooxygenase from Rat Liver Microsomes[a]

Treatments of microsomes	Additions	μg	Relative activity (%)
None	None	—	100[b]
Phospholipase A_2	None	—	41.5
Phospholipase A_2	Asolectin	125	63.4
Phospholipase A_2	Asolectin	250	107.3
5% Na deoxycholate	None	—	100[c]
Gel filtration	None	—	26.3
Gel filtration	Asolectin	125	76.8
Gel filtration	Asolectin	250	107.3
Gel filtration	Total microsomal phospholipids	5	55.4
Gel filtration	Total microsomal phospholipids	10	105.2
Gel filtration	Phosphatidylcholine	20	46.5
Gel filtration	Phosphatidylethanolamine	20	30.4
Gel filtration	Phosphatidylserine	20	36.4
Gel filtration	Phosphatidylinositol	20	29.6
Gel filtration	Phosphatidylglycerol	20	122.1
Gel filtration	Phosphatidic acid	20	36.4
Gel filtration	Diphosphatidylglycerol	20	111.7
Gel filtration	Sphingomyelin	20	27.3
Gel filtration	Lysophosphatidylcholine	20	32.4
Gel filtration	Lysophosphatidylethanolamine	20	27.3
Partially purified[e]	None	—	100[d]
Partially purified	Asolectin	125	257.7

[a]These activities were obtained under condition of first order kinetics.

[b,c,d]The enzyme activity of each enzyme with no addition of phospholipid is 100%. The actual activities of b, c and d were 7.5, 4.3 and 61.5 units/mg protein, respectively.

[e]This column was cited from our previous report (10).

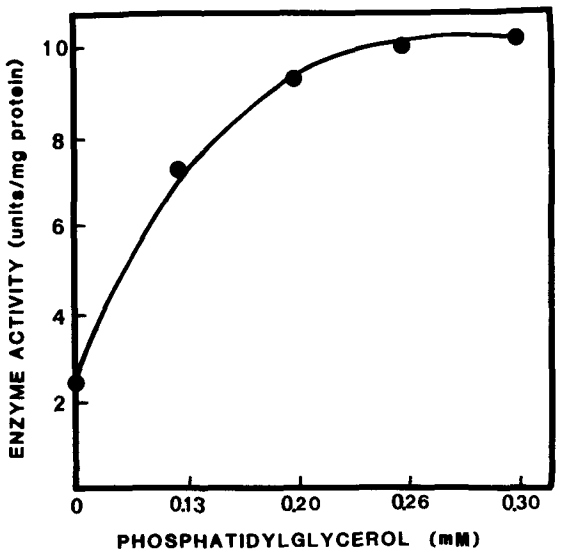

FIG. 2. Stimulation of alkylglycerol monooxygenase activity in delipidated-microsomal protein by phosphatidylglycerol. Enzyme activity was determined as described in Experimental Procedures, except that the indicated amount of phosphatidylglycerol was added with 70 μg protein of microsomal protein.

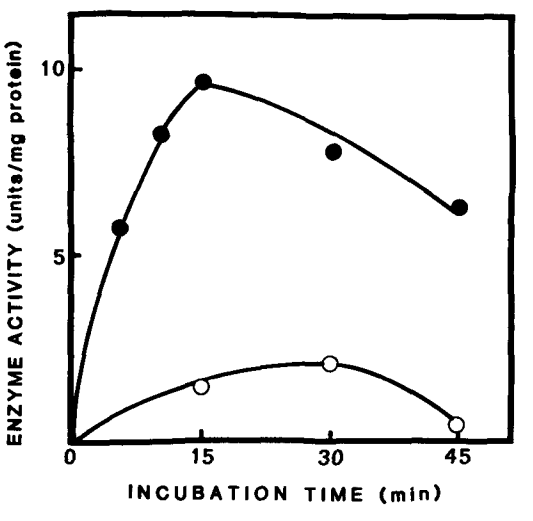

FIG. 3. Effect of phosphatidylglycerol on alkylglycerol monooxygenase activity in delipidated-microsomal protein. Enzyme activity was determined as described in Experimental Procedures, except that 70 μg protein of microsomal protein was used and incubation time changed as indicated with (●) or without (○) phosphatidylglycerol (0.26 mM).

enzyme dihydroxyacetone phosphate acyltransferase, which is a key enzyme in the biosynthesis of glycerol ether lipid (32), was stimulated by the addition of phospholipid. On the other hand, rat liver phenylalanine hydroxylase, a pteridine-requiring enzyme, also was reported to be stimulated by lysophosphatidylcholine (33). It is now possible to use the purified proteins and artificial liposomes consisting of known molecular species of phospholipid to explore the lipid dependence of alkylglycerol monooxygenase in more detail.

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Brown Adipose Tissue Triacylglycerol Fatty Acids of Obese and Lean Mice: In Situ and in Transplants

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The triacylglycerols of white adipose tissue (WAT) from animals with high rates of lipogenesis, such as obese hyperglycemic mice or hypothalamically lesioned rats, contain high proportions of palmitoleic acid (16:1) and low proportions of linoleic acid (18:2). These differences appear to result from dilution of dietary 18:2 by synthesized fatty acids, particularly 16:1. To test this we have investigated the triacylglycerol fatty acid composition of brown and white adipose tissue of lean and obese mice, as brown adipose tissue (BAT) has a higher lipogenic rate than WAT and lipogenesis is faster in obese than in lean mice. Between three and eight weeks of age the proportions of fatty acids in the tissues changed, with a marked fall in milk-derived lauric and myristic acids. From 8 to 16 weeks they were more stable and the proportions of 16:1 and 18:2 in the different tissues were as expected, with the highest and lowest proportions, respectively, in BAT from obese mice. When BAT from obese mice was transplanted under the kidney capsule of lean mice, or vice versa, for one month, the fatty acid composition of the grafts changed toward that of the host BAT. The proportions of 18:2 and, to a lesser extent, 16:1 were slightly higher in the grafts than in the hosts but since this also occurred in lean-to-lean and obese-to-obese grafts it was probably a transplantation artifact. Overall, the results confirm that the physiological environment, rather than the source of the adipose tissue, is the major determinant of its fatty acid composition.
Lipids 21, 195-201 (1986).

Animals with high rates of lipogenesis, such as obese hyperglycemic mice or hypothalamically lesioned rats, show characteristic differences in the fatty acid composition of the triacylglycerol stores in their WAT compared with lean animals given the same diet (1-3). The proportion of dietary linoleic acid (18:2) falls as a result of dilution by synthesized fatty acids, and elevated activities of fatty acyl-CoA Δ^9 desaturase increase the proportion of monoenoic acids, particularly palmitoleic acid (16:1) (4-7). Because BAT in mice has a higher rate of fatty acid synthesis than WAT (8-10), it too might be expected to show such differences. However, it has been reported to have lower concentrations of both linoleic acid and monounsaturated fatty acids, while the concentration of saturated fatty acids is higher (11). The high rate of fatty acid synthesis in BAT is related to thermogenesis (9,10), which also may affect the type of fatty acids deposited. Thermogenesis is defective in the BAT of obese-hyperglycemic mice (12,13), but the tissue still has higher rates of lipogenesis than the WAT (14-16). We have analyzed, therefore, the triacylglycerol fatty acid compositions of BAT and WAT in obese mice and compared them to those of lean mice. We aimed to determine if the decreased thermogenesis in the BAT of obese mice resulted in an altered fatty acid composition and, if so, whether the composition resembled that of nonthermogenic WAT. As the rate of fatty acid synthesis in BAT changes during growth (15), we have studied mice from 3 to 16 weeks of age. In general, we found that the concentration of 18:2 was lower and that of 16:1 was higher in BAT compared with WAT in both lean and obese mice. Compared with lean mice, the BAT of obese mice was lower in 18:2 and had an exceptionally high concentration of 16:1.

It is not clear whether the defects in the BAT of obese mice such as low thermogenesis (13), decreased lipolysis (17) and adenylate cyclase activity (18), low GDP binding (12) and abnormal mitochondria (19) result from a decreased sympathetic nervous stimulation (20-22), different hormonal environments or specific genetic differences expressed within the tissue. To determine whether the physiological environment or genetic differences in the adipose tissue produced the differences between the fatty acid composition of BAT from obese and lean mice, we used the transplantation technique previously used with WAT (23,24). BAT from obese mice was transplanted under the kidney capsule of lean mice and vice versa. The disruption to BAT is potentially greater than that to WAT because there is not only sympathetic innervation of the blood vessels, but a separate sympathetic supply to the individual adipocytes (25). However, to compensate for this we determined the fatty acid composition of lean and obese BAT transplanted into host mice of the same type. We also compared these transplants with the adjacent perirenal adipose tissue to distinguish whether changes in the fatty acid composition were site-specific and whether transplanted BAT might resemble WAT, a possibility if reinnervation failed to occur.

METHODS AND MATERIALS

Male C57Bl/6J obese *(ob/ob)* and lean (+/+) mice were from the colony at the AFRC Food Research Institute, Bristol, which was derived from stock supplied by the Jackson Laboratory (Bar Harbor, Maine). The lean mice were obtained by selecting out the *ob* gene and were not more than two generations removed from the obese line. Mice were weaned at 21 days and were housed in groups of 2 to 8 in boxes with solid bottoms and sawdust bedding. They were fed ad libitum on Oxoid breeding diet (3.1% lipid; for fatty acid content, see Table 1) and had unlimited access to water. Temperature was maintained at 21 C normally, 20 C minimum. Obese and lean mice used for the age study were aged 3, 4, 5, 6, 8, 10, 12 and 16 weeks. The young obese mice were selected visually and their genotypes confirmed by the body fat content revealed on dissection. Mice were killed by decapitation, and interscapular BAT and WAT were sampled for subsequent fatty acid analysis. Samples of 100 to 200 mg were taken from the center of the left suprascapular lobe of

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TABLE 1

Fatty Acid Composition (% by Wt) of Diets

Fatty acid	Oxoid breeding diet	Spratts laboratory diet No. 1
C14:0	0.8	1.1
C16:0	13.7	14.8
C16:1	1.0	0.9
C18:0	2.2	1.4
C18:1	23.8	21.2
C18:2	52.1	53.8
C18:3	3.8	2.9
Others	2.5	3.9

BAT and from within the suprascapular WAT posterior and lateral to the BAT.

Mice used in the two transplantation experiments were transferred to Cambridge 7 to 11 days before the operations, housed there at 22 ± 2 C and received Spratts laboratory diet No. 1 ad libitum (3.4% lipid; for fatty acid content see Table 1). They were housed singly after the operations. The ages and weights of donor and host mice are shown in Table 2. The technique for transplantation was as described previously for WAT (23,24). Interscapular BAT from obese or lean donor mice was transplanted under the kidney capsules of obese and lean host mice. Samples were also taken for determination of fatty acids. After one mo, host mice were killed and the grafts were removed, together with samples of the host interscapular BAT. In the second experiment, transplants were between the same type of animal only, and host perirenal adipose tissue adjacent to the site of the graft was also sampled after one mo.

Lipid was extracted from all samples of tissue with chloroform/methanol (2:1, v/v) containing 2,6-di-*tert*-butyl-*p*-cresol as antioxidant, and triglyceride fatty acids were prepared as described previously (24). Fatty acid methyl esters were analyzed on a glass WCOT capillary column (25 m × 0.32 mm i.d.), coated with CP™ Sil 88, film thickness 0.2 μm (Chrompak U.K. Ltd., London, United Kingdom), at 150 C for 10 min, then rising at 3 C/min to 210 C, or on a fused silica WCOT capillary column (25 m × 0.2 mm i.d.), coating BP20, film thickness 0.25 μm (SGE [U.K.] Ltd., Milton Keynes, United Kingdom), isothermally at 185 C. Comparable results were obtained with these systems, and peak areas were measured by an Infotronics 304-50 computing integrator (LDC Div., Milton Roy Inc., Stone, Staffs., United Kingdom).

Analysis of variance, paired t-tests and simple linear regression were used to analyze the results.

RESULTS

Fatty acid compositions of scapular BAT and WAT from obese and lean mice. The triacylglycerol fatty acid composition of BAT and WAT from the interscapular depot of obese and lean mice between three and 16 weeks is shown in Figure 1.

At three weeks both tissues from lean and obese mice contained relatively large amounts of lauric acid (12:0) and myristic acid (14:0). Obese tissues contained the most 12:0

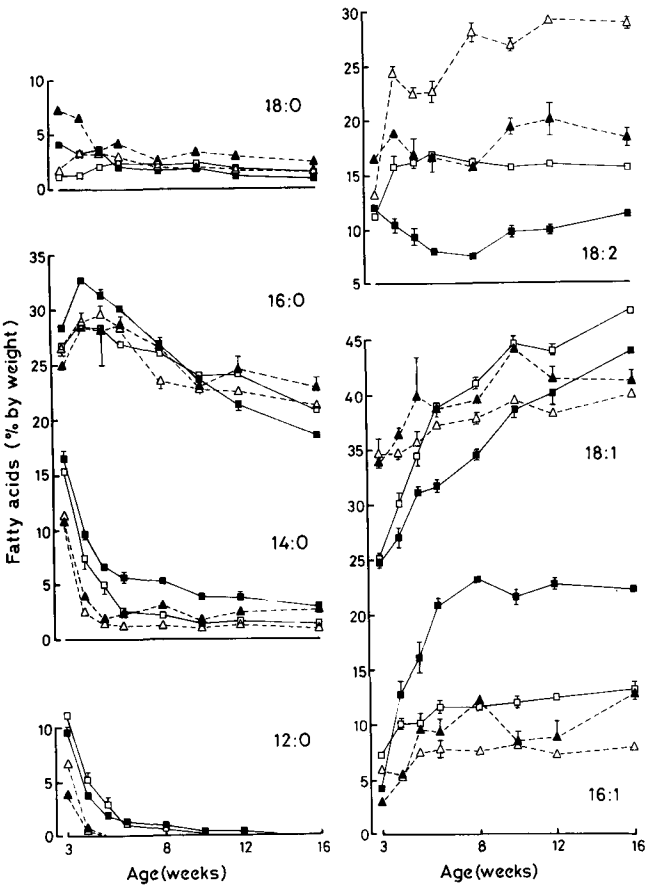

FIG. 1. Changes with age in the proportions of the major fatty acids in brown and white scapular adipose tissue of obese and lean mice. Symbols show mean percent by weight ± SEM for the following numbers of obese animals, with lean the same as obese unless in parentheses: ages 3 wk, 6; 4 wk, 7(8); 5 wk, 6(3); 6 wk, 8; 8 wk, 6; 10 wk, 4; 12 wk, 7(5); 16 wk, 10. ■, Obese brown; □, obese white; ▲, lean brown; △, lean white.

and 14:0, and obese WAT also contained 2% capric acid (10:0). By four weeks, the proportions of these fatty acids were halved in all four groups.

The proportion of palmitic acid (16:0) reached a maximum at the age of 4–5 weeks, with obese BAT having the most at 33%; thereafter it decreased in all groups until the age of 10 weeks, when some leveling-out occurred. Obese BAT showed the steepest fall in 16:0 and was lowest at 16 weeks with 19%. Stearic acid (18:0) was significantly higher in lean BAT than in any other group for most of the period studied.

At three weeks, 16:1 was low though significantly different in the tissues examined. Up to six weeks, 16:1 in both lean tissues and obese WAT increased; after this age, the proportion stabilized in the WAT, obese WAT having more (at 11–13%) than lean WAT (at 8%), but in lean BAT it varied between 8 and 12% after the age of six weeks. In obese BAT, 16:1 increased until about eight weeks of age, when 23% was present, which was double or even treble that in the other three types of tissue.

At three weeks, the obese tissues had considerably less oleic acid (18:1) (at 25%) than the lean tissues (at 34%), but this pattern was reversed by 16 weeks. The concentration of 18:1 increased in all groups; obese WAT showed

the greatest gain. Obese BAT had significantly less 18:1 than obese WAT after 5 weeks of age, while lean WAT gained least.

Lean WAT and BAT had significantly more 18:2 at weaning. In lean WAT, within a week of weaning, linoleic acid had increased from 13% to over 22%, and later increased to about 29%, significantly higher than in the other groups. In lean BAT, 18:2 fluctuated during the period studied, while obese WAT gained a small amount by the fourth week, then stabilized at a proportion of about 16%. Obese BAT differed significantly from the other groups in showing a decrease in 18:2 until eight weeks of age; thereafter there was a slight increase to 11%, which was still significantly lower than in the other groups.

Transplantation: first experiment. Ages and weights of donor and host mice are shown in Table 2. Mean fatty acid compositions of host BAT and grafts from the first transplantation experiment were compared by analysis of variance (Table 3). Lean host BAT differed, as expected, from obese host BAT.

Grafts of obese BAT removed from lean host mice had similar amounts of 14:0, 16:1 and 18:1 to the host BAT, but significantly less 16:0 and 18:0 and significantly more 18:2. Comparison of the graft with the initial fatty acid composition of the obese donor tissue (Table 3) showed that 14:0 and particularly 16:1 had decreased considerably, while the proportions of 16:0, 18:0 and 18:2 had increased.

Lean BAT grafted into obese hosts also resembled the host tissue in the percentage of 14:0 and 16:0, but 16:1 was significantly higher. Stearic acid and oleic acid were lower compared with the host BAT and 18:2 was higher. During the hosting period, the proportion of 16:1 apparently increased in the graft while those of 16:1 and 18:0 decreased along with 18:1.

Grafts of obese BAT in obese hosts were similar to the host BAT in the proportions of 14:0, 16:0 and 16:1. Stearic and oleic acids in the grafts were slightly lower than in the host BAT and 18:2 was higher. Comparison of the graft with the initial donor tissue revealed that 16:1 was lowered, 18:0 and 18:1 did not change and 18:2 increased.

As with the obese-BAT-into-obese-host transplants, the proportions of 14:0, 16:0 and 16:1 in lean BAT grafted into lean hosts were not different between grafts and hosts; 18:0 and 18:1 were lower in grafts, while 18:2 was higher. The grafts appear to have gained 16C fatty acids, lost 18:0 and 18:1 and retained the proportion of 18:2. The proportions of fatty acids in grafts from either lean or obese donors into lean hosts were not significantly different. Likewise, grafts from either lean or obese donors into obese hosts were not different except for the proportion of 14:0. Conversely, grafts taken from lean hosts differed significantly from grafts taken from obese hosts, irrespective of donor.

Transplantation: second experiment. Because "self" transplants differed in fatty acid composition from the host BAT but only numbered four of each type, a second experiment was performed to confirm the results. WAT also was sampled from the perirenal depot adjacent to the site of the grafts in the hosts. Ages and weights of mice are shown in Table 2. Analysis of variance (Table 4) showed that lean and obese host BAT differed except for 14:0 and 18:2. Lean hosts were similar to those in the previous experiment but obese hosts had lower 16:0 and 16:1 and higher 18:2 than those in the first experiment. Obese host perirenal WAT and scapular BAT had similar proportions of 16:1 and 18:2 but different 16:0. Lean host perirenal WAT differed from lean host BAT for all fatty acids except 18:1.

Compared to obese host BAT, the proportions of the major fatty acids in grafted obese BAT tissue showed no differences except for having lower 18:1 and higher 18:2 than host BAT. Compared with the fatty acid composition of the donor BAT, 14:0, 16:0 and 16:1 were lowered, while 18:1 and 18:2 were increased. Grafts differed from the host perirenal WAT in having higher 14:0 and lower 16:0.

Lean BAT grafted into lean hosts had less 16:0 but more 16:1 and 18:2 than the host BAT; all other fatty acids were similar. As the initial donor tissue was mostly similar to the host BAT, these differences represented

TABLE 2

Ages and Body Weights of Donor and Host Mice in Transplantation Experiments

Experiment	Type of animal	Stage	Lean		Obese	
			Age (wk)	Weight (g)	Age (wk)	Weight (g)
First	Donor	Initial	8.5	24.0 ± 1.0(2)[a]	8.5	38.0(1)
	Hosts for lean grafts	Initial	4.5-6.5	16.7 ± 0.9(3)	4.5-6.5	20.1 ± 1.0(6)
		Final	9-11	23.8 ± 0.7	9-11	40.8 ± 0.6
	Hosts for obese grafts	Initial	4.5-6.5	18.1 ± 0.8(7)	4.5-6.5	19.4 ± 1.4(3)
		Final	9-11	25.3 ± 0.5	9-11	39.5 ± 1.3
Second	Donor	Initial	8	23.0(1)	7	28.4(1)
	Hosts for lean grafts	Initial	8	23.0 ± 0.2(3)	—	—
		Final	12.5	27.3 ± 0.4	—	—
	Hosts for obese grafts	Initial	—	—	5-7	25.3 ± 2.0(3)
		Final	—	—	9.5-11.5	41.7 ± 2.1

[a]Means ± SEM for the number of mice in parentheses.

TABLE 3

Proportions of Major Fatty Acids (% by Wt) in the Donor, Graft and Host Brown Adipose Tissue: Experiment 1

	Grafts from obese donors					Grafts from lean donors				
		Lean hosts		Obese hosts			Lean hosts		Obese hosts	
Fatty acid	Donor (1)	Graft (11)	Host (7)	Graft (4)	Host (3)	Donor (2)	Graft (4)	Host (3)	Graft (11)	Host (6)
14:0	5.3*	2.7 ± 0.2^{ab,†}	2.9 ± 0.1^{abc}	3.6 ± 0.3^{c}	4.0 ± 0.1^{c}	1.9	2.9 ± 0.1^{abc}	2.6 ± 0.4^{a}	3.4 ± 0.2^{bc}	3.4 ± 0.1^{bc}
16:0	24.0	29.7 ± 0.8^{b}	34.7 ± 1.0^{d}	26.0 ± 1.2^{a}	25.1 ± 0.6^{a}	26.8	31.5 ± 0.4^{bc}	33.7 ± 1.3^{cd}	26.4 ± 0.5^{a}	26.1 ± 0.3^{a}
16:1	23.3	9.4 ± 0.3^{b}	7.5 ± 0.3^{ab}	17.9 ± 1.3^{d}	18.9 ± 1.8^{d}	5.8	7.8 ± 1.2^{ab}	7.0 ± 0.7^{a}	18.1 ± 0.8^{d}	15.4 ± 0.5^{c}
18:0	1.4	2.5 ± 0.2^{d}	5.0 ± 0.2^{e}	1.3 ± 0.1^{a}	1.9 ± 0.2^{bc}	3.5	2.6 ± 0.3^{d}	5.0 ± 0.2^{e}	1.4 ± 0.1^{ab}	2.1 ± 0.1^{cd}
18:1	37.1	36.8 ± 0.7^{a}	38.5 ± 1.0^{ab}	37.0 ± 1.2^{a}	41.3 ± 1.0^{cd}	43.0	36.6 ± 1.0^{a}	40.1 ± 1.5^{bc}	38.4 ± 0.9^{ab}	44.0 ± 0.4^{d}
18:2	8.8	18.8 ± 0.9^{d}	11.5 ± 0.4^{b}	14.3 ± 1.0^{c}	8.8 ± 1.4^{a}	19.1	18.6 ± 1.3^{d}	11.6 ± 0.8^{b}	12.4 ± 0.5^{bc}	9.1 ± 0.4^{a}

*Results are means (± SEM) for the number of mice or grafts in parenthesis; donor and host tissues were sampled in duplicate.
†Numbers within a line with different superscript letters differ significantly, $P < 0.05$.

TABLE 4

Proportions of the Major Fatty Acids (% by Wt) in the Donor, Graft and Host Brown Adipose Tissue and Host White Adipose Tissue: Experiment 2

	Obese				Lean			
Fatty acid	Donor (1)	Graft (6)	Host BAT (3)	Host WAT (3)	Donor (1)	Graft (6)	Host BAT (3)	Host WAT (3)
14:0	4.7*	2.5 ± 0.2^{b,†}	2.9 ± 0.1^{b}	1.6 ± 0.1^{a}	3.4	2.9 ± 0.3^{b}	2.7 ± 0.1^{b}	1.6 ± 0.1^{a}
16:0	29.9	23.7 ± 1.1^{a}	22.1 ± 0.5^{a}	29.1 ± 1.2^{b}	32.5	27.3 ± 1.1^{b}	33.9 ± 0.9^{c}	28.2 ± 0.3^{b}
16:1	20.8	14.0 ± 0.3^{c}	13.8 ± 1.0^{c}	12.9 ± 0.5^{c}	10.9	10.1 ± 0.3^{b}	8.2 ± 0.7^{a}	10.8 ± 0.7^{b}
18:0	3.2	3.0 ± 0.6^{a}	2.9 ± 0.1^{a}	3.0 ± 0.4^{a}	5.3	6.2 ± 0.8^{b}	6.5 ± 0.2^{b}	2.9 ± 0.3^{a}
18:1	34.8	40.8 ± 1.4^{c}	46.1 ± 0.8^{d}	38.4 ± 0.2^{bc}	36.5	36.8 ± 1.5^{ab}	36.7 ± 0.6^{ab}	34.5 ± 0.4^{a}
18:2	6.7	16.0 ± 0.6^{b}	12.3 ± 1.0^{a}	15.1 ± 1.3^{ab}	11.5	16.6 ± 1.3^{b}	12.0 ± 1.0^{a}	22.0 ± 0.6^{c}

*Results are means (± SEM) for the number of mice or grafts in parenthesis; donor and host tissues were sampled in duplicate.
†Numbers within a line with different superscript letters differ significantly, $P < 0.05$.

an apparent loss of 16:0, retention of 16:1 and gain of 18:2 by the grafted tissue over the period of transplantation. Grafts were similar to lean host perirenal WAT in the proportions of 16C fatty acids and 18:1, but 14:0 and 18:0 were higher and 18:2 was lower in the grafts.

To isolate and examine the effects of transplantation in the two experiments more closely and eliminate the effects of age, the percentage value for each fatty acid in the initial donor was subtracted from each related graft value to give "change in graft" (ΔG) and from the related host value to give the difference between host and donor or (H-D) (Fig. 2). Paired t-tests showed that obese tissue grafted into obese hosts was most similar in fatty acid composition to the host BAT, while obese grafts in lean hosts displayed significant differences ($p < 0.05$) in all five major fatty acids. All four types of graft had significantly more 18:2 than their host BAT ($p < 0.01$), but regression analysis of ΔG on H-D showed a good relationship ($r = 0.934$, $p < 0.001$) (Fig. 3). Only obese tissue grafted into obese hosts had similar 16:1 to host BAT ($p > 0.05$). However, this fatty acid yielded the best relationship when regression analysis was carried out on the ΔG and H-D values ($r = 0.978$, $p < 0.001$) (Fig. 3).

DISCUSSION

The proportions of the triacylglycerol fatty acids in the scapular BAT of obese mice clearly differ from those in lean mice, and they also differ from those of the WAT in the adjacent depot. At 16 weeks of age the order of increasing concentration of 16:1 in the tissues—lean WAT, lean BAT, obese WAT, obese BAT—is similar to the reported relative rates of fatty acid synthesis (14–16). The concentration of 18:2 decreases in the same tissue order, which also suggests dilution of this dietary fatty acid by fatty acids synthesized in vivo.

Fatty acid compositions differed as early as three weeks of age. Differences between obese and lean mice at this age could be expected in that *ob/ob* pups already have four times as much adipose tissue as lean pups (6). The high proportions of 12:0 and 14:0 presumably reflect the diet of milk which is reported to contain 13% of each of these fatty acids in this strain of mice (26). The higher proportions of these fatty acids in the adipose tissues of obese mice are presumably related to their decreased energy expenditure (27). This makes more of the milk fatty acids available for deposition, even though *ob/ob* pups do not have a higher intake of milk (28).

The marked changes in the fatty acid compositions of all the tissues studied after three weeks coincide with the change from a high fat to a high carbohydrate diet, with increased rates of fatty acid synthesis (6) and with the

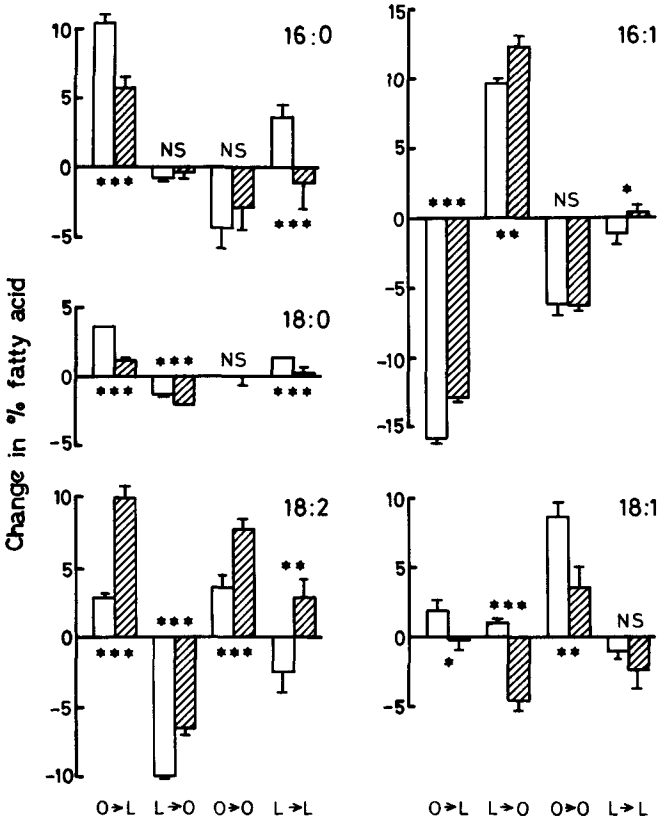

FIG. 2. Comparison of the changes in the proportions of the major fatty acids in the grafted tissue (ΔG) with the difference between the proportions in host and donor tissues (H-D). Results from the two experiments are expressed as means ± SEM for H-D (= % by weight in host − % in donor), open, and ΔG (= % in graft − % in donor), hatched, for the four types of graft (numbers in parentheses): O → L, obese tissue grafted into lean host (11); L → O, lean tissue grafted into obese host (11); O → O, obese tissue grafted into obese host (10); L → L, lean tissue grafted into lean host (10). Significance of difference between H-D and ΔG: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significantly different, $P > 0.05$, by paired t-test.

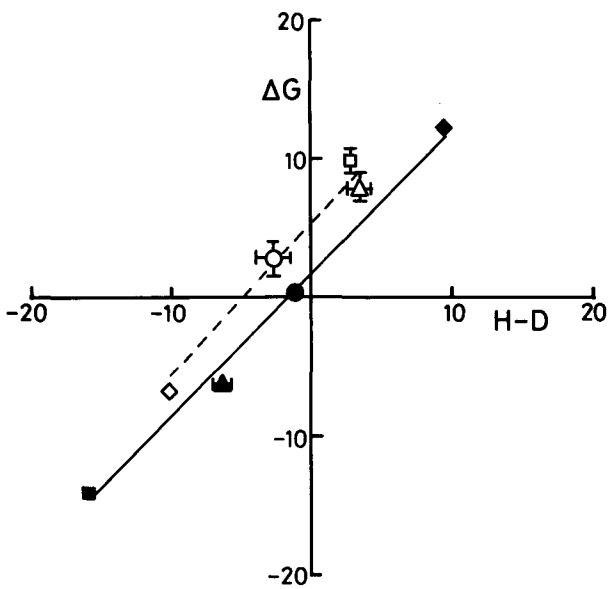

FIG. 3. Relationship between change in proportion of fatty acid in grafted tissue (ΔG) and the difference between the proportions in host and donor tissue (H-D) for 16:1 and 18:2. For origin of ΔG and H-D see legend to Figure 2. Regression constants were for 16:1, $r = 0.978$, $P < 0.001$; $y = 1.644 + 1.028x$ (——) and for 18:2, $r = 0.934$, $P < 0.001$; $y = 5.200 + 1.091x$ (— — —). Symbols show means (± SEM) for 16:1 (closed) and 18:2 (open) (numbers in parentheses): ■, □, obese tissue grafted into lean host (11); ◆, ◇, lean tissue grafted into obese host (11); ▲, △, obese tissue grafted into obese host (10); ●, ○, lean tissue grafted into lean host (10).

growth of adipose tissue. Thus the proportions of the medium chain fatty acids fall as they are lost by turnover and/or diluted by synthesized fatty acids. In obese mice where rates of fatty acid synthesis and fat deposition are higher, we see large increases in the monounsaturated fatty acids. The high proportion of 16:1 in the *ob/ob* BAT presumably reflects decreased palmitoyl-CoA elongation and/or the preferential desaturation of palmitoyl-CoA, compared to *ob/ob* WAT. An initial slow development of elongation might account for the peak in 16:0 at 4 weeks and the lag in 18:1 production in the BAT of obese mice. High rates of fatty acid synthesis de novo in obese mice soon after weaning (15) may overload the elongating system.

The BAT of obese mice has a higher proportion of monounsaturated fatty acids than the other tissues that we studied, and even after 16 weeks there could be a further increase in both BAT and WAT, indicative of high rates of synthesis and in agreement with reported rates (14-16). BAT in the Aston strain of obese mice is reported to have lower rates of fatty acid synthesis after five weeks of age compared to lean controls (15), but the Aston mice are at least 40% heavier (6,15) and have about 60% less scapular BAT than C57Bl mice. The relative weights of the interscapular BAT for C57Bl mice as used in these studies (obese, 16 g/kg body weight [BW]; lean, 7.4 g/kg BW, age 16 weeks) could well be double the values for Aston mice at similar age (from data in ref. 15 for 13-week-old mice: obese, ca. 6.5 g/kg BW; lean, ca. 3.0 g/kg BW). This suggests that the BAT of C57Bl mice would carry out a much greater proportion of the whole body lipid synthesis.

Overall, the differences in fatty acid composition between obese and lean BAT appear to be an exaggeration of those between obese and lean WAT and do not suggest any particular effects due to the abnormalities of obese mouse BAT. Other studies on mice and rats tend to report a lower proportion of monoenes or a higher proportion of saturated fatty acids in BAT compared with WAT (11,29-31). However, the ratio of monounsaturated to saturated fatty acids (M/S) for WAT and BAT from our lean mice was similar at a given age.

Transplantation of obese BAT into lean mice was more effective in normalizing the proportion of 16:1 than the proportion of 18:2. Grafts of lean tissue into obese mice and "self" grafts also appeared able to adapt their metabolism with respect to 16:1. This would indicate that the tissue was functioning correctly under conditions of both high and low fatty acid synthesis. However, the proportion of 18:2 in all grafts was consistently higher than in hosts: the increment was 6-7% for grafts into lean hosts and 3-4% for grafts into obese hosts. The residual differences between graft and host for 18:2 and the much smaller differences for 16:1 both appear to be transplantation effects, as indicated by the highly significant linear regression of ΔG on H-D (Fig. 3). Conditions of restricted feeding or starvation, when the rates of fatty acid synthesis may be low and rates of lipolysis increased, tend to cause conservation of 18:2 in the lipids of both obese and lean mice (3,32). A lowering of 18:1 can also occur under these conditions, and as many grafts had lower proportions of 18:1 than hosts, grafts of BAT may need a longer period than one mo to integrate with their hosts and reverse a possible initial loss of lipid.

We do not yet know the extent of the restoration of the vascular system and the sympathetic innervation in the grafted BAT; lack of reinnervation, particularly the sympathetic innervation of the cells, could also explain the residual graft-host differences of 16:1 and 18:2. However, in the rat, sympathetic innervation appears important only in regulating fatty acid synthesis during increased thermogenesis in response to cold or overfeeding and not during normal conditions (36). Grafts of WAT, which lack the cell innervation, do not show residual differences in fatty acid composition from the host after one month (24). By then they are well-vascularized (33), presumably with the accompanying sympathetic innervation of the blood vessels. Suitable sympathetic innervation for BAT grafts is available in the area of the kidney since functional perirenal BAT with dual innervation has been demonstrated (8,34,35).

Our conclusion from these studies is that there are significant differences between the fatty acid composition of BAT from lean and obese mice kept at 20-24 C which are akin to the differences between the composition of WAT. The differences appear to arise from differences in the rates of fatty acid synthesis within the tissues. The acquisition of the fatty acid composition of the host by transplanted BAT indicates that there are no expressed genetic differences in fatty acid synthesis and deposition between obese and lean BAT. The reported absence of an effect of denervation on the rate of fatty acid synthesis in BAT of normal chow-fed rats (36) suggests that sympathetic stimulation may not be the mediator of increased lipogenesis. A more likely candidate is the greater sensitivity of BAT than WAT to insulin-stimulated lipogenesis (37) and the hyperinsulinemia of obese mice.

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Desaturation and Chain Elongation of Essential Fatty Acids in Isolated Liver Cells from Rat and Rainbow Trout

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Isolated hepatocytes from rainbow trout and rat were incubated with ^{14}C-labeled linoleic acid, linolenic acid, dihomogammalinolenic acid or eicosapentaenoic acid. The most striking difference in the desaturase activity was the lower level of Δ5 desaturase in trout than in rat. No Δ4 desaturation of 22:4(n-6) to 22:5(n-6) was observed in either of the two species, while the conversion of 22:5(n-3) to 22:6(n-3) was significant in both groups and highest in rainbow trout. The chain-elongating activity was remarkably similar in the two species, except for the "dead-end" elongation which was distinctly more important in fish. *Lipids 21,* 202–205 (1986).

Fish contain a higher proportion of n-3 polyunsaturated fatty acids than land mammals, which have high concentrations of n-6 fatty acids, especially arachidonic acid and linoleic acid. The metabolism of essential fatty acids, including the desaturation reactions in mammals, are known to be regulated by both dietary and hormonal factors (1–4).

Except for studies of the influence of temperature on the metabolism of fatty acids (5–8), little is known about the regulation of the desaturation, elongation and esterification enzyme systems in fish.

We have compared the metabolism of some essential fatty acids in isolated liver cells from rainbow trout *(Salmo gairdneri)* and rat. The different desaturation and chain elongation steps were studied by using linoleic acid and dihomogammalinolenic acid of the n-6 series and linolenic acid and eicosapentaenoic acid of the n-3 series as substrates.

MATERIALS AND METHODS

Parenchymal liver cells were prepared (9) from male weanling rats of the Wistar strain (from Møllegaard Laboratory, Havdrup, Denmark) fed a commercial pellet diet and from rainbow trout maintained on a commercial pellet diet at 6 C. The rat diet was from Ewas-Alab (Soedertaelje, Sweden) and contained, by weight, 6% lipids, 40% protein and 25% carbohydrates. The trout diet was from Felleskjoepet (Stavanger, Norway) with a similar composition of protein and carbohydrates as in the rat diet, but with 15% lipids by weight. Trout liver cells were isolated using the same method and perfusion fluids as for the rat, but with the following exceptions: the liver was perfused in situ, the perfusion solution containing collagenase was not recirculated and the temperature of the perfusion solutions was 12–15 C (10).

The concentration of cells in the stock suspension was approximately 6×10^6 cells/ml, and 90–95% were viable, as measured by resistance to uptake of trypan blue.

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Cells were incubated in an oxygenated suspension medium (11) with 1.5% (v/v) bovine serum albumin and 10 mM (+) lactate, final concentrations. One ml of the cell suspension (in a total volume of 2 ml) was incubated with 172 nmol of [1-^{14}C]linoleic acid, [1-^{14}C]linolenic acid (both from Radiochemical Centre, Amersham, United Kingdom), [1-^{14}C]dihomogammalinolenic acid or [1-^{14}C]eicosapentaenoic acid (both from New England Nuclear, Boston, Massachusetts). The specific activity of labeled fatty acid was 7 mCi/mmol.

The incubation temperature was 37 C for rat hepatocytes and 12 C for trout hepatocytes.

The measurement of radioactive soluble products and of radioactive CO_2 was performed as described by Christiansen (12). The lipids were extracted by the method of Folch et al. (13) and separated on silicic acid thin layer plates (Stahl H+) (hexane/diethylether/glacial acetic acid, 80:20:1, v/v/v). Aliquots of the total lipid extracts and the free fatty acid, triacylglycerol and phospholipid fractions were transmethylated (14) and analyzed by radiogas chromatography (15). Cellular protein was determined according to the method of Lowry et al. (16).

RESULTS AND DISCUSSION

Δ4 Desaturase activity was studied by using eicosapentaenoic acid [20:5(n-3)] as the initial substrate. In trout hepatocytes the fraction of labeled 22:5(n-3) converted to 22:6(n-3) was nearly two times higher (23%) than with rat hepatocytes (13%) (Table 1). With 20:3(n-6) as the initial substrate, little 22:4(n-6) was formed, and no Δ4 desaturation could be demonstrated in either of the two species (Table 2).

In isolated rat liver cells, conversion of 22:4(n-6) to 22:5(n-6) previously has been demonstrated only in animals fed an essential fatty acid-deficient diet, known to stimulate the Δ4 desaturase activity (15). In fish, a very low conversion of 22:4(n-6) to 22:5(n-6) has previously been shown in isolated hepatocytes incubated with labeled linoleic acid (1–3).

When Δ5 desaturase activity was estimated by using [1-^{14}C]dihomogammalinolenic acid [20:3(n-6)] as substrate for trout hepatocytes, only a small fraction (13%) was converted to arachidonic acid [20:4(n-6)], with a subsequent and partial chain elongation to docosatetraenoic acid [22:4(n-6)] (Table 2). With rat hepatocytes, three to four times more (48%) of the 20:3(n-6) substrate was desaturated. Previous work with isolated liver microsomes has also demonstrated a lower Δ5 desaturase activity in a species of fish *(Bagre amarillo)* than in the rat (17).

Also with 18:2(n-6) as substrate, the Δ5 desaturase activity was studied by calculating the sum of [^{14}C]labeled 20:4 and 22:4 in percent of the sum of 20:4, 22:4 and 20:3. Although the result may be influenced by different rates of Δ6 desaturation and C_{18} chain elongation in the two species, the Δ5 desaturase activity was found by this method to be higher in rat (48%) than in trout (41%).

TABLE 1

[^{14}C]Labeled Fatty Acids in Isolated Hepatocytes from Rainbow Trout and Rat, Incubated with [^{14}C]Linolenic Acid [18:3(n-3)] or [^{14}C]Eicosapentaenoic Acid [20:5(n-3)]

	18:3		20:5	
Fatty acid substrate	Rainbow trout	Rat	Rainbow trout	Rat
Fatty acid in phospholipid + triacylglycerol				
22:6	0.24 ± 0.05	0.04 ± 0.01	0.65 ± 0.11	0.16 ± 0.05
22:5	0.13 ± 0.06	0.17 ± 0.04	1.95 ± 0.04	1.15 ± 0.02
20:5	0.37 ± 0.09	0.49 ± 0.15	6.82 ± 0.15	4.08 ± 0.20
20:4	0.65 ± 0.13	0.18 ± 0.02		
20:3	1.13 ± 0.16	0.14 ± 0.03		
18:4	0.11 ± 0.03	0.11 ± 0.03		
18:3	8.71 ± 0.39	4.79 ± 0.38		
Phospholipids	2.25 ± 0.14	0.79 ± 0.19	2.16 ± 0.09	1.50 ± 0.39
Triacylglycerol	7.08 ± 0.24	5.06 ± 0.22	7.05 ± 0.24	3.95 ± 0.41
Free fatty acids	2.20 ± 0.22	0.05 ± 0.00	1.89 ± 0.11	0.06 ± 0.00
Oxidation products	traces	1.24 ± 0.04	traces	1.09 ± 0.02

Incubation conditions as described in the text. Labeled fatty acids (0.07 mM) and hepatocytes from rat (23.0–25.8 mg of protein) and rainbow trout (13.6–15.9 mg of protein) were incubated for 180 min. The results are expressed as nmol/mg of protein of [^{14}C]labeled fatty acid esterified, oxidized or remaining as free fatty acids. Means ± SD of two parallel incubations from hepatocytes from four different livers are given for each species.

TABLE 2

[^{14}C]Labeled Fatty Acids in Isolated Hepatocytes from Rainbow Trout and Rat, Incubated with [^{14}C]Linoleic Acid [18:2(n-6)] or [^{14}C]Dihomogammalinolenic Acid [20:3(n-6)]

	18:2		20:3	
Fatty acid substrate	Rainbow trout	Rat	Rainbow trout	rat
Fatty acid in phospholipid + triacylglycerol				
22:4			0.11 ± 0.06	0.09 ± 0.03
22:3			0.28 ± 0.05	
20:4	0.18 ± 0.02	0.17 ± 0.02	0.85 ± 0.16	2.73 ± 0.18
20:3	0.26 ± 0.07	0.18 ± 0.05	6.45 ± 0.39	3.01 ± 0.2
20:2	0.96 ± 0.11	0.12 ± 0.03		
18:3	0.10 ± 0.07	0.10 ± 0.02		
18:2	3.70 ± 0.20	5.57 ± 0.07		
Phospholipids	2.12 ± 0.03	2.22 ± 0.11	2.37 ± 0.22	3.40 ± 0.21
Triacylglycerol	2.72 ± 0.38	4.38 ± 0.07	5.77 ± 0.28	2.46 ± 0.20
Free fatty acids	6.70 ± 0.42	0.06 ± 0.00	3.90 ± 0.55	0.05 ± 0.00
Oxidation products	traces	0.57 ± 0.05	traces	0.70 ± 0.01

Incubation conditions as described in the text. Labeled fatty acids (0.07 mM) and hepatocytes from rat (23.0–25.8 mg of protein) and rainbow trout (13.6–15.9 mg of protein) were incubated for 180 min. The results are expressed as nmol/mg of protein of [^{14}C]labeled fatty acid esterified, oxidized or remaining as free fatty acids. Means ± SD of two parallel incubations from hepatocytes from four different livers are given for each species.

With linolenic acid [18:3(n-3)] as substrate, the Δ5 desaturase activity calculated as the conversion of labeled 20:4(n-3) to 20:5(n-3) (Table 2) was also higher in rat hepatocytes (77%) than in trout hepatocytes (55%).

Δ6 Desaturase appeared to be nearly equally active in the two species. Approximately 10% of the 18:2(n-6) substrate metabolized was desaturated in both trout and rat hepatocytes under the conditions used. A larger fraction of the substrate was Δ6 desaturated with 18:3(n-3) (17%) than with 18:2(n-6) as the substrate in rat liver cells, in agreement with the view that the enzyme prefers the n-3 substrate (18).

The chain elongations of 18:3(n-6) to 20:3(n-6), of 18:4(n-3) to 20:4(n-3) and of 20:5(n-3) to 22:5(n-3) were remarkably similar in rat hepatocytes and rainbow trout (Tables 1 and 2). The elongated fatty acids thus formed are all normal intermediates in the synthesis of polyunsaturated n-6 and n-3 fatty acids. In contrast, the chain elongation of 20:4(n-6) to 22:4(n-6) was more efficient in fish than in rat when labeled 20:3(n-6) was used as substrate.

When fish hepatocytes were incubated with labeled 18:2(n-6) or 18:3(n-3), 15–20% of labeled fatty acid was recovered as 20:2(n-6) and 20:3(n-3), respectively. In rat hepatocytes only traces of such "dead-end" chain elongation products were recovered. With 20:3(n-6) as substrate,

22:3(n-6) was formed in distinct amounts with trout hepatocytes but was not detectable with rat liver cells.

The dead-end elongation products 22:3(n-6), 20:2(n-6) and 20:3(n-3) were preferentially incorporated into triacylglycerol (data not shown), indicating that such fatty acids are not preferred as membrane components. It is suggested that dead-end chain elongation products are incorporated into triacylglycerols, stored and later liberated for retroconversion (5) and subsequent utilization by desaturating and chain-elongating enzyme systems. However, very little of 22:3(n-6) and 20:2(n-6) are present in liver lipids of rainbow trout (6,7), indicating a rapid turnover.

Some of these results could be slightly modified by the presence of 5,11,14-20:3(n-6) and 5,11,14,17-20:4(n-3), which are the Δ5 desaturated products of 20:2(n-6) and 20:3(n-3), respectively (7,20,21). However, several studies have shown that these isomers represent only small parts of the total 20:3(n-6) and 20:4(n-3) fractions, both in rat (21) and trout (7).

Very little fatty acid was oxidized in trout hepatocytes (Tables 1 and 2).

The free fatty acid substrate was nearly completely metabolized in the experiments with rat hepatocytes. Tables 1 and 2 show that with trout hepatocytes, the amount of labeled free fatty acid recovered, as a measure of the rate of total fatty acid metabolism, was 15–55% of the total amount of substrate available. The slower fatty acid metabolism in trout cells is probably explained by the lower incubation temperature used. 18:3(n-3) was metabolized more rapidly than 18:2(n-6) in the trout cells (Tables 1 and 2). In previous experiments with rat hepatocytes we found that 18:3(n-3) is metabolized more rapidly than 18:2(n-6) in these cells also. Acyl-CoA synthetase in rat liver microsomes has a higher activity with 18:3(n-3) than with 18:2(n-6) as substrate (19).

A complicating factor in the interpretation of the above results is the difference in incubation temperature used for rat and trout cells. In the present study, trout hepatocytes also were incubated at 20 C (results not shown), but only minor changes were observed in desaturating and chain elongating activities compared with incubations at 12 C. With temperatures above 20 C, the viability of trout hepatocytes decreased rapidly with temperature increase. The present incubation temperatures were thus chosen to maintain conditions as physiological as possible for the two cell types.

The different pattern of endogenous fatty acids in liver phospholipids of fish and land mammals (Table 3) may be caused to a large extent by dietary factors. The ratio of n-6 to n-3 fatty acids was 0.1 in trout liver phospholipids and 3.0 in the rat liver phospholipids, while the ratios were 0.16 and 5.0 in trout and rat diets, respectively (Table 4).

The trout food contained very little linoleic acid [18:2(n-6)], only 1.5% of total fatty acids, while the rat diet contained 42%. Still, the trout phospholipids contained 5% 18:2(n-6), compared to 12.4% in rat liver. The 20:4(n-6) content in trout liver was only 3.9%, compared to 16% in the rat. This illustrates that the dietary fatty acid pattern such as the n-6/n-3 fatty acid ratio in the diet is not the only factor affecting the endogenous fatty acid pattern.

The rates of desaturation and chain elongation, which were remarkably similar in the rat and the trout, apparently cannot explain the very different patterns of endogenous phospholipid fatty acids in the two species. It is, however, possible that the higher rate of Δ4 desaturation in the trout may facilitate the formation of 22:6(n-3) in this species.

It is possible that factors other than diet and, to a smaller extent, desaturase activities are important in determining liver phospholipid fatty acid patterns. Different rates of acylation/deacylation of individual fatty acids in fish and mammals may favor the esterification of very long chain n-3 fatty acids in the trout liver phospholipids.

TABLE 3

Pattern of Endogenous Fatty Acids in Liver Phospholipids from Rainbow Trout and Rat

Fatty acid	Rainbow trout	Rat
16:0	22.2 ± 0.2	21.0 ± 0.4
16:1	3.9 ± 0.3	3.6 ± 0.3
18:0	16.7 ± 0.4	22.6 ± 2.3
18:1	2.8 ± 0.4	8.4 ± 1.5
18:2(n-6)	5.0 ± 0.1	12.4 ± 1.0
18:3(n-3)	1.7 ± 0.8	
20:1	2.7 ± 1.1	
20:3(n-9)		0.5 ± 0.1
20:3(n-6)		1.8 ± 0.7
20:3(n-3)	3.3 ± 0.1	
20:4(n-6)	3.9 ± 0.1	16.0 ± 0.3
20:5(n-3)	8.3 ± 0.3	
22:5(n-6)		0.2 ± 0.1
22:5(n-3)	1.3 ± 0.3	0.6 ± 0.1
22:6(n-3)	25.0 ± 2.4	9.9 ± 1.0
Unidentified	3.2 ± 0.1	0.8 ± 0.4
Ratio n-6/n-3	0.1	3.1

The quantity of each fatty acid is given in percent of total fatty acids. Means ± S.D. of two parallel analyses from three different livers are given.

TABLE 4

Fatty Acid Composition of Diets

Fatty acid	Rainbow trout diet	Rat diet
14:0	8.8	
14:1	1.0	
16:0	11.0	21.0
16:1	10.0	2.2
18:0	1.5	5.0
18:1	18.5	21.0
18:2(n-6)	1.5	42.0
18:3(n-3)	0.5	8.2
18:4(n-3)	1.5	
20:1	22.0	
20:5(n-3)	3.5	
22:1	15.0	
22:5(n-3)	1.0	
22:6(n-3)	3.0	
Ratio n-6/n-3	0.2	5.0

The quantity of each fatty acid is given as mol % of total fatty acids.

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Properties of Diacylglycerol Kinase Purified from Bovine Brain

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A nearly homogeneous but somewhat unstable diacylglycerol kinase (ca. MW 72,000 daltons) was purified from bovine brain by modification of the procedure of Kanoh et al. (Kanoh, H., Kondoh, H., and Ono, T. [1983] *J. Biol. Chem. 258,* 1767-1774). The purification consisted of four steps (brain cytosol isolation and successive chromatography on DEAE-cellulose, Sephadex G-25 for desalting and ATP-agarose) carried out in buffers stabilized with EDTA, ATP and dithiothreitol (DTT). Specific activities, determined within 4 hr of purification, ranged from 908-1857 nmol ATP incorporated/min/mg protein, with the variation reflecting the instability. Optimal activities required deoxycholate (0.1%), one of the phosphoglycerides [phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylserine (PS)] (0.025-0.25 mM), ATP (5 mM, apparent K_m = 0.57 mM), 1,2-dioleoyl-*rac*-glycerol (5 mM, apparent K_m = 1 mM) and Mg^{2+} (10 mM, apparent K_m = 2.2 mM). Phosphatidylinositol (PI) was slightly less effective than PC, PE or PS and noninhibitory in combination with PC, PE or PS. Relative to PC phosphatidic acid (PA) (52%), sphingomyelin (48%), lyso-PC (1.5%) and lyso-PI (28.6%) were less effective activators. The sulfhydryl reagents, *p*-chloromercuribenzoic acid (PCMB) (1.0 mM), *N*-ethylmaleimide (NEM) (1.0 and 2.0 mM) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (1.0 mM), showed strong inhibition of activity which was prevented by 0.5 mM DTT. In contrast to other reports, this purified enzyme showed no monoacylglycerol kinase activity. Comparison of diacylglycerols of varying fatty acid composition indicated that the enzyme showed a preference for substrates with at least one unsaturated fatty acid, particularly in the 2-position. With saturated fatty acids the order of preference was C_{10} and $C_{12} > C_{14} > C_{16} > C_{18}$. Such a pattern indicates that the enzyme shows little selectivity that favors the generation of particular molecular species of PA.
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Diacylglycerol kinase (EC 2.7.1.-) catalyzes the reaction

1,2-diacylglycerol + ATP → phosphatidic acid (PA) + ADP [I]

Activity of this enzyme was first reported in brain (1,2) and since then has been shown to occur widely in animal tissues (see [3] for refs.). Evidence also exists for the presence of monoacylglycerol kinase activity in brain (4,5) and liver (6) which in studies to date seems to be closely associated with the diacylglycerol kinase activity (5,6). Either 1-monoacyl- or 2-monoacylglycerol substrates are phosphorylated to form lyso-PA according to the reaction

1-monoacylglycerol or 2-monoacylglycerol + ATP → lyso-PA + ADP [II]

A previous study from this laboratory (5) has shown that these two kinase activities have a wide intracellular distribution.

Our interest in diacylglycerol kinase arises out of the two different ways in which this enzyme may play important roles in the metabolism of PI (5). The first is the requirement for this enzyme in what has proven to be the cycle of phosphatidylinositol 4,5 bis-phosphate, rather than PI, breakdown and resynthesis that occurs in intact tissues or cells in the response of certain cell receptors to external stimuli (7-10). In the more recent form of this cycle, there is a resultant formation of inositol triphosphate, which functions as a possible second messenger for Ca^{2+} release, and of diacylglycerol, which may be reused for PA formation by reaction I or function as an activator of protein kinase C. The second way relates to the possibility of this enzyme playing a role in the generation of selective molecular species of PA which might, for example, be used to generate the predominant 1-stearoyl, 2-arachidonoyl molecular species of PI that occurs in most tissues. In an earlier study (5) designed to assess this possibility, it was shown that the diacylglycerol and monoacylglycerol kinases of microsomal and cytosolic fractions of rat brain showed no pattern of selectivity or specificity that favored the generation of PAs or lyso-PAs with a preponderance of the 1-stearoyl, 2-arachidonoyl molecular species. However, some selectivity was shown for diacylglycerols and monoacylglycerols with unsaturated fatty acids, particularly if they are located in the 2-position.

In an extension of the above work, attempts were made to purify the acylglycerol kinase activities of brain to assess (i) whether the purified preparation exhibits both monoacylglycerol and diacylglycerol kinase activities and (ii) the properties, requirements and substrate specificity of such a preparation. Initial attempts were directed toward purification from rat brain cytosol and then, because more quantities were needed, toward bovine brain cytosol. As commented on later, maintenance of stability of the enzyme activity became the major problem to overcome. While this work was in progress, Kanoh et al. reported first the partial purification of a diacylglycerol kinase activity from rat liver cytosol which still possessed monoacylglycerol kinase activity (6) and then the purification of diacylglycerol kinase from pig brain cytosol (3), but mention is not made of whether any monoacylglycerol kinase activity exists in the latter preparation. In the study reported here we have, using a modification of the steps of Kanoh et al. (3), obtained a highly purified preparation from bovine brain which, although very unstable (necessitating measurement within 3-4 hr), exhibits only diacylglycerol kinase activity. Some of the properties, the lipid requirements and the substrate specificity of this purified diacylglycerol kinase are reported here.

MATERIALS AND METHODS

Materials. The [γ-^{32}P]ATP was purchased from the Amersham Corp. (Arlington Heights, Illinois) as the sodium salt of adenosine 5′-[γ-^{32}P]triphosphate (0.5-3.0 mCi/mmol). The monoacylglycerol, 2-oleoyl-*rac*-glycerol and the 1,2-diacylglycerols *sn* 1,2-di, 10:0, 12:0, 14:0, 16:0,

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18:1; *rac* 1,2-di 18:0, 18:1; *sn* 1-16:0, 2-18:1; *sn* 1-18:1, 2-16:0; *sn* 1-18:0, 2-18:1; *sn* 1-18:0, 2-18:2, and those derived from egg PC and pig liver PC were purchased from Serdary Research Lab. (London, Ontario, Canada) and prepared for use and used as described earlier (5). The following phospholipids (chromatographically pure) also were obtained from Serdary Research Lab.: PC from bovine brain, PE from pig liver, PS from bovine brain, PI from pig liver, PA from bovine brain, sphingomyelin from bovine brain, lyso-PC prepared from pig liver PC and lyso-PI prepared from pig liver PI. The phospholipid mixture from bovine brain cytosol was purified from a total lipid extract obtained by the method of Folch et al. (11). The phospholipids were separated from the neutral lipids using florisil columns as described by Jato-Rodriguez et al. (12). The proportions of individual phospholipids were determined by separating the individual phospholipids on thin layer chromatography, as carried out by Gray and Strickland (13), scraping off the individual bands (identified by authentic markers) and estimating their phosphorus content as described by Rouser et al. (14). DEAE-cellulose (DE 52, Whatman) was obtained from Mandel Scientific Co. (Rockwood, Ontario, Canada). The Sephadex G-25 and Sephadex G-150 came from Pharmacia (Canada) Ltd. (Dorval, Quebec, Canada) and the Ultragel AcA 44 (LKB) from Fisher Scientific Co. (Toronto, Ontario, Canada). ATP-agarose with ATP attached through the N^6-amino group with an 8-carbon spacer, DTT, PCMB and NEM were supplied by the Sigma Chemical Co. (St. Louis, Missouri). The SH reagent (Ellman's), DTNB, was obtained from the Aldrich Chemical Co. (Milwaukee, Wisconsin).

Preparation of bovine brain cytosol. Bovine cerebral hemispheres, obtained from the Thorndale Abattoir (Thorndale, Ontario, Canada), were placed on ice within 10 min after decapitation of the animal and transported on ice to the laboratory. The blood vessels and connective tissue were removed and the grey matter was dissected out. The grey matter fraction then was homogenized in a Waring blender with 2.5 ml buffer A/g tissue and centrifuged at 10,000 × g for 20 min and 100,000 × g for 1 hr essentially as described by Kanoh et al. (3). The buffer A of Kanoh et al. consisted of 0.25 M sucrose, 25 mM Tris-HCl (pH 7.4), 0.5 mM DTT, 50 μM ATP, 1 mM EDTA and 0.02% (w/v) sodium azide. The resulting 100,000 × g supernatant corresponding to the cytosol was stored in batches of 200 ml at −20 C until used.

Purification of diacylglycerol kinase. The cytosol (200 ml) was thawed and applied directly to a column (2.6 × 25 cm) of DEAE-cellulose previously equilibrated with 500 ml of buffer A. The column then was washed with 700 ml buffer A containing 50 mM NaCl, and the enzyme was eluted with a linear gradient of 100 mM-300 mM NaCl in 1000 ml buffer A. Fractions of 20 ml were collected into tubes containing 20 μmol EDTA and 1 μmol ATP in 1 ml buffer A. The active fractions were stored at −20 C until used for further purification.

Two of the active fractions from the DEAE column were thawed, pooled and desalted on a Sephadex G-25 column (1.5 × 12 cm). The desalted active enzyme fractions then were loaded onto an ATP-agarose column (1.5 × 8 cm) previously washed with 30 ml 7 M urea in 2 M KCl and 100 ml of buffer A. The flow rate for loading the ATP-agarose was carefully maintained at 15 ml/hr. After all of the enzyme fraction had passed onto the column, the flow was stopped for 30 min before washing with 100 ml buffer A containing 50 mM NaCl. The enzyme was eluted with a linear gradient of 50–450 mM NaCl in 150 ml buffer A. Fractions of 2 ml were collected into tubes containing 2 μmol EDTA, 1 μmol DTT and 0.1 μmol ATP in 0.1 ml of buffer A. The active fractions were pooled and used for enzyme studies within 4 hr because of the extreme instability of the preparation.

Diacylglycerol and monoacylglycerol kinase assays. Both assays were carried out essentially as described previously (5) with the exception that the incubation time at 37 C was reduced from 5 to 3 min. The phospholipids were added to the assay tubes in chloroform and the latter was removed by a stream of nitrogen before the addition of deoxycholate buffer and other components. The mono- and diacylglycerols were dispersed in deoxycholate-phosphate buffer (at 2.5 times the final concentrations) by sonication in three 15-sec bursts at room temperature using a microtip properly tuned and set at 4 (Branson Sonifier, W-350, Danbury, Connecticut).

Extraction and analysis of ^{32}P-incorporation into PA and lyso-PA. Further assessment showed that the methods of extraction used previously (5) were the most satisfactory. Accordingly, the method of McMurray (15) was used for the diacylglycerol kinase assay and that of Bremer (16) for the monoacylglycerol kinase assay. For each sample, one-quarter (1 of 4 ml) of the washed chloroform lipid extract (diacylglycerol kinase assay) and one-half (0.5 of 1 ml) of the washed *n*-butanol lipid extract (monoacylglycerol kinase assay) were placed in counting vials. After evaporation of the chloroform extract (butanol does not quench), 5 ml aqueous counting cocktail (Formula 963, Dupont NEN Research Products, Boston, Massachusetts) was added to each vial; counting in the ^{32}P channel was carried out in a Beckman LS 6800 liquid scintillation counter (Beckman Instruments, Irvine, California). The results have been expressed in terms of nmol [γ-^{32}P]ATP incorporated/min/mg protein.

Protein estimation. Protein was estimated using the Bio-Rad protein assay kit (Bio-Rad Lab. [Canada], Mississauga, Ontario, Canada) for cell fractions and where possible for fractions from the column runs. Where the amount of protein per fraction was small, as for the active peak from the ATP-agarose column, the active peaks were pooled and concentrated and the protein was estimated by the method of Lowry et al. (17).

RESULTS

The purified diacylglycerol kinase obtained from the ATP-agarose affinity column proved to be nearly homogeneous (some preparations gave a single band and others a major band of MW ca. 72,000 daltons on SDS polyacrylamide gel electrophoresis) but very unstable (commented on later), necessitating that all measurements be made within 4 hr. Assessment of requirements for optimal activity in addition to those for the substrates, ATP and diacylglycerol and the divalent cation, Mg^{2+}, showed a need for deoxycholate (already commonly used in tissue and tissue fraction assays) (3,5) and for phospholipid (e.g., PC). With neither deoxycholate nor PC present there is only low activity (e.g., 116 nmol ATP incorporated/min/mg protein); with deoxycholate or PC added separately

there is a four-fold increase in activity; and with both added there is a synergistic action resulting in an overall nine- to 10-fold increase in activity. As a result, unless otherwise noted, all assays of diacylglycerol and monoacylglycerol kinases were done with both activators added.

The effect of the addition of a variety of phospholipids either singly (all at ca. 0.25 mM) or as mixtures is shown in Tables 1 and 2. The activation by PE and PS were equal to or slightly greater than that of PC. PI showed 71%, PA 48% and sphingomyelin 52% of the activation of PC. Lyso-PI was only slightly effective (28.6%) and lyso-PC was ineffective (possibly even inhibitory) as an activator. With most enzyme preparations (a representative experiment is shown in Table 2), only small amounts (10 μg or 0.025 mM) of PC, PE or PS were required for maximal activation. Most assays were subsequently done with 50 μg PC added. The addition of mixtures of PC/PE/PS or PC/PE/PS/PI in the ratio present for the phospholipids of brain cytosol showed an activation slightly in excess of that for PC, but comparable to those for PE and PS. Brain cytosolic phospholipids added as a mixture also gave optimal activation of the purified diacylglycerol kinase. Apart from the requirement for phospholipid, the optimal conditions for the purified diacylglycerol kinase agreed well with those reported earlier (5) for microsomal and cytosolic activities. Kinetic parameters, obtained from Lineweaver-Burk plots, for ATP, diacylglycerol and $MgCl_2$ are listed in Table 3. Apparent K_m values of 0.57 mM, 1.09 mM and 2.17 mM were obtained for ATP, 1,2-dioleoyl-*rac*-glycerol and Mg^{2+}, respectively. The V_{max} values ranged from 1220–1905 nmol ATP incorporated/min/mg protein.

As DTT was one of the cofactors that helped to stabilize the diacylglycerol kinase activity, a study was made of the effect of a number of sulfhydryl reagents on the activity of this enzyme. The data obtained are presented in Table 4. PCMB at 0.5 mM (data not in table) showed some inhibition and at 1.0 mM showed strong inhibition of activity, which in each instance was almost completely prevented by the addition of 0.5 mM DTT. Similar observations for NEM were obtained for concentrations of 1.0 (data not in table) and 2.0 mM. With DTNB at 1.0 and 2.0 mM strong inhibition resulted, which was overcome

TABLE 1

Effects of the Addition of Various Phospholipids on Diacylglycerol Kinase Activity Purified from Bovine Brain

Phospholipid added (100 μg unless otherwise noted)	Specific activity[a] (expressed as percent of activity when PC is added)			
	Expt. 1	Expt. 2	Expt. 3	Average
None added	23.5	1.8	8.4	11.2
PC[b]	100	100	100	100
PE	118	98	—	108
PS	119	113	104	112
PI	84	69	61	72.3
PA	53	43	—	48
Sphingomyelin	71	33	—	52
Lyso-PC, 50 μg	0	2.9	—	1.5
Lyso-PI, 50 μg	18.8	38.3	—	28.6

[a]Diacylglycerol kinase was purified from brain cytosol as described in the Materials and Methods. Specific activities, expressed as nanomoles ATP incorporated/min/mg protein, ranged from 908–1857 with 1,2-dioleoyl-*rac*-glycerol as substrate and PC 100 μg or 0.25 mM from bovine brain added.

[b]In two separate experiments, PC from egg was shown to be equally effective to that from bovine brain. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid. Sources of phospholipids given in Materials and Methods.

TABLE 2

Effects of Varying Phospholipid Mixtures on the Activity of Diacylglycerol Kinase Purified from Bovine Brain

Phospholipids[a] and amounts added	Specific activity (nmol ATP incorporated/min/mg protein)
None added	435
PC, 10 μg	806
PC, 50 μg	875
PC, 100 μg	908
PE, 10 μg	1058
PE, 50 μg	1048
PS, 10 μg	1035
PS, 50 μg	1048
PI, 10 μg	960
PI, 50 μg	907
PC, 10 μg + PE, 5 μg + PS, 5 μg	1031
PC, 20 μg + PE, 10 μg + PS, 10 μg	1035
PC, 10 μg + PE, 5 μg + PS, 2.5 μg + PI, 2.5 μg	1085
PC, 20 μg + PE, 10 μg + PS, 5 μg	1112
Bovine brain cytosol phospholipids[b], 23 μg (PC/PE/PS/PI, 2:1:0.5:0.5)	977

[a]Abbreviations and sources of phospholipids as in Table 1.

[b]Phospholipids from bovine brain cytosol extracted and analyzed as described in Materials and Methods.

TABLE 3

Kinetic Parameters Obtained for Substrates and Cofactors of Diacylglycerol Kinase Purified from Bovine Brain

Substrate or cofactor	Kinetic parameters[a]	
	Apparent K_m (mM)	V_{max}
ATP	0.57	1639
Diacylglycerol (1,2-dioleoyl-*rac*-glycerol)	1.09	1905
$MgCl_2$[b]	2.17	1220

[a]The K_m and V_{max} values were determined from Lineweaver-Burk plots. Each substrate or cofactor was assessed with the other substrates or cofactor at optimum concentrations. V_{max} is expressed as nanomoles ATP incorporated/min/mg protein.

[b]In this study the concentration of $MgCl_2$ was corrected for any chelation by EDTA already present in the enzyme preparation.

by 0.5 mM only at the lower concentration. It is conceivable that higher concentrations of DTT would have prevented the inhibition at 2.0 mM. Interestingly, it was observed that iodoacetate at 2.0 and 4.0 mM was non-inhibitory.

Table 5 summarizes data obtained on the diacylglycerol and monoacylglycerol kinase activities of fractions obtained at various stages of purification. With the exception of the 100,000 × g pellet (data not shown) where the diacylglycerol kinase to monoacylglycerol kinase ratio averaged 13.4, this ratio for the other fractions, including the whole homogenate and cytosol in Table 4, fell within a range of 4–6. These ratios are a little lower than those reported for rat brain (5) but still are consistent with the possibility that the same enzyme possesses both activities. However, as purification was achieved on DEAE-cellulose and ATP-agarose, this ratio increased dramatically. Fractions from DEAE-cellulose gave an average ratio of 17.3 for diacylglycerol kinase to monoacylglycerol kinase. With ATP-agarose there was no monoacylglycerol kinase measurable in three of the preparations and there were very low (due to net counts of 2 to 3) activities in the other two preparations. This resulted in a very high ratio (1381 with the range from 399–∞ (Table 5). These findings strongly suggest that the purified diacylglycerol kinase possesses no monoacylglycerol kinase activity.

A number of the diacylglycerols compared in the previous study on rat brain fractions (5) again were compared using the purified diacylglycerol kinase from bovine brain (Table 6). The pattern observed is quite similar in the two studies. Under the conditions of assay the racemic *(rac)* and sterospecific *(sn)* synthetic 1,2-dioleins gave identical results. The *rac* preparation was, because of its ready availability, used as the reference diacylglycerol and was arbitrarily set at 1.0. It is evident that the presence of unsaturation in at least one of the fatty acids is necessary for optimal activity. In the one comparison made

TABLE 4

Effects of Sulfhydryl Inhibitors on the Activity of Diacylglycerol Kinase Purified from Bovine Brain

Inhibitor[a] and concentration	Specific activity (nmol ATP incorporated/min/mg protein)	Percent of control
Control	1288 (1159, 1416)	100
Control + DTT[b], 0.5 mM	1314 (1126, 1501)	100
PCMB, 1.0 mM	136 (0, 271)	9.6
PCMB, 1.0 mM + DTT, 0.5 mM	1205 (1156, 1254)	93.1
NEM, 2.0 mM	247 (286, 211)	20.2
NEM, 2.0 mM + DTT, 0.5 mM	1259 (1226, 1292)	97.5
DTNB, 1.0 mM	194 (194, 193)	15.2
DTNB, 1.0 mM + DTT, 0.5 mM	1290 (1209, 1371)	99.4
DTNB, 2.0 mM	122 (143, 101)	9.7
DTNB, 2.0 mM + DTT, 0.5 mM	217 (232, 202)	17.0

[a]Inhibitor abbreviations are as in footnote.

[b]DTT (dithiothreitol) is added to overcome inhibitions by the above sulfhydryl reagents.

TABLE 5

Diacylglycerol (DG) and Monoacylglycerol (MG) Kinase Activities in Fractions Obtained at Various Stages of Purification

Enzyme[a] source	Specific activity[b] (nmol ATP incorporated/min/mg protein)		
	DG kinase	MG kinase	Ratio DG kinase/ MG kinase
Homogenate	2.44 (3)	0.53 (3)	4.60
Cytosol (100,000 × g supernatant)	3.90 (3)	0.63 (3)	6.19
DEAE-cellulose column (from active peak)	18.6 (3)	1.08 (3)	17.30
ATP-agarose column (from active peak)	1160 (5)	0.84[c] (5)	1381

[a]The original of each enzyme source is described in Materials and Methods.

[b]Expressed as an average with the number of estimates given in parentheses.

[c]Three of the five estimates were equal to zero and the other two were very low values.

TABLE 6

Comparison of Diacylglycerols of Varying Fatty Acid Composition as Substrates for Phosphatidic Acid Formation by Diacylglycerol Kinase[a] Purified from Bovine Brain Cytosol

Diacylglycerol (5 mM)	Relative incorporation of [γ-^{32}P]ATP into lipid[b]		
	Number	Range	Average
1,2-dioleoyl-*rac*-glycerol	7	1.00	1.00
1,2-dicaproyl-*sn*-glycerol	2	0.63–0.77	0.70
1,2-dilauroyl-*sn*-glycerol	3	0.59–0.84	0.71
1,2-dimyristoyl-*sn*-glycerol	2	0.25–0.26	0.26
1,2-dipalmitoyl-*sn*-glycerol	2	0.14–0.16	0.14
1,2-distearoyl-*racp*glycerol	3	0.04–0.09	0.07
1-palmitoyl,2-oleoyl-*sn*-glycerol	3	0.93–0.94	0.94
1-oleoyl,2-palmitoyl-*sn*-glycerol	3	0.42–0.77	0.61
1-stearoyl,2-oleoyl-*sn*-glycerol	2	0.92–0.96	0.94
1-stearoyl,2-linoleoyl-*sn*-glycerol	4	0.86–1.10	0.96
1,2-dioleoyl-*sn*-glycerol	4	0.91–1.12	0.98
1,2-diacyl-*sn*-glycerol derived from egg PC	4	1.01–1.40	1.26
1,2-diacyl-*sn*-glycerol derived from pig liver PC	3	1.27–1.41	1.32

[a]Diacylglycerol kinase was purified from bovine brain cytosol as described in Materials and Methods. Specific activities, expressed as nanomoles ATP incorporated/min/mg protein, ranged from 862–1501 with 1,2-dioleoyl-*rac*-glycerol as substrate and phosphatidylcholine from bovine brain (50–100 μg) added as activator.

[b]Relative activity denotes the activity relative to that of 1,2-dioleoyl-*rac*-glycerol set at 1.00.

(palmitoyl, oleoyl), some preference is shown for the unsaturated fatty acid being in the 2-position. The 1,2-diacyl *sn* preparations derived from natural sources (egg and pig liver PC) were about 30% more effective than the synthetic 1,2-dioleins. In the small series shown for disaturated diacylglycerols (all *sn* except 1,2-distearin), C_{10} and C_{12} showed the highest activities (ca. 70% of the activity of 1,2-diolein) followed by 1,2-dimyristin (26%), 1,2-dipalmitin (14%) and 1,2-distearin (7%).

DISCUSSION

The study reported here represents an extension of work previously reported from this laboratory on diacylglycerol and monoacylglycerol kinase activities in rat brain (5). The study in part complements and adds to and in part is at variance with that of Kanoh et al. (3) reported on pig brain. A major problem encountered in both studies was the extreme instability of the diacylglycerol kinase, particularly as it is carried through the final steps of purification. With pig brain, Kanoh et al. have obtained or realized conditions for purifying the enzyme to homogeneity through six steps with sufficient stability to store it for use in further studies (3 and especially 18). Purification of bovine brain to homogeneity has been more difficult. In this work a variety of approaches were tried, including the six steps of Kanoh et al. (3). Extreme loss of activity occurred with $(NH_4)_2SO_4$ fractionation, and a large loss of activity, possibly due to the resulting extreme instability, occurred with all attempts to use gel exclusion chromatography (Sephadex G-150 and Ultragel AcA 44). The best highly purified preparations (nearly homogeneous to homogeneous, as judged by SDS polyacrylamide gel electrophoresis) of diacylglycerol kinase (ca. MW 72,000 daltons) from bovine brain were obtained with the four-step purification (brain cytosol isolation and successive column chromatography on DEAE-cellulose, Sephadex G-25 for desalting and ATP-agarose) described here. The purified enzyme, even when stabilized with EDTA, ATP and DTT and kept in the frozen state (at −20 or −80 C), lost at least 90% of its activity in 24 hr. Further attempts to stabilize by the addition of glycerol (10% final), the return of unabsorbed (void volume) protein or the addition of bovine serum albumin were unsuccessful. This problem required that both the localization of the peak and subsequent enzymatic studies be done within 3–4 hr of elution from ATP-agarose keeping the enzyme at 4 C. Specific activities (nmol ATP incorporated/min/mg protein) of individual fractions (measured within 2 hr) were in excess of 2,000 and of pooled peaks (measured within 3–4 hr) ranged from 908–1857 (footnote *a*, Table 1). Instability created through protease action possibly remains a contributing factor, even though use of the protease inhibitor, phenylmethylsulfonylfluoride, added before or after elution of the enzyme from ATP-agarose failed to help reduce the rapid loss of activity.

Some of the requirements for optimal activity of the purified diacylglycerol kinase from bovine brain are very similar to those of Kanoh et al. (3), while others differ. The apparent K_m for ATP and optimum concentration of Mg^{2+} are in general agreement with those of Kanoh et al. In this study an initial lag seen with Mg^{2+} was removed when appropriate correction was made for chelation by EDTA present in the enzyme preparation. The apparent K_m found for diacylglycerol was somewhat higher than the value reported by Kanoh et al. This difference quite possibly is related to differences in the sonication procedures used. This preparation, like that of Kanoh et al., showed a strong activation by PC. However, some of the features of the activation differed, again possibly because of certain procedural differences (e.g., sonication and in the addition and vortexing of the phosphoglycerides). In this system the synergistic action of deoxycholate and PC were so great as to warrant doing all assessments of phospholipid activation in the presence of deoxycholate. Kanoh et al., on the other hand, found no activation by lipids in the presence of deoxycholate and did all their assessments in the absence of deoxycholate and usually in the presence of PC. This basic difference between the two systems no doubt accounts for a number of the additional differences seen in the activation by phospholipids. Thus, while PC is a good activator, it is not preferred over PE or PS and is only slightly better than PI. Furthermore, PI is not inhibitory when added with PC, PE or PS, nor does lyso-PC activate as observed by Kanoh et al. (3). The failure of lyso-PC to activate may reflect an inhibitory detergent action at the concentration examined. With PC, PE or PS, maximal activation was achieved in each instance with quite small amounts (10 μg or 0.025 mM). Mixtures of phospholipids (PC, PE and PS or PC, PE, PS and PI) in the proportion found in brain cytosol showed good activation but only marginally above that observed for PC, PE or PS added singly. It is of some interest to note that the mixtures of phospholipids from bovine brain cytosol (consisting primarily of PC, PE, PS and PI) showed almost the same activation as artificial mixtures of the same proportions. This study supports the requirement of a phosphoglyceride for optimal diacylglycerol kinase activity, but does not support the contention that PC is necessarily the best activator, nor that PI is an inhibitor regulator as suggested by Kanoh et al. (3). What the real answer is in vivo must remain open, for in either of the above studies the conditions are far removed from those existing in vivo.

The experiments with sulfhydryl reagents give strong support to the view that one or more SH groups is essential for diacylglycerol kinase activity. This possibility initially was indicated by the stabilization effects of DTT. These effects warranted the routine addition of DTT to buffers used throughout the purification and to assay carried out. As a result there is a small contribution of DTT (~0.4 mM) by the added enzyme to the control of Table 4. The fact that the three sulfhydryl reagents, PCMB, NEM and DTNB, all showed strong inhibition at 1.0 mM, which was prevented by the addition of 0.5 mM DTT, gives strong credence to the above view. The only inconsistency is the observation that iodoacetate at 4.0 mM was ineffective. Clarification of this situation should come from proposed studies on the amino acid content and, in particular, on the cysteine residues present in the purified enzyme.

The finding that there is no apparent monoacylglycerol kinase activity associated with the purified diacylglycerol kinase of bovine brain puts into question the earlier suggestion that the two activities are carried on by one enzyme (5,6). Since Kanoh et al. (3) do not report an activity for their purified pig brain diacylglycerol kinase, it is

possible that their preparation also is devoid of monoacylglycerol kinase activity. We are beginning to assess the possibility that there exists a separate enzyme possessing only monoacylglycerol kinase or possibly both kinase activities. Alternatively, it may be that the substrate requirements for the fully purified diacylglycerol kinase are no longer met by monoacylglycerol, in this case more specifically 2-oleoyl-*rac*-glycerol.

In the final aspect of this study some assessment, but not as detailed as reported previously for rat brain microsomes and cytosol (5), has been made of the effect of varying fatty acid composition in the diacylglycerol substrate. The findings obtained with the purified enzyme are consistent with those obtained earlier. There is no pattern evident that favors the selection of a particular molecular species. A preference is shown for diacylglycerols having at least one unsaturated fatty acid, particularly in position-2. Also, shorter chain saturated fatty acids in the diacylglycerol result in higher activities than longer chain acids. Much of the latter observation may be explained on the basis that substrates with shorter chains sonicate more easily. These findings agree with the earlier conclusion of Holub and Piekarski (19) and this lab (5) that diacylglycerol kinase does not show a strong selectivity for diacylglycerols of a specific molecular species which would favor the generation of specific PAs. This, however, does not preclude the use by this enzyme of diacylglycerols of selective composition in specific locations that are accessible to the enzyme. For example, its cytosolic location might allow this kinase to be accessible to the inner side of plasma membranes, where the phosphatidylinositol 4,5 bis-phosphate cycle occurs, or to the outer side of the endoplasmic reticulum. The recent unusual observation that Rous sarcoma virus-transforming gene product, pp60, phosphorylates substrates of wide specificity (glycerol, casein, PI and diacylglycerol) including diacylglycerol (20) raises interesting questions: what sort of regulation exists with respect to diacylglycerol metabolism in not only the cells infected by this virus, but other cells, and what role may be played by the more specific diacylglycerol kinase (such as the one purified in this study which shows no activity towards glycerol or histone-1 used in the assay of protein kinase C) present in these cells?

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Dexamethasone Blocks Arachidonate Biosynthesis in Isolated Hepatocytes and Cultured Hepatoma Cells

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The effect of dexamethasone on the incorporation and conversion of [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid in isolated hepatocytes and in hepatoma tissue culture (HTC) cells was studied. In both kinds of cells, no changes in the exogenous acid incorporation were found when the hormone was added to the incubation media at 0.1 or 0.2 mM concentration, while the biosynthesis of arachidonic acid was significantly depressed. The effect on the biosynthesis was faster in isolated normal liver cells (60 min) than in tumoral cells (120 min) and reached an inhibition of ca. 50% after 3 hr of treatment. The addition of cycloheximide (10^{-6} M) also caused a marked decrease in the biosynthesis of this polyunsaturated fatty acid, but when dexamethasone was added to the media simultaneously with cycloheximide, a synergistic action was not observed. The results obtained show that protein synthesis would be involved in the modulation of the biosynthesis of arachidonic acid by glucocorticoids. The changes in the Δ5 desaturation of labeled 20:3ω6 to arachidonic acid correlated with changes in the fatty acid composition in isolated cells.
Lipids 21, 212-219 (1986).

It has been demonstrated that fatty acid Δ6 and Δ5 desaturases play an important regulatory function in the metabolism of polyunsaturated fatty acids. Both desaturases are under nutritional and hormonal controls (1-4). The Δ5 desaturase that converts eicosa-8,11,14-trienoic acid (20:3ω6) to arachidonic acid is depressed when epinephrine or dibutyryl cyclic AMP is administered to rats (5-8) or added to the medium of cultured cells (9). The injection of natural and synthetic glucocorticoids, such as dexamethasone, also produces a depression in Δ5 desaturation activity of rat liver microsomes (10). However, the use of living animals is not a very adequate model to elucidate the mechanism of action of the hormone at the cellular level. With this model it is difficult to determine if the effect is produced directly on the liver or through an steroid-induced process in the whole animal that evokes a depression on hepatic Δ5 desaturation activity as a secondary effect (10). For this reason, the aim of the present work was to investigate comparatively the direct effect of dexamethasone on two types of cells: normal rat hepatocytes and HTC cells. HTC cells were chosen as an example of malignant cells that still preserve hormonal receptors (11,12) and have an active Δ5 desaturase (13). Fatty acid metabolism of these tumoral cells has been studied extensively in our laboratory (9,13-17).

MATERIALS AND METHODS

Isolated cells. Liver cells were isolated from female Wistar rats (160-180 g body weight), fed Purina chow ad libitum. Purina chow consisted of (in calories) 56.7% carbohydrates, 10.4% lipids and 32.9% protein, vitamins and minerals. The relative percentages of fatty acids of this diet were 21.4, palmitic; 2.1, palmitoleic; 8.2, stearic; 24.9, oleic; 37.7, linoleic; and 0.2, arachidonic. Parenchymal cell isolation was carried out according to Seglen (18) except that the operational temperature was maintained at 37 C. To minimize glycogenolysis, 0.2 g percent glucose was added to all media employed during perfusion and isolation (19). The liver cells were suspended in oxygenated Hank's buffer solution (20) containing 1.0 g percent of bovine albumin. After 30 min they were centrifuged and resuspended in IMEM-Zo medium (21) from which linoleic acid was omitted.

HTC cells came from an ascites tumor which had been derived from a solid hepatoma (7288 c) (22). The cells were grown at 37 C in confluent layer attached to glass on Swim's 77 (S 77) medium supplemented with 10% calf serum using sterile conditions as previously described (23). The tumoral cell suspension was obtained from surface cultures of HTC cells as it was described elsewhere (24).

Cell viability (90%) was assessed using exclusion of trypan blue (25).

Chemicals. Radioactive [1-^{14}C]eicosa-8,11,14-trienoic acid (54.9 mCi/mmol, 98% radiochemically pure) was provided by New England Nuclear Corp. (Boston, Massachusetts). Unlabeled eicosa-8,11,14-trienoic acid was supplied by Nu-Chek-Prep (Elysian, Minnesota). Bovine albumin, essentially fatty acid-free bovine albumin, N-2-hydroxyethyl-piperazin-N-2-ethanosulphonic acid (HEPES), collagenase type IV, cycloheximide and trypsin soybean inhibitor were provided by Sigma Chemical Co. (St. Louis, Missouri). Dexamethasone (phosphate salt) was purchased from Merck Sharp Dome Lab (Buenos Aires, Argentina). Radioactive L-[3,4,5]^{3}H-leucine (1.0 mCi/mmol, 95% radiochemically pure) was provided by CNEA (Saclay, France).

Experimental procedure. HTC cells and isolated liver cells were counted in a hemocytometer. Aliquots (3.0×10^6 cells) were incubated with 5 ml modified IMEM-Zo medium in 25-ml siliconized Erlenmeyer flasks under an atmosphere of 95% oxygen and 5% carbon dioxide in a metabolic shaker (70 strokes per min).

To measure the incorporation and conversion of exogenous eicosa-8,11,14-trienoic acid in both kinds of cells under dexamethasone treatment, three types of experiments were carried out. In the first one, HTC cells and normal hepatocytes were incubated for 6 hr with an 80 μM mixture of labeled and unlabeled eicosa-8,11-14-trienoic acid (containing 0.5 μCi of [1-^{14}C]eicosa-8,11,14-trienoic acid) per flask. The acid was added as sodium salt bound to defatted albumin according to Spector et al. (26). The amount of albumin used was 200 nmol per flask. Dexamethasone phosphate was added simultaneously with the substrate at two final concentrations, 0.1 mM or 0.2 mM.

In the second experiment, both types of cells were incubated with 0.2 mM dexamethasone phosphate for

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1-6 hr. Fifteen min before the end of each period of incubation, all media were supplemented with 20:3ω6 acid (80 μM concentration).

In the third experiment, HTC cells were incubated with dexamethasone (0.2 mM) for 3 or 6 hr in the presence or not of cycloheximide (10^{-6} M). Eicosa-8,11,14-trienoic acid was added as in the second experiment.

At the end of the incubations, cell suspensions were transferred into ice-cold tubes and centrifuged. Each pellet was washed twice (3 ml each time) and resuspended (5 ml) using cold 0.85 g percent NaCl. An aliquot of the suspension was used to determine cell viability and the amount of cellular protein (27). The rest was centrifuged at 2,000 × g for 5 min and the saline solution was decanted. Cell pellets were saponified for 45 min at 85 C with 2.0 ml of 10% KOH in ethanol plus 500 μl of methanol to facilitate the extraction step. The fatty acids were extracted after acidification with HCl with light petroleum (bp 30-40 C) and esterified with 3 N HCl in methanol for 3 hr at 65 C.

Chromatographic measurements. The radioactivity of the recovered methyl esters was determined in a Beckman liquid scintillation counter (model LS-3133 P) with 97% efficiency for ^{14}C. The distribution of radioactivity among the fatty acid methyl esters was determined by gas liquid radiochromatography using an Acromat CG-100 equipped with a Packard proportional counter. Percentage conversion was calculated from the distribution of radioactivity between the substrate and product measured directly on the radiochromatogram (28). The column was packed with 15% EGSS-X (ethyleneglycolsuccinate) coated on chromosorb WHP (80-100 mesh) (Supelco, Bellefonte, Pennsylvania). The composition of the fatty acid methyl esters was determined by gas liquid chromatography (GLC) in a Hewlett-Packard 5840-A with a terminal computer integrator system. The column was packed with 10% SP-2330 on 100-200 Chromosorb WAW (Supelco). The oven was programmed from 140 C to 220 C at 3 C/min after 1 min initial hold. The acids were identified by comparison of their relative retention times of standards, and the mass distribution was determined by GLC in the presence of an internal standard of eicosa-11-monoenoic acid.

In some experiments additional flasks were incubated (3 to 6 hr) without exogenous fatty acid, with or without dexamethasone 0.1 or 0.2 mM and/or cycloheximide 10^{-6} M. In this way, tyrosine aminotransaminase (TAT) activity used as a test of hormone response or protein biosynthesis as a test of cycloheximide action was determined. TAT activity was assayed (29) on the soluble cytosolic fraction obtained from sonicated cells. Protein biosynthesis activity was estimated by determining the incorporation of ^{3}H leucine into the trichloroacetic (TCA) precipitable material of the hepatoma cells. Briefly, 0.2 mCi/ml of L-[3,4,5]^{3}H-leucine was added to each flask in the last 30 min of incubation. The cells were washed twice with 0.85% (w/v) NaCl cold solution, suspended in distilled water and disrupted by sonication with two 30-sec bursts at medium power in a Heat Systems-Ultrasonic sonicator model W-220F (Plainview, New York) equipped with microtip. The proteins were precipitated with 5 vol ice-cold TCA solution (6% w/v) and after 3 min were collected by centrifugation at 5,000 × g for 10 min. The precipitate was washed twice with TCA solution and once with 5 vol ethanol/ether (1:1, v/v). The final pellet solubilized with 2 N NaOH was counted in a Beckman liquid scintillation counter with 60.0% efficiency for ^{3}H. The total amount of protein was determined by the method of Lowry et al. (27).

Incubation of rat liver microsomes with dexamethasone. A possible direct effect of dexamethasone on the microsomal desaturation system was examined. Rat liver microsomes were separated by differential centrifugation at 100,000 × g (8). They were preincubated for 15 or 30 min with or without 0.2 mM dexamethasone phosphate, after which desaturation of fatty acid was measured by estimation of the percentage conversion of [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid. Three nmol (54.9 mCi/mmol) of the labeled acid and 97 nmol of unlabeled acid were incubated with 5 mg of microsomal protein in a shaker at 37 C for 15 min. The composition of the incubation medium, the procedure to obtain the fatty acid methyl esters and the analysis of radioactive fatty acid methyl esters have been described elsewhere (8).

Student's t-test was used for the statistical treatment of the data.

RESULTS

Simultaneous incubation of dexamethasone and labeled eicosa-8,11,14-trienoic acid. The incorporation of exogenous [1-^{14}C]eicosa-8,11,14-trienoic acid in isolated cells after 6 hr of incubation with dexamethasone was not modified by the hormone. The amount of exogenous acid incorporated expressed as nmol/mg of cellular protein was higher in HTC cells (229 ± 6) than in normal hepatocytes (91 ± 6). The percent of the added radioactivity recovered in the methyl esters was higher in hepatoma cells (54%) than in isolated liver cells (22%). On the contrary, dexamethasone treatment significantly depressed the conversion of eicosa-8,11,14-trienoic acid into arachidonic acid in hepatoma cells and isolated hepatocytes using labeled substrate (Figs. 1A and 1B). The effect was similar in both types of cells.

As expected, both kinds of cells also were sensitive to other corticoid-dependent stimulations, as dexamethasone caused a marked increase in TAT activity. This effect was more pronounced in isolated hepatocytes (ranging from 155% to 0.1 mM dexamethasone to 250% at 0.2 mM) than in tumoral cells (from 55 to 90%).

Tables 1 and 2 provide some information on the changes produced by dexamethasone on the fatty acid composition of HTC cells and isolated liver cells. They confirm earlier data (24) revealing that fatty acid composition of tumoral cells is significantly different from that of normal liver cells (Table 2). In malignant cells, one of the most characteristic alterations shown is the very low level of the essential fatty acids, arachidonic and linoleic, and the abundance of monoenoic acids, especially oleic. In both cells studied, exogenous eicosa-8,11,14-trienoic acid was incorporated, but HTC cells showed the greatest avidity for this acid, reaching very high levels. Eicosa-8,11,14-trienoic acid incorporation also modified the fatty acid composition and caused a decrease in the amount of saturated and monoenoic acids. The exogenous substrate also was converted into arachidonic acid.

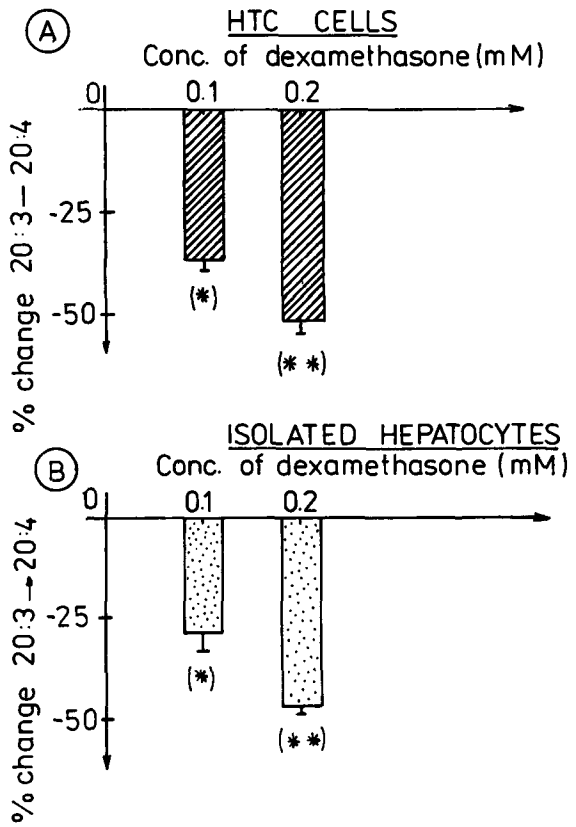

FIG. 1. Effect of dexamethasone after 6 hr of incubation with HTC cells (A) or isolated hepatocytes (B) on the change of [1-^{14}C]eicosa-8,11,14-trienoic acid conversion to arachidonic acid. Zero points correspond to percentage conversion of 20:3 → 20:4 of cells incubated for 6 hr without hormonal treatment (29.3 ± 1.8 for HTC cells and 14.1 ± 0.3 for isolated hepatocytes). The eicosatrienoic acid and the hormone were added simultaneously to the incubation media. Conditions of incubations and measurement of the arachidonic acid formed are described in Experimental Procedures. Values are the mean of 3 incubation flasks; vertical lines represent 1 SEM. Significance compared to the cells incubated with exogenous acid and without hormonal treatment: *, $P < 0.02$, **, $P < 0.01$.

The incubation of hepatoma cells for 6 hr in IMEM-Zo medium produced no changes on total fatty acid composition (Table 1). However, after the same period of incubation, the fatty acid composition of normal liver cells showed an increase in the amounts of palmitic, stearic and oleic acids (Table 2). These alterations probably reflected an adaptation to nutritional conditions in vitro; in a previous work we demonstrated that this phenomenon occurs when isolated hepatocytes are incubated in chemically defined IMEM-Zo medium (24).

The analysis of the fatty acid composition of isolated cells incubated in the presence of dexamethasone was in accordance with results showing that the hormone produces a depression in the conversion of eicosa-8,11-14-trienoic acid to arachidonic acid. In fact, the comparison of cells incubated with and without dexamethasone addition showed that, when 0.2 mM dexamethasone was added to the incubation medium simultaneously with eicosa-8,11,14-trienoic acid (80 μM conc.), the amount of this acid increased significantly while arachidonic acid was depressed (Tables 1 and 2). This effect was not evident when the exogenous acid was omitted in the incubation media.

Time course of dexamethasone action. In all the above experiments the hormone was added to the incubation media at zero time, simultaneous with the exogenous eicosa-8,11,14-trienoic acid. To account for the possible existence of a rapid effect of the hormone on Δ5 desaturation activity, the conversion of eicosa-8,11,14-trienoic acid to arachidonic acid was measured during 15 min after different periods of dexamethasone treatment.

Figure 2A shows that in HTC cells a period of at least 2 hr was necessary to obtain a significant inhibition in the conversion of the exogenous substrate. This depression reached maximum values (50%) after 3 hr of treatment and then remained apparently constant until the end of the assay period. The behavior of isolated liver cells in the presence of the hormone was similar. Nevertheless, in this case the depressive action was observed as early as 1 hr after the treatment (Fig. 2B).

TABLE 1

Effect of 6 Hr of Dexamethasone (Dx) Treatment on the Fatty Acid Composition of HTC Cells[a]

Fatty acids	Without incubation	Incubated for 6 hr				
				+ 20:3ω6, 80 μM		
		None	Dx 0.2 mM	No Dx	Dx 0.1 mM	Dx 0.2 mM
14:0	1.2 ± 0.2[b]	1.3 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.2 ± 0.2	0.7 ± 0.2
16:0	19.6 ± 0.7	19.0 ± 0.5	18.8 ± 0.4	8.8 ± 0.3	8.9 ± 0.8	10.0 ± 0.7
16:1	6.0 ± 0.9	6.0 ± 0.2	5.3 ± 0.5	2.5 ± 0.2	2.9 ± 0.5	2.9 ± 0.3
18:0	19.5 ± 0.8	20.0 ± 0.7	19.6 ± 0.4	8.1 ± 0.5	7.9 ± 0.3	8.9 ± 0.4
18:1	45.0 ± 1.2	44.3 ± 1.1	44.5 ± 1.1	18.1 ± 0.5	18.8 ± 0.9	20.8 ± 0.8
18:2	6.9 ± 0.1	6.9 ± 0.4	6.5 ± 0.2	2.5 ± 0.2	2.4 ± 0.1	2.8 ± 0.2
20:3ω6	1.4 ± 0.3	1.7 ± 0.2	1.5 ± 0.2	48.0 ± 0.3	50.1 ± 0.2	67.5 ± 0.3
20:4ω6	4.9 ± 0.4	4.9 ± 0.1	4.6 ± 0.5	6.0 ± 0.3	4.1 ± 0.2	4.1 ± 0.2
22:4ω6	tr	tr	tr	3.0 ± 0.3	2.6 ± 0.5	1.8 ± 0.5
22:5ω6	tr	tr	tr	1.4 ± 0.5	2.5 ± 0.6	1.8 ± 0.5
22:5ω3	1.9 ± 0.1	2.0 ± 0.3	1.7 ± 0.2	1.5 ± 0.5	2.8 ± 0.6	1.9 ± 0.2
22:6ω3	2.1 ± 0.2	1.9 ± 0.2	1.9 ± 0.1	1.0 ± 0.1	1.9 ± 0.4	1.8 ± 0.2

Dexamethasone phosphate and 20:3ω6 acid were added simultaneously to the incubation media.

[a]For details, see Experimental Procedures.

[b]Results were expressed as μg of fatty acids per mg of cellular protein and are the mean of 3 incubation flasks ± 1 SEM.

TABLE 2

Effect of 6 Hr of Dexamethasone (Dx) Treatment on the Fatty Acid Composition of Isolated Hepatocytes[a]

Fatty acids	Without incubation	Incubated for 6 hr				
		None	Dx 0.2 mM	+ 20:3ω6, 80 μM		
				No Dx	Dx 0.1 mM	Dx 0.2 mM
14:0	0.5 ± 0.05[b]	0.9 ± 0.05	0.8 ± 0.1	0.9 ± 0.1	0.6 ± 0.05	0.6 ± 0.1
16:0	27.7 ± 0.3	33.3 ± 0.1	32.1 ± 0.4	24.2 ± 0.3	26.0 ± 1.1	25.7 ± 1.0
16:1	3.0 ± 0.1	3.3 ± 0.1	3.6 ± 0.1	3.0 ± 0.1	2.5 ± 0.1	2.3 ± 0.2
18:1	50.5 ± 1.0	53.5 ± 0.3	54.9 ± 0.5	48.6 ± 1.3	45.3 ± 1.5	47.4 ± 1.1
18:1	19.9 ± 0.2	21.0 ± 0.2	21.9 ± 0.3	19.3 ± 0.5	16.3 ± 0.3	18.1 ± 0.9
18:2	27.6 ± 0.5	26.4 ± 0.6	28.0 ± 0.7	26.3 ± 1.6	21.1 ± 0.9	24.1 ± 0.8
20:3ω6	2.1 ± 0.1	2.3 ± 0.2	2.7 ± 0.2	9.0 ± 0.1	9.8 ± 0.4	10.9 ± 0.5
20:4ω6	48.2 ± 0.9	48.0 ± 0.3	47.5 ± 0.5	55.2 ± 0.2	56.0 ± 0.4	49.8 ± 0.5
22:4ω6	2.6 ± 0.2	3.0 ± 0.2	3.8 ± 0.2	3.0 ± 0.9	4.0 ± 0.5	3.3 ± 0.1
22:5ω6	2.1 ± 0.2	2.4 ± 0.3	2.0 ± 0.2	2.0 ± 0.1	2.3 ± 0.1	1.9 ± 0.1
22:5ω3	3.3 ± 0.2	3.5 ± 0.2	3.2 ± 0.5	3.1 ± 0.5	3.2 ± 0.2	2.8 ± 0.1
22:6ω3	12.3 ± 0.8	13.4 ± 0.5	11.6 ± 1.0	10.4 ± 1.0	9.3 ± 1.5	9.8 ± 1.0

Only relevant fatty acids are considered.

Dexamethasone phosphate and 20:3ω6 were simultaneously added to the incubation media.

[a]For details, see Experimental Procedures.

[b]Results were expressed as μg of fatty acids per mg of cellular protein and are the mean of 3 incubation flasks ± 1 SEM.

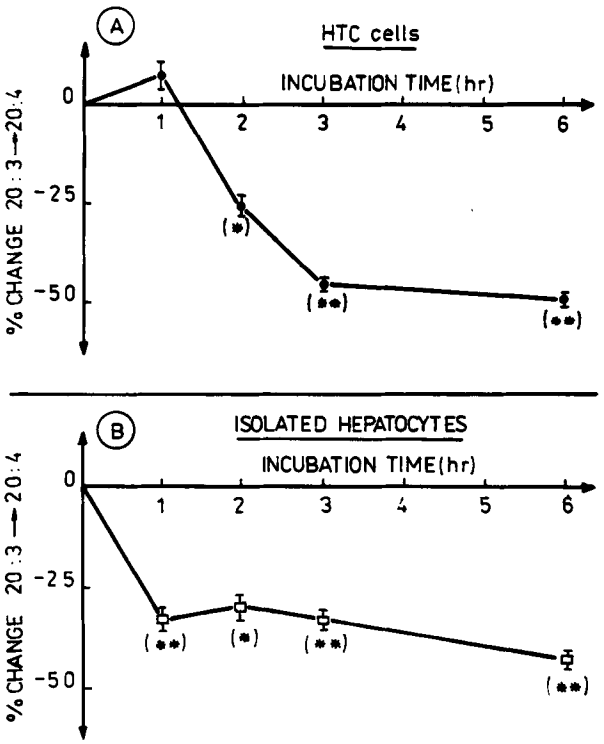

FIG. 2. Effect of different periods of dexamethasone incubation on the percent change of eicosa-8,11,14-trienoic acid conversion to arachidonic acid in HTC cells (A) or isolated hepatocytes (B). 20:3ω6 acid was added in the last 15 min of each period studied. Zero points correspond to percentage conversion of 20:3 → 20:4 obtained with cells incubated during the corresponding period without dexamethasone addition (4.4 ± 0.4 for HTC cells and 13.5 ± 0.8 for isolated hepatocytes). Conditions of incubations and measurement of arachidonic acid formed are described in Experimental Procedures. Values are the mean of 3 incubation flasks; vertical lines represent 1 SEM. *, Results significantly different, P < 0.02. **, Results significantly different, P < 0.01.

The profile of the induction of TAT activity by dexamethasone in 6 hr of incubation was also similar in both kinds of cells. However, the magnitude of the activities obtained at 3 or 6 hr of incubation in the case of normal hepatocytes was higher than in hepatoma cells (data not shown).

To check the effect produced by dexamethasone on the fatty acid composition of the cells, GLC analyses of the samples were carried out. Figures 3 and 4 show the variations of the amount of the principal fatty acids during different periods of incubation in the presence or not of dexamethasone. In both types of isolated cells the level of eicosa-8,11,14-trienoic acid was raised simultaneously with a decrease in the amount of arachidonic acid as a function of time of hormonal action. The effect started 2 hr after hormonal treatment in HTC cells and 1 hr in normal hepatocytes. After 6 hr, arachidonic acid was 1.3-fold decreased and 20:3ω6 was 1.4-fold increased. The results are similar to data shown in Table 2. Between 0 and 6 hr the hormone produced no significant changes in the amount of the other fatty acids. Linoleic acid was increased in normal liver cells but this change was not significantly different from that of control values.

Direct effect of dexamethasone on microsomal Δ5 desaturation activity. When rat liver microsomal fractions were preincubated with 0.2 mM dexamethasone for 15 or 30 min and compared to microsomes preincubated in hormone absence, no significant changes in the Δ5 desaturation activity were observed (data not shown).

Effect of cycloheximide. To study the mechanism by which dexamethasone modified the conversion of eicosa-8,11,14-trienoic acid to arachidonic acid, cycloheximide was added to the incubation medium. The effect of cycloheximide in the concentration range from 0 to 10^{-4} M and during different periods of time on the viability of HTC cells was studied. When the concentration of the antimetabolite in the media was 10^{-6} M or lower, no

significant changes in the percentage of cell viability during the incubation periods assay were observed.

The results obtained on the incorporation of ^{3}H-leucine into TCA-precipitable material of HTC cells after 3 or 6 hr of incubation with cycloheximide 10^{-6} M in the presence or absence of dexamethasone are shown in Fig. 5. As expected, cycloheximide caused a marked decrease in the incorporation of the labeled amino acid in protein molecules. Conversely, dexamethasone treatment did not modify this parameter either at 3 or 6 hr of incubation.

Histograms in Figure 6 show the effects produced by dexamethasone and cycloheximide on the incorporation and conversion of exogenous 20:3ω6 acid to arachidonic acid. After 3 or 6 hr of incubation the incorporation of the acid was not modified by the glucocorticoid. However, cycloheximide produced a decrease in the incorporation of the exogenous acid, but this decrease was not potentiated when it was added to the media simultaneously with the steroid hormone. In relation to arachidonic acid biosynthesis, Figure 6 shows that once again 3 hr of dexamethasone treatment produced a significant decrease on its biosynthesis. Cycloheximide treatment also caused a marked decrease in the biosynthesis of this polyunsaturated acid, but when dexamethasone was added to the media simultaneously with cycloheximide, a synergistic action was not observed. A similar pattern of effects was obtained after 6 hr of incubation.

DISCUSSION

Δ5 Desaturase is a regulatory enzyme situated in a key position in the sequence of reactions that leads to the biosynthesis of arachidonic acid and eicosapentaenoic acid from linoleic and α-linolenic acids, respectively (1-4,13). Glucocorticoids modify the activity of fatty acid desaturases, and it has been demonstrated in our laboratory that their injection in rats evokes a depression of Δ5 desaturase (10). However, due to the physiological complexity of the whole animal it is very difficult to elucidate in animals in vivo the intimate mechanism of glucocorticoid action. Isolated cells are the more appropriate model for this investigation.

The results obtained in this experiment demonstrate that the addition of dexamethasone to the incubation media of isolated cells leads to a decrease in the biosynthesis of arachidonic acid. That the effect may be produced by the hormone at the level of the Δ5 desaturase is evidenced by the decreased conversion of labeled eicosa-8,11,14-trienoic acid to arachidonic acid, a decrease evoked without alteration of eicosa-8,11,14-trienoic acid incorporation in the cells. Besides, the effect was dose-dependent, because the increase of hormone concentration from 0.1 mM to 0.2 mM increased the inhibition (Fig. 1). In the case of HTC cells, a short lag period was necessary for the manifestation of the hormonal response (Fig. 2). This lag was not evident in the case of isolated hepatocytes, where after 1 hr of hormonal treatment it

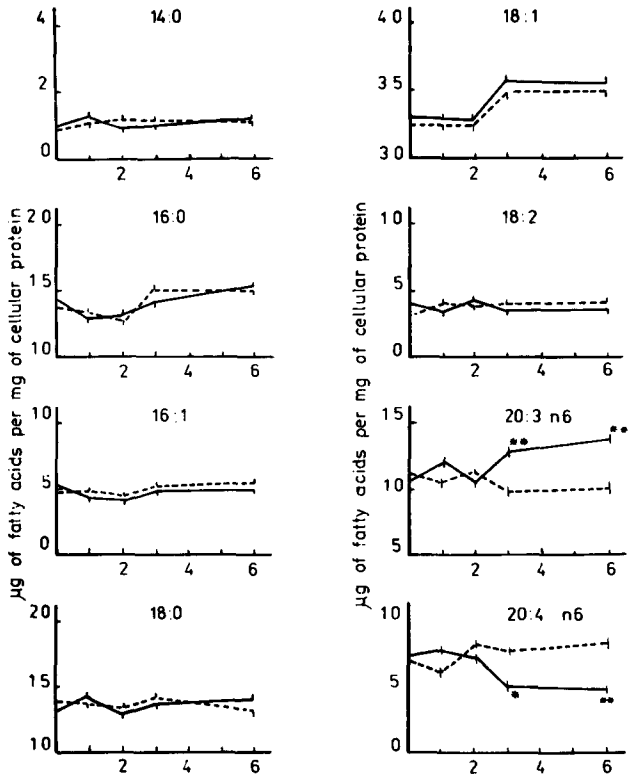

FIG. 3. Effect of different periods of dexamethasone action on the composition of the principal fatty acids of HTC cells. 20:3ω6 acid was added in the last 15 min of each period studied. For details, see Experimental Procedures. Without dexamethasone (– – – –). With dexamethasone (——). Results are the mean of 3 incubation flasks; vertical lines represent 1 SEM. *, Values significantly different, $P < 0.02$. **, Values significantly different, $P < 0.01$.

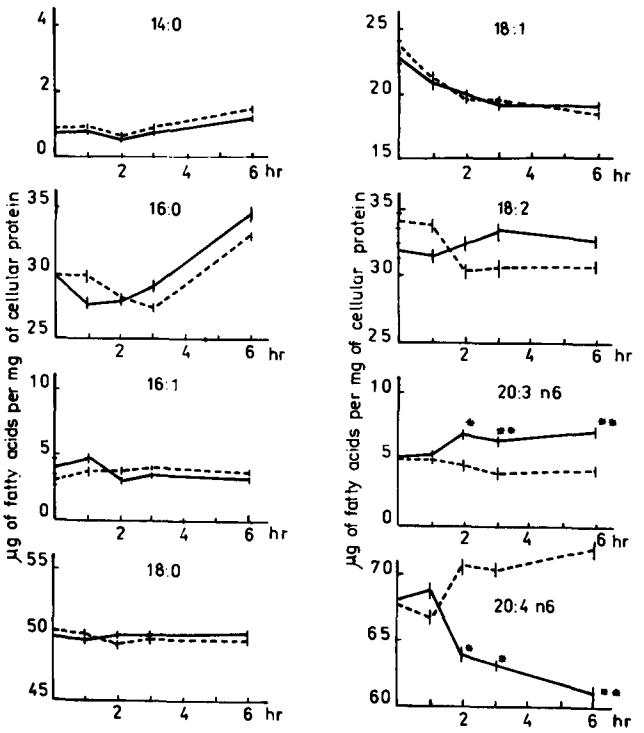

FIG. 4. Effect of different periods of dexamethasone action on the composition of principal fatty acids of isolated hepatocytes. 20:3ω6 acid was added in the last 15 min of each period studied. For details, see Experimental Procedures. Without dexamethasone, (– – – –); with dexamethasone, (——). Results are the mean of 3 incubation flasks; vertical lines represent 1 SEM. *, Values significantly different, $P < 0.02$. **, Values significantly different, $P < 0.01$.

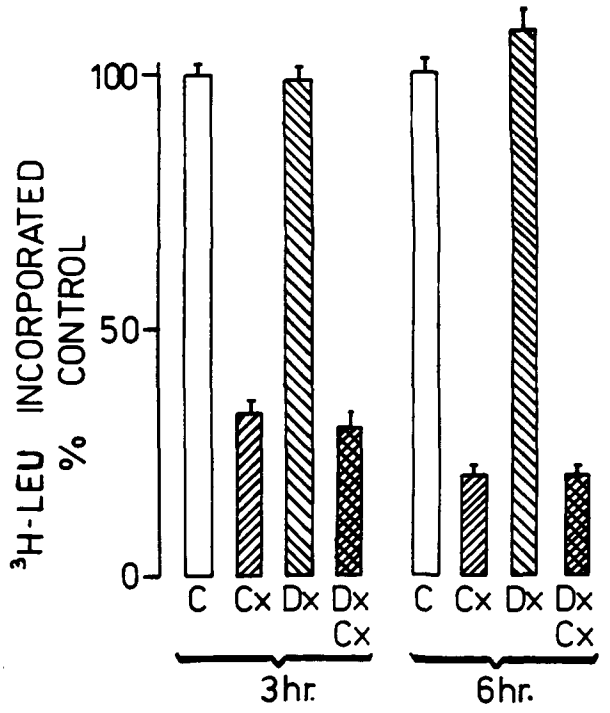

FIG. 5. Effect of cycloheximide 10^{-6} M (▨), dexamethasone 2.10^{-4} M (▧) and cycloheximide plus dexamethasone (▩) compared to control cells (□) on the incorporation of ^{3}H-leucine in trichloroacetic precipitable material of HTC cells. For details, see Experimental Procedures. Results are the mean of 3 incubation flasks and are expressed as percent changes from the control response (cpm per mg protein). Vertical lines represent 1 SEM.

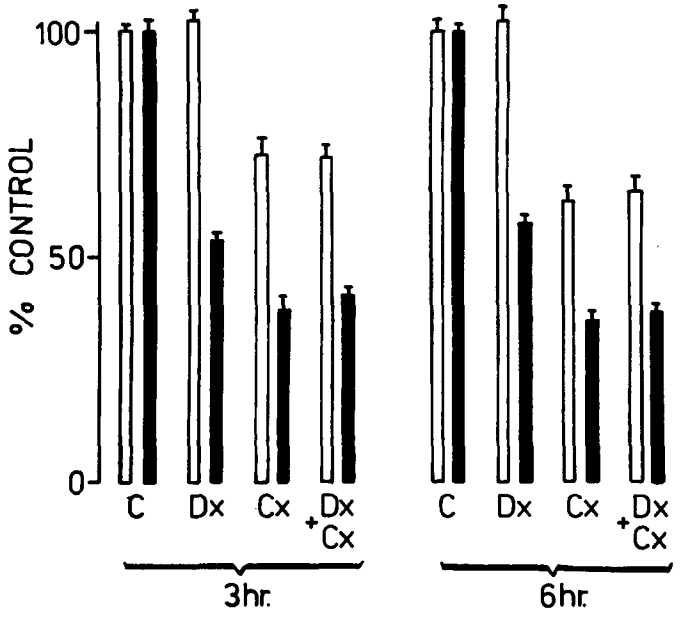

FIG. 6. Effect of 10^{-6} M cycloheximide (Cx), 2.10^{-4} M dexamethasone (Dx) and cycloheximide plus dexamethasone (Dx + Cx) compared to controls (C) on the incorporation and conversion of eicosa-8,11,14-trienoic acid in HTC cells. □, Eicosa-8,11,14-trienoic acid incorporated; ■, arachidonic acid formed. For details, see Experimental Procedures. Results are the mean of 3 incubation flasks and are expressed as percent changes from the control response. Vertical lines represent 1 SEM.

was already possible to detect ca. 35% inhibition in the arachidonic acid production. In both kinds of cells the inhibition increased following an apparently hyperbolic curve, and a steady inhibition of ca. 50% was reached after 6 hr of hormonal treatment.

A remarkable parallelism exists between the changes of Δ5 desaturation and the fatty acid composition of the cells. Actually, a decrease of Δ5 desaturase activity after 6 hr of dexamethasone treatment evoked a change in the fatty acid composition of the cells, diminishing the amount of arachidonic acid and raising the amount of 20:3ω6 acid (Tables 1 and 2). In accordance with these results, Holloway and Margolis (30) have shown that dexamethasone treatment of adrenalectomized rats decreased the arachidonic/linoleic acid ratio after 6 hr of the hormone administration. Analytical alterations of the principal fatty acids after different periods of hormonal action also indicated that dexamethasone provokes a significant increase of eicosa-8,11,14-trienoic acid and at the same time a concomitant decrease in the level of arachidonic acid after a lag period (Figs. 3 and 4). Therefore, these alterations in the fatty acid composition of the cells are in accordance with an impairment in the biosynthesis of arachidonic acid, and in both types of cells they were produced a short time after the effect of dexamethasone on arachidonate biosynthesis was evident (Tables 1 and 2, Fig. 2). Therefore, the results obtained indicate that glucocorticoids apparently can exert their effect on polyunsaturated acid metabolism by a direct action on isolated target cells.

The mechanism by which glucocorticoids modify the desaturation of fatty acid can be explained in different ways. The mechanism could be genomic or nongenomic. A possible nongenomic mechanism of dexamethasone action could be evoked by direct binding of the hormone to the microsomes as already reported for rat liver (31). This effect of the hormone could be produced directly on the Δ5 desaturation system or mediated through changes of the physicochemical properties of the biomembrane that, as we know, may modulate desaturase activity (32,33). However, the preincubation of rat liver microsomes with dexamethasone did not produce any significant and specific effect on the Δ5 desaturase activity in the conditions of our experiment. Therefore, we may discard rather confidently a direct effect of dexamethasone on the Δ5 desaturation system.

In the classic mechanism generally accepted, steroids enter the cells by simple diffusion and combine with a high-affinity cytoplasmic receptor. Then, the steroid-protein complex undergoes activation and is transferred to the nucleus where it binds to selective sites in the chromatin, leading to a modulation of RNA and protein biosynthesis (34–38).

In the present experiment, we found that a short lag period is necessary in HTC cells to detect the action of the steroid hormone. It is known that the effect of glucocorticoids is produced after a lag period ranging from 20 min to several hours, between the entry of the molecule into the cell and the manifestation of the hormonal response (39). In our experiments the lag period was not evident in normal hepatocytes. Similarly, no lag period for TAT activity induction was detected (data not shown). However, the lag period has been observed repeatedly by other authors (12,40,41). Therefore, it is

possible that using a shorter period of time the lag period could be detected for TAT and Δ5 desaturase in normal liver cells. Glucocorticoid regulation of TAT activity has been shown to be produced by the classic mechanism already described (39). If the action of dexamethasone on Δ5 desaturase activity of isolated cells were produced through the classic scheme, it is reasonable to assume that it would be due to the biosynthesis of a factor that, after a lag period, in a second step would evoke the effect on the microsomal desaturase system. In this respect, it has been reported that when rats were injected with natural and synthetic glucocorticoids the liver microsomal desaturase measured by in vitro assays showed a very low activity (10).

Cycloheximide inhibits protein biosynthesis in eucaryotic cells and may be used as a tool to pinpoint regulatory mechanisms in which protein biosynthesis is involved. The experiments carried out to investigate the role played by protein synthesis in the mechanism of glucocorticoid action on the biosynthesis of arachidonic acid demonstrate that cycloheximide blocked the inhibitory effect of dexamethasone (Fig. 6). This result implies that the regulatory action of the glucocorticoid would be produced through the biosynthesis of a protein involved in the modulation of Δ5 desaturation activity. Cycloheximide per se produced both a decrease in the incorporation of eicosa-8,11,14-trienoic acid and a decrease in eicosa-8,11,14-trienoic acid conversion to arachidonic acid. In consequence, proteins would be involved in eicosa-8,11,14-trienoic incorporation and in the desaturation reaction. These inhibitory effects would not be due to a decrease in cell viability, because after 3 or 6 hr of incubation with 10^{-6} M cycloheximide, the percent of cells that excludes trypan blue was very similar to that obtained on the control cells. The protein involved in 20:3ω6 incorporation in the cells would be independent of glucocorticoid induction, as dexamethasone produced no changes in the fatty acid incorporation.

Different mechanisms can explain the results found in the present experiment with cycloheximide. One is that dexamethasone promotes the biosynthesis of a protein inhibitory of arachidonate production. Therefore, the addition of cycloheximide to the incubation medium would inhibit its biosynthesis and eliminate the effect of dexamethasone on the eicosatrienoic acid conversion. However, because cycloheximide per se inhibits the biosynthesis of arachidonic acid in the same proportion as dexamethasone plus cycloheximide (Fig. 6), it would imply that cycloheximide also inhibits the biosynthesis of another protein of the reaction that could be the Δ5 desaturase itself. However, the inhibition produced by cycloheximide on either the dexamethasone-dependent protein or the Δ5 desaturation does not progress with time after 3 hr of treatment. Therefore, the dexamethasone-dependent protein would have a very short life. Besides, results show that the progress of the deactivation of arachidonate production by cycloheximide with time is not detected in our experiment at the interval between 3 and 6 hr. In consequence, this previous interpretation is not in accordance with data showing that the life of Δ5 desaturase is rather long (Gómez Dumm, I.N.T. de, and Alaniz, M.J.T. de, unpublished results). Another mechanism could be that dexamethasone promotes the inhibition of a protein of rapid turnover directly or indirectly necessary for the Δ5 desaturation of fatty acids. This inhibition would be evoked by the modulation of the biosynthesis of this protein. In this case, cycloheximide and cycloheximide plus dexamethasone would produce the same inhibition on the Δ5 desaturase when added to the incubation medium. Besides, this mechanism would require that the half-life of the Δ5 desaturase be long enough not to observe a measurable decay in the short period from 3 to 6 hr (Fig. 6). This interpretation fits better the experimental results since the Δ5 desaturase has a long life (Gómez Dumm, I.N.T. de, and Alaniz, M.J.T., unpublished results).

In previous works we have demonstrated that c-AMP produces a depression in the fatty acid Δ6 and Δ5 desaturase system (5-9) but not on the Δ9 desaturase. Moreover, several experiments have revealed that glucocorticoids can produce an increase in the c-AMP levels (42-45). Consequently, we must recognize the possibility that glucocorticoids increasing the intracellular c-AMP levels could evoke, at least partially, the depressive action found on the biosynthesis of arachidonic acid in both types of isolated cells studied.

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Effects of Dietary Triolein and Sunflower Oil on Insulin Release and Lipid Metabolism in Zucker Rats

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Obese and lean male Zucker rats were fed ad libitum on diets containing either 50 (L) or 200 (H) g/kg diet of either triolein (T) or sunflowerseed oil (S). The specific activity of the hepatic microsomal Δ9 desaturase enzyme was depressed in both lean and obese rats fed the HS diet compared with the other three diets. The fatty acid composition of liver and subcutaneous white adipose tissue lipids were consistent with a lower Δ9 desaturation activity in rats fed the H diets, particularly for the HS diet. In both genotypes, microsomal Δ9 desaturase activity and the ratio of 16:1/(16:0 + 16:1) fatty acids in liver lipids were inversely related to the proportion of 18:2 in liver lipid. Plasma insulin concentrations and rates of glucose-stimulated insulin release in vivo were higher in obese rats compared with lean rats, and plasma insulin levels were higher in rats fed S compared with T. There was no relationship between Δ9 desaturase activity and either plasma insulin concentration or rates of insulin release in vitro. These findings suggest that hepatic Δ9 desaturase activity of Zucker rats is responsive to changes in the proportion of 18:2 in liver lipids but is not affected by changes in insulin secretion.

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The genetically obese Zucker rat (fa/fa) is characterized by hyperphagia, excessive weight gain and lipid deposition in liver and adipose tissue, hyperlipemia and hyperinsulinemia (1). Rates of de novo FA synthesis are increased in liver and adipose tissue of obese Zucker rats as compared to lean (1), as is the activity of the hepatic Δ9 desaturase enzyme (EC 1.14.99.5) (2). Lipogenesis is known to be inhibited by high levels of dietary fat, PUFA generally having the greatest effect (3,4). Dietary PUFA also decreases hepatic Δ9 desaturase activity in obese Zucker (2) and non-obese (5,6) rats. Effects of saturated FA on Δ9 desaturase activity are less clear, with both increased (5,7) and decreased (6) activities being reported.

Both fatty acid synthesis and Δ9 desaturase (8,9) are insulin-dependent. Previous studies concerning the effects of dietary fat on insulin secretion show varying results. High levels of dietary fat have been reported to increase (10), decrease (11,12) and have no effect (13-16) on plasma insulin concentration in rats. In one study, corn oil tended to have a greater depressing effect than hydrogenated fat (11).

The purpose of the present work was to determine the effects of level and type of dietary fat on insulin secretion in lean and obese Zucker rats and to investigate the hypothesis that changes in insulin secretion may mediate the effects of PUFA on FA metabolism, particularly hepatic desaturase activity. An alternative hypothesis is that control by insulin is only significant in extreme conditions, tissue levels of esterified linoleic acid being the primary regulator of Δ9 desaturase activity (17). Sunflowerseed oil (65% 18:2) was used as a source of PUFA and was compared to triolein; it was used in preference to saturated FA because of its higher digestibility. A preliminary account of part of this work has been presented (18).

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MATERIALS AND METHODS

Rats and diets. Genetically obese (fa/fa) and lean (Fa/−) male Zucker rats were obtained from the Rowett Institute colony. Four groups of 12 rats each (six obese and six lean littermates), aged 42-50 days, were fed ad libitum on one of the diets described in Table 1. Diets contained either a low (L) level (50 g/kg) or a high (H) level (200 g/kg) of fat, supplied as either sunflowerseed oil (S) or triolein (T). The fatty acid compositions of the diets are given in Table 2. The low fat diets (LS, LT) supplied 12% and the high fat diets (HS, HT) 40% of calculated metabolizable energy as fat.

Rats were weighed twice weekly and were slaughtered in a randomized order after 27-50 days. The variation in feeding period was caused by the need to measure pancreatic insulin release rapidly after slaughter.

Sampling of tissues and assay procedures. Animals were not fasted before slaughter, so that maximal desaturase activity could be measured (5). Between 9 and 11 a.m. on the day of slaughter rats were anesthetized with ether and blood was collected by cardiac puncture into heparinized tubes; plasma was separated and stored frozen until analyzed. After slaughter the liver was removed, blotted and weighed, and subcutaneous white adipose tissue was sampled from the shoulder region.

TABLE 1

Composition (g/kg) of Diets[a]

Ingredient	LS	HS	LT	HT
Sunflowerseed oil	50	200	—	—
Triolein	—	—	50	200
Dextrose monohydrate	601	451	601	451
Glycerol	150	150	150	150
Casein	150	150	150	150
Mineral mix[b]	39	39	39	39
Vitamin mix[c]	10	10	10	10

[a]L, low level; H, high level; S, sunflowerseed oil; T, triolein.

[b]Contained (g) $CaCO_3$, 10.6; KH_2PO_4, 12.4; NaCl, 9.0; $MgSO_4 \cdot 7\ H_2O$, 3.61; $FeSO_4 \cdot 7\ H_2O$, 0.175; $CuSO_4 \cdot 5\ H_2O$, 0.055; $MnSO_4 \cdot 4\ H_2O$, 0.058; $ZnSO_4 \cdot 7\ H_2O$, 0.012; KIO_3, 1.95×10^{-4} made up to 39 g with maize starch.

[c]Contained (g) choline chloride, 4.93; ascorbic acid, 2.96; inositol, 0.329; p-aminobenzoic acid, 0.329; menadione, 0.329; niacin, 0.296; retinol, 0.296; calcium pantothenate, 0.197; α-tocopherol, 0.099; riboflavin, 0.066; pyridoxine hydrochloride, 0.066; thiamine hydrochloride, 0.066; cholecalciferol, 0.016; cobalamin, 0.009; folic acid, 0.006; biotin, 0.001.

TABLE 2

Fatty Acid Composition of Experimental Diets (% by Wt of Total Fatty Acids)

Fatty acid[a]	Diet[b] LS	HS	LT	HT
14:0	tr[c]	tr	1.7	1.0
16:0	8.4	6.8	6.5	5.4
16:1	tr	tr	6.0	4.9
18:0	4.9	4.2	1.8	1.6
18:1	24.9	21.9	69.1	75.4
18:2	57.8	65.3	9.3	6.5
18:3	1.4	0.7	tr	tr

[a]Chain length: number of double bonds.

[b]For details of diets, see Table 1.

[c]tr: <0.5% of total fatty acids.

TABLE 3

Effect of Dietary Fat on Body Weight, Growth Rate and Liver Weight of Lean and Obese Zucker Rats[a]

Rat	Diet	Body weight (g)	Growth rate (g/27 days)[b]	Liver weight g	Liver weight g/100 g rat
Lean	LS	342	117	11.6	3.4
	HS	332	118	10.1	3.1
	LT	317	112	10.7	3.4
	HT	326	104	11.6	3.5
Obese	LS	465	170	25.0	5.4
	HS	463	196	17.8	3.9
	LT	459	160	20.6	4.5
	HT	448	150	16.2	3.6
	SEM[c]	32	21	1.5	0.2
Source[d]		Level of significance[e]			
Genotype (G)		***	***	***	***
Fat type (T)		NS	NS	NS	NS
Fat level (L)		NS	NS	**	***
G × T		NS	NS	NS	**
G × L		NS	NS	*	***
T × L		NS	NS	NS	*

[a]For details of diets, see Table 1. Mean values given for six rats per group.

[b]Growth rate calculated over first 27 days of experiment before any rats were slaughtered.

[c]Pooled standard error of mean for all column means.

[d]Statistical analysis as a 2^3 factorial design, with main effects and listed interactions tested.

[e]***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant.

The pancreas was removed rapidly after slaughter and placed in ice-cold Krebs Ringer bicarbonate buffer, pH 7.4. Portions of tissue, drawn from all regions of the gland, were freed of adherent fat and chopped into small pieces. Rates of glucose-stimulated insulin release were determined at 37 C by incubating the pieces in Krebs Ringer bicarbonate buffer, pH 7.4, containing 5 mg bovine serum albumin/ml and either 0.5 (low) or 3.0 (high) mg glucose/ml (19). Incubation media were stored frozen prior to insulin assay. Rates of insulin release are given as mean values for four successive 15-min incubation periods in either the low- or high-glucose medium. The viability of the pancreatic pieces was assessed by their ability to maintain stable rates of insulin output over the final three incubation periods.

Hepatic microsomes were prepared and the extent of microsomal desaturation of [1-^{14}C]stearic acid was determined as described previously (20).

The fatty acid compositions of frozen samples of liver and adipose tissue and of the diets were determined as methyl esters (21) by gas liquid chromatography (GLC), which was carried out at 170 C with a Pye 104 instrument, using a 2 m × 4 mm glass column packed with 15% polyethylene glycol succinate on Chromosorb W-AW (80–100 mesh). The proportion of each fatty acid present was determined from the peak areas using a CS1 Supergrator 1 computing integrator. Only the fatty acids present in greater than 0.5% proportions are reported.

Insulin was estimated in plasma and incubation media by radioimmunoassay (22), using rat insulin (Novo Industri, Copenhagen, Denmark) as standard. Plasma glucose was measured using a YSI Model 23A Glucose Analyzer. Commercial assay systems (Boehringer Corp. [London], Lewes, Sussex, United Kingdom) were used to measure plasma levels of total lipids, nonesterified fatty acids, triglycerides (23) and total cholesterol. The latter method used cholesterol esterase and cholesterol oxidase, liberating hydrogen peroxide, which oxidized methanol to formaldehyde in the presence of catalase. Formaldehyde reacted with ammonia and acetylacetone to form a colored complex, measured spectrophotometrically at 410 nm.

Statistical analysis. The data were analyzed as a 2^3 factorial analysis of variance, which included genotype (obese or lean), fat type (S or T) and level of fat (L or H) as main effects, together with their interactions.

RESULTS

As Table 3 shows, body weight at slaughter and growth rate over the first 27 days of the experiment were similar for all rats of a given genotype, regardless of diet. The HT diet initially caused digestive disturbances, but growth rate recovered after a few days (unpublished).

Food intake was not measured in this experiment. In further experiments using rats of similar age, groups of four rats of each genotype were fed ad libitum on one of the diets and energy intake was recorded over a 14-day period. Growth rates of these animals were very similar to the values given in Table 3 (unpublished). Daily energy intake (kcal/day) was 65 ± 4 (SEM), 70 ± 4, 64 ± 2 and 63 ± 1 for lean rats fed LS, HS, LT and HT, respectively. For the obese rats the corresponding energy intake was 91 ± 8, 82 ± 5, 89 ± 4 and 84 ± 2 for rats receiving diets LS, HS, LT and HT, respectively. Differences in energy intake between obese and lean genotypes were highly significant ($P < 0.001$), but there were no significant effects of diet on intake.

The livers of obese rats were heavier than those of lean rats, both in absolute terms and relative to body weight. Liver weight was decreased by the higher level of dietary fat, particularly for obese rats. Feeding S, compared with

TABLE 4

Effect of Dietary Fat on Plasma Glucose and Insulin Levels and Glucose-Stimulated Insulin Release from Pancreases of Lean and Obese Zucker Rats[a]

Rat	Diet	Plasma level		Insulin release in vitro[b]	
		Glucose (mg/100 ml)	Insulin (μU/ml)	Low glucose	High glucose
Lean	LS	215	48	199	364
	HS	185	35	277	302
	LT	174	34	221	334
	HT	159	23	87	182
Obese	LS	140	279	438	632
	HS	269	433	441	685
	LT	134	255	326	727
	HT	164	194	569	928
	SEM	25	45	75	95
Source		Level of significance			
Genotype (G)		NS	***	***	***
Fat type (T)		*	*	NS	NS
Fat level (L)		NS	NS	NS	NS
G × T		NS	NS	NS	NS
G × L		**	NS	NS	NS
T × L		NS	NS	NS	NS

[a]See Tables 1 and 3 for further details.

[b]Rates of glucose-stimulated insulin release measured in vitro (see Materials and Methods) in presence of either 0.5 (low glucose) or 3.0 (high glucose) mg glucose/ml. Results expressed as μU insulin released/g tissue/min.

T, resulted in a greater relative liver weight for the obese rats, but not for the lean rats.

The plasma concentrations of total lipid, triglyceride, nonesterified fatty acids and cholesterol were all significantly higher in the obese rats than in the lean littermates ($P < 0.001$ in all cases, results not presented in detail), only cholesterol levels being additionally affected by diet. Cholesterol concentration was lower in animals fed the higher level of fat ($P < 0.05$), but the type of fat had no effect.

The S diets resulted in a raised plasma glucose concentration compared with T (Table 4). The higher fat level (and lower dietary glucose level) resulted in a lower plasma glucose concentration in lean animals but caused an elevation in the obese rats. Both plasma insulin concentration and rates of insulin release in vitro were markedly higher in the obese rats than in the lean littermates. The only significant dietary effect was a higher insulin level in plasma of rats fed S compared with T. Plasma insulin concentrations tended to be higher in obese rats fed the HS diet as opposed to LS, but the effect was not statistically significant.

The specific activity of the hepatic microsomal Δ9 desaturase system was depressed in both lean and obese rats fed the HS diet, with little difference between the other three diets (Table 5). There was no relationship between desaturase activity and either plasma insulin concentration or rates of insulin release in vitro.

Both diet and genotype had marked effects on the fatty acid composition of liver (Table 6) and subcutaneous

TABLE 5

Effect of Dietary Fat on Δ9 Desaturase Activity of Hepatic Microsomes of Lean and Obese Zucker Rats[a]

Rat	Diet	Desaturase activity[b]
Lean	LS	17.6
	HS	10.9
	LT	17.1
	HT	22.9
Obese	LS	19.7
	HS	11.8
	LT	19.6
	HT	17.2
	SEM	3.0

[a]See Tables 1 and 3 for further details.

[b]Desaturase activity = nmol 18:0 fatty acid converted to 18:1/30 min/mg microsomal protein. Statistical analysis as a 2^3 factorial design testing main effects and interactions listed in Table 3 indicated significant effects of fat type and fat type × fat level interaction (both $P < 0.05$).

adipose tissue (Table 7) lipids, with adipose tissue being the more responsive to diet. Thus for rats fed either T or S diets, the proportions of the respective principal dietary fatty acid 18:1 (T) or 18:2 (S) were greater in the adipose tissue than in the liver. Proportions of the fatty acids 14:0, 16:0, 16:1 and 18:1 were higher and proportions of 18:0, 18:2 and 20:4 were lower in liver lipids of obese rats than in their lean littermates. In adipose tissue, obese animals had higher proportions of 14:0, 16:0 and 16:1 and a lower proportion of 18:2 than lean rats.

Hepatic microsomal Δ9 desaturase activity was inversely related to the proportion of 18:2 in liver lipids, both when data for obese and lean rats were combined ($r = -0.36$, $P < 0.01$, $n = 48$) and when the two genotypes were examined separately (obese $r = -0.39$, $P < 0.05$, $n = 24$; lean $r = -0.41$, $P < 0.05$, $n = 24$). The proportion of the fatty acid 16:1 and the ratio 16:1/(16:0 + 16:1) give indirect indices of Δ9 desaturase activity. Both parameters were significantly lower in both liver and adipose tissue of animals fed the higher level of dietary fat ($P < 0.001$ in all cases), particularly when S was fed (significant fat type × fat level interactions, $P < 0.05$). In each tissue the ratio 16:1/(16:0 + 16:1) was inversely related to the proportion of 18:2 present (liver $r = -0.665$, $P < 0.001$, $n = 48$; adipose tissue $r = -0.538$, $P < 0.001$, $n = 48$). When the two genotypes were examined separately, the gradients of the linear regression lines relating the ratio 16:1/(16:0 + 16:1) to the proportion of 18:2 in liver lipids did not differ significantly between genotypes. The regression intercept was significantly greater for the obese rats (0.289 ± 0.017) than for the lean animals (0.234 ± 0.023, $P < 0.01$), indicating a higher value for this index of desaturase activity in obese rats at all levels of 18:2.

DISCUSSION

The present study clearly shows that changes in plasma insulin concentration are not primarily responsible for diet-induced variations in the activity of the hepatic

TABLE 6

Effect of Dietary Fat on the Fatty Acid Composition (Wt %) of Liver Lipids of Lean and Obese Zucker Rats[a]

		Gas chromatographic analysis							Ratio
Rat	Diet	14:0	16:0	16:1	18:0	18:1	18:2	20:4	16:1/(16:0 + 16:1)
Lean	LS	0.8	23.0	4.3	16.0	21.1	12.8	15.6	0.16
	HS	0.4	17.1	1.5	15.2	14.3	29.2	15.9	0.08
	LT	0.7	23.3	5.7	16.3	29.6	6.4	10.4	0.19
	HT	0.6	19.9	3.8	11.8	40.4	7.9	9.8	0.16
Obese	LS	1.8	29.3	13.2	7.5	37.8	4.7	2.5	0.31
	HS	1.0	24.8	4.4	8.6	25.6	23.2	6.8	0.15
	LT	1.6	30.9	11.2	9.2	38.1	3.2	2.4	0.27
	HT	1.3	27.3	7.3	8.2	41.5	6.2	3.9	0.21
	SEM	0.2	1.5	0.7	0.8	1.8	0.8	1.5	0.02
Source		Level of significance							
Genotype (G)		***	***	***	***	***	***	***	***
Fat type (T)		NS	NS	*	NS	***	***	***	*
Fat level (L)		**	***	***	**	NS	***	*	***
G × T		NS	NS	NS	*	***	***	NS	NS
G × L		NS	NS	***	**	*	NS	*	NS
T × L		NS	NS	**	**	***	***	NS	*

[a]See Tables 1 and 3 for further details.

TABLE 7

Effect of Dietary Fat on the Fatty Acid Composition (Wt %) of Subcutaneous Adipose Tissue Lipids of Lean and Obese Zucker Rats[a]

		Gas chromatographic analysis						Ratio
Rat	Diet	14:0	16:0	16:1	18:0	18:1	18:2	16:1/(16:0 + 16:1)
Lean	LS	1.9	27.2	8.6	5.0	34.9	19.8	0.25
	HS	1.2	15.8	2.7	3.3	27.1	47.1	0.14
	LT	1.9	28.1	8.3	5.3	45.5	7.4	0.23
	HT	1.7	17.6	5.4	2.5	60.3	8.8	0.23
Obese	LS	2.0	29.3	10.1	5.0	37.5	13.2	0.26
	HS	1.3	22.1	5.1	3.8	32.6	33.3	0.19
	LT	2.7	28.0	11.2	5.4	43.4	5.6	0.29
	HT	2.1	24.4	7.4	3.5	51.6	7.6	0.23
	SEM	0.2	1.1	0.6	0.4	1.3	1.2	0.02
Source		Level of significance						
Genotype (G)		*	***	***	NS	NS	***	*
Fat type (T)		***	NS	***	NS	***	***	**
Fat level (L)		***	***	***	***	***	***	***
G × T		NS	NS	NS	NS	***	***	NS
G × L		NS	***	NS	NS	NS	NS	NS
T × L		NS	NS	**	NS	***	***	*

[a]See Tables 1 and 3 for further details.

microsomal Δ9 desaturase enzyme in Zucker rats. Thus, although the obese Zucker rat is hyperinsulinemic, differences in plasma insulin levels or rates of glucose-stimulated insulin release do not directly mediate either the higher Δ9 desaturase activity of the obese Zucker rat reported earlier (2) or the lower desaturase activity of animals fed the HS diet. While Δ9 desaturase activity of rat liver can be reduced by chemical diabetes (8,9) and increased by insulin injection (5), changes in insulin secretion within the physiological range are not the primary regulator of enzyme activity. This is not the only aspect of hepatic lipid metabolism which is unresponsive to changes in plasma insulin. Equalization of plasma insulin levels in lean and obese Zucker rats did not prevent excessive lipid deposition in the obese rats, although body weight gain and food intake per unit body weight were equalized (24).

Hepatic Δ9 desaturase activity could depend on the

sensitivity and responsiveness of the liver to insulin, rather than simply on the level of insulin secretion. Insulin binding to liver plasma membranes and insulin receptor number decreased by over 50% in non-obese rats fed a high fat, carbohydrate-free diet (25), but changes would likely be smaller with less extreme diets such as used in the present study. Although the obese Zucker rat is generally considered to be insulin-resistant, glucose oxidation and fatty acid synthesis in hepatocytes from obese Zucker rats aged 2–3 mo may be as responsive to insulin as are hepatocytes from lean rats (26).

Changes in tissue insulin sensitivity are more clearly important in regulating lipid metabolism by extrahepatic tissues. Adipose tissue and muscle of adult obese Zucker rats are markedly insulin-resistant (27). In obese rats, but not in lean Zucker rats, insulin resistance was further increased by a high fat diet (27). Such diets increase insulin resistance in non-obese rats (12), affecting both skeletal muscle (10) and adipose tissue (28). The elevated plasma glucose levels we observed in obese rats fed HS and HT diets, in the face of a reduced dietary glucose intake, suggest that peripheral sensitivity to insulin was further impaired at the higher level of dietary fat.

Dietary PUFA had a clear inhibitory effect on hepatic Δ9 monodesaturation, in agreement with other studies (2,6,29). Substitution of dietary starch by T has been reported to reduce hepatic Δ9 desaturase activity, although to a lesser extent than with corn oil (6). In the present work the Δ9 desaturase activity of obese and lean rats was similar for animals fed HT compared with LT, but the ratio of fatty acids 16:1/(16:1 + 16:0) in liver lipid indicated a significant fall in desaturase activity in animals fed the HT diet. Similarly, genotype did not significantly affect hepatic microsomal Δ9 desaturase activity, but the proportion of the fatty acid 16:1 and the ratio 16:1/(16:1 + 16:0) in liver and adipose tissue lipids were consistent with greater desaturation of 16:0 in obese rats compared with lean, as reported previously (2). Other workers reported clear effects of diabetes on fatty acid composition of liver phospholipids associated with only a 5–15% decrease in Δ9 desaturase activity (9). Measured Δ9 desaturase activities in the present study showed considerable variability, raising the possibility that either analytical problems or short-term variations in microsomal desaturase activity partially masked the longer term effects of diet and genotype on fatty acid monodesaturation which were apparent when the FA composition of tissue lipids was examined. Results were not affected by the variable feeding period involved, since use of age at slaughter as a covariate did not affect the conclusions obtained from the statistical analysis.

Transplantation of adipose tissue between lean and obese (ob/ob) mice demonstrated that adipose tissue fatty acid composition depends on the physiological environment of the animal in which the fat is implanted, rather than the genotype of the adipose tissue itself (30). Our results suggest that plasma insulin levels are a less important component of the hepatic environment regulating Δ9 desaturase activity than is the availability of PUFA. Both hepatic Δ9 desaturase activity and the indirect index of Δ9 desaturation in liver and adipose tissue were inversely related to the proportion of 18:2 in tissue lipids, suggesting that desaturase activity is directly inhibited by 18:2 levels. A similar conclusion was reached by Enser (17,29), who studied the effects of food restriction, diet and diabetes on plasma insulin levels and Δ9 desaturase activity of obese (ob/ob) and lean mice. A close inverse relationship existed between Δ9 desaturase and the concentration of esterified 18:2 in liver lipid, but not in adipose tissue. The proportion of 18:2 in adipose tissue varied within a narrow range, however, compared with the present study. The mechanisms by which 18:2 inhibits Δ9 desaturase remain uncertain; inhibition of enzyme synthesis or of enzyme activity have been proposed (31).

Hepatic Δ9 desaturase activity was also reduced by 18:2 in both lean and obese (ob/ob) mice, but at all levels of 18:2 in liver lipids, activity was greater in the obese mice, provided they were fed ad libitum (29). The effect was ascribed to the hyperphagia in the obese animals, providing additional substrates for desaturation. The difference between genotypes in the intercept of the regression relating 16:1/(16:0 + 16:1) to the proportion of 18:2 in liver lipids would be consistent with a similar effect of hyperphagia in the obese Zucker rat. However, in the absence of data from obese rats pair-fed to the intake of lean littermates, we cannot differentiate between responses to hyperphagia and other consequences of the obese genotype.

The intake data, obtained from animals similar to those used in the main experiment, do not suggest that differences in energy intake were a major confounding factor in interpreting the effects of diet, within a genotype. Thus, indices of Δ9 desaturase activity were markedly reduced in both lean and obese rats fed a high level of fat, particularly for the HS diet. Energy intake, on the other hand, was only slightly and not significantly lower at the high level of dietary fat in the obese animals, while in the lean animals energy intake was very similar for all diets.

Dietary carbohydrate intake also influences Δ9 desaturase activity (6,9). Glucose intake was probably lower for rats fed the higher level of dietary fat, but we do not believe that this was responsible for the effects reported. Hepatic Δ9 desaturase activity is similar in obese and lean rats fed a 56% sucrose, 5% sunflowerseed oil diet compared with a laboratory chow (2), and dietary 18:2 was 18 times as potent per unit of weight in reducing desaturase activity than was dietary sucrose in inducing the enzyme (6). Glucose is a weaker inducer of Δ9 desaturase than sucrose (32), so it is unlikely that the difference between 60 and 45% dietary glucose would have markedly influenced desaturase activity when compared with responses to a fourfold change in fat level.

The effects of dietary fat on insulin secretion in rats are unclear (see introduction). Age and strain of rat and type of dietary fat could contribute to the inconsistent responses reported. Rates of glucose-stimulated insulin release in vitro may be expected to be a more sensitive measure than single plasma insulin values taken at slaughter. Thus insulin release in vitro was depressed in obese and lean Zucker rats fed a high level of hydrogenated vegetable oil compared with a high starch diet, while plasma insulin levels were not significantly affected by diet (27). In the present work the only significant dietary effect was a higher plasma insulin level in rats fed S compared with T. Dietary lipid appears to have less effect on plasma insulin levels in obese Zucker rats (13–16,33) than in other strains of rat (10–12). Diet also had surprisingly little effect on plasma lipid level,

compared with the large effect of genotype.

In conclusion, we have demonstrated that hepatic Δ9 desaturase activity of both lean and obese Zucker rats is responsive to changes in the proportion of 18:2 in liver lipids but is not affected by changes in insulin secretion within the physiological range in either genotype.

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Effects of Pre- and Postweaning Undernutrition on Polyphosphoinositide Pools in Rat Kidney

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The effects of undernutrition on polyphosphoinositide levels in rat kidneys removed and frozen immediately after animal death or 10 min later were determined. Weanling (21-day-old) rats of dams fed a 5 or 22% protein diet and litters fed either normal or protein-deficient diets for an additional six wk were used. Nutritional deprivation lowered phosphatidylinositol-4-phosphate (PtdIns4P) preferentially (35–40%) but preserved phosphatidylinositol-4,5-bisphosphate (PtdIns4,5P_2) at weaning. This effect was not completely reversed in animals nutritionally rehabilitated after weaning. Postweaning protein deficiency did not reduce the levels of these lipids. Postmortem loss was the same for all five groups, minimal for PtdIns4P and about one-third for PtdIns4,5P_2. *Lipids 21,* **226–229 (1986).**

Kidney tissue, like brain, has the capacity to synthesize myoinositol and incorporate it into phosphoinositides (1). Although concentrations of polyphosphoinositides are by far the highest in brain, kidney is the organ containing the second highest levels of these compounds (2–4). Further, metabolism of polyphosphoinositides in kidney is unique in that there is rapid labeling of these compounds with $^{32}P_i$ in vivo followed by a rapid decline, which has not been observed in any other organ examined (5). Several authors have suggested that polyphosphoinositides in kidney may play a role as important functional components of the tubule membranes during secretion and reabsorption of solutes from the lumen of the tubule (6–9).

Kidneys of newborn animals are less adaptable to nutritional insults than adult kidneys. Maternal protein restriction has been shown to retard cell division as well as the morphological and possibly the functional development of the kidney in newborn rats. These early changes may be permanent since cell division is compromised and the structural changes may never be fully reversed (10). Undernutrition during the pre- and postweaning periods causes reduction in kidney weight, and only partial recovery is obtained on subsequent rehabilitation (11). Of possible importance for inositol phospholipid metabolism is the observation that patients with chronic renal failure exhibit dramatic elevations in serum levels of free myoinositol which, in turn, may contribute to the pathogenesis of uremic polyneuropathy (12,13). A decreased glomerular filtration rate and disturbed inositol reabsorption also have been shown to be present in advanced forms of glomerulonephritis (14).

We have reported the apparent existence of at least two pools of polyphosphoinositides with different metabolic activities in rat brain (15,16) and also have studied the effect of undernutrition and rehabilitation on these pools in whole brain, cerebral cortex, brainstem and cerebellum at two ages (17–19). Because the high concentration and active metabolism of polyphosphoinositides in kidney may be related to functional and structural features of this organ which are adversely affected by undernutrition and because of our findings in brain, we determined the levels of polyphosphoinositides at two times postmortem in weanling and adult rat kidneys after nutritional deprivation.

EXPERIMENTAL METHODS

The following experimental design was used: (i) After giving birth the dams were given access to either a 22% protein diet (diet I, composed of 26.0% casein, 50.3% dextrin, 8.7% sucrose, 8.0% hydrogenated cottonseed oil, 5.0% Ralston Purina mineral mix, 2.0% Ralston Purina vitamin mix and 0.15% DL-methionine) or a 5% protein diet (diet II) where the proportions of casein (6.0%), dextrin (61.0%) and sucrose (18.0%) were altered. Pups (eight per litter) were used at weaning (21 days). After decapitation of the animals, kidneys were removed either immediately (one min) or after 10 min at room temperature (10 min) and dropped into liquid nitrogen. Kidneys were pooled as needed and designated L^+ (control animals) or L^- (undernourished animals). Both male and female animals were used throughout this study. (ii) Postweaning, three groups of animals were carried for an additional six wk as follows: (a) L^+ animals were fed either diet I (L^+P^+, control) or diet III, a 3% protein diet (casein, 3.5%; dextrin, 81.5%; sucrose, 0%; other constituents identical to diet I [L^+P^-, postweaning undernutrition]); (b) L^- animals were fed diet I (L^-P^+, rehabilitation). The increased amount of sucrose in diet II served to enhance the food intake of the lactating mothers. Sucrose was omitted from diet III to reduce the food intake in order to achieve maximum undernutrition. Removal of the kidneys at one or 10 min postmortem was as described above for the 21-day-old animals. Less than 60 sec elapsed between death and freezing of the tissue in liquid nitrogen. The frozen tissue samples were weighed and homogenized in $CaCl_2$ containing $CHCl_3/CH_3OH$ (1:1, 15 ml/g of tissue) (4). After being filtered and washed, the tissue residue was re-extracted three times with acidified solvents to remove polyphosphoinositides (4). After washing of the extract the lipids were isolated by thin layer chromatography (20) and analyzed by phosphorus estimation (21). Data were analyzed by the two-tailed Student's t-test.

RESULTS AND DISCUSSION

When dams were undernourished, there was a considerable reduction in the body weights of the pups at weaning (Table 1). Their kidneys were decreased in weight almost to the same extent as body weight. In adult rats where undernutrition was begun at weaning, body weights were dramatically lower and kidney weights

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TABLE 1

Body and Kidney Weights

Nutritional state[a]	Whole animal (g)	Change (%)	Kidneys (g/animal)	Change (%)
L^+	43.2 ± 2.8 (36)		0.500 ± 0.038 (8)	
L^-	18.1 ± 1.2 (44)[b]	−58	0.255 ± 0.019 (8)[b]	−49
L^+P^+	279.7 ± 36.6 (16)		2.345 ± 0.305 (8)	
L^+P^-	50.4 ± 5.6 (29)[b]	−82	0.565 ± 0.073 (8)[b]	−76
L^-P^+	240.2 ± 36.9 (16)[c]	−14	1.914 ± 0.083 (8)[c]	−18

Values are means ± S.D. (number of observations).

[a]See text for definitions of symbols.

[b]Values significantly different from control, $p < 0.001$.

[c]Values significantly different from control, $p < 0.01$.

TABLE 2

Levels and Disappearance after Death of Kidney Polyphosphoinositides in Undernourished Weanling and Adult Rats

	PtdIns4,5P$_2$			PtdIns4P		
Nutritional state[a]	1 Min (nmol/g wet wt)	10 Min (nmol/g wet wt)	Decrease (%)	1 Min (nmol/g wet wt)	10 Min (nmol/g wet wt)	Decrease (%)
L^+	39.4 ± 3.9	26.7 ± 7.7[c]	32.2	74.1 ± 7.8	63.6 ± 16.9	14.2
L^-	32.3 ± 3.6[e]	20.0 ± 3.5[b]	38.1	48.9 ± 3.1[d]	39.4 ± 4.1[c,e]	19.4
L^+P^+	111.9 ± 38.2	57.2 ± 6.5[c]	49.9	99.7 ± 10.7	87.7 ± 23.8	12.0
L^+P^-	90.5 ± 7.8	54.4 ± 6.5[b]	39.9	115.1 ± 13.8	107.3 ± 9.4	7.0
L^-P^+	108.2 ± 26.1	40.9 ± 7.8[b]	62.2	80.8 ± 9.4[e]	71.9 ± 19.7	11.0

One min refers to tissues frozen as rapidly as possible after death, 10 min to tissues frozen 10 min later (see text). Values are means ± S.D.; n = 4 at 1 min and 10 min except n = 5 for L^+P^+.

[a]See text for definition of symbols.

[b]Values significantly different from 1 min values, $p < 0.005$.

[c]Values significantly different from 1 min values, $p < 0.05$.

[d]Values significantly different from control (L^+ or L^+P^+), $p < 0.001$.

[e]Values significantly different from control, $p < 0.05$.

showed again a parallel deficit. In rehabilitated animals, small but significant weight reductions remained in both the whole animal and in the kidneys.

The time required for dissection and freezing of kidneys was 0.75–1 min, so that the results for the kidneys removed immediately represent 1-min postmortem levels of polyphosphoinositides but not 2-sec levels, as in the case of whole brain where heads were frozen in liquid N_2 after decapitation (17). Because freezing of the whole animal and removal of the kidney from bodies frozen in liquid N_2 was difficult, the metabolically highly active pool lost during the first minute could not be determined in this tissue. However, polyphosphoinositide levels at 10 min post mortem were also estimated. Significant losses (35–40%) during this time period (1–10 min postmortem) could be detected only in PtdIns4,5P$_2$ in all five groups (L^+, L^-, L^+P^+, L^+P^- and L^-P^+) of animals (Table 2). It is not known if the postmortem lability of these lipids in kidney is due to a diesteratic or monoesteratic cleavage or a combination of both enzymes. The activities of the hydrolases are, however, higher in the brain when compared to kidney. Further, the extent of losses postmortem in polyphosphoinositide levels would depend on the Ca^{2+} ion concentration which regulates the activities of these phosphohydrolases and also the accessibility of the different pools to these enzymes in the membrane.

The levels of both polyphosphoinositides determined in this study were considerably higher than those reported for kidney by some earlier authors (22,23). This presumably results from the use of procedures giving more complete extraction. However, Tou et al. calculated concentrations of both lipids about 50% greater, using specific and total radioactivity data from separations in two different chromatographic systems (3). In one of the previous investigations the changes in levels postmortem were also examined (2). Although decreases of 50% or more were found in both lipids within 10 min, the concentrations given are only 1–4% as high as those found in the present study and seem to represent only a small fraction of the total.

Nutritional deprivation had a negative effect only during the lactation period, an effect more pronounced for PtdIns4P than for PtdIns4,5P$_2$. It was incompletely reversed by nutritional rehabilitation during the subse-

TABLE 3

Effect of Varying Nutritional Conditions on the 1-Min Postmortem Levels of Polyphosphoinositides in Brain and Kidney and 10-Min Postmortem Levels of Polyphosphoinositides in Kidney

	PtdIns4,5P_2			PtdIns4P		
	L^-	L^+P^-	L^-P^+	L^-	L^+P^-	L^-P^+
	As % of control (L^+ or L^+P^+)					
1 min						
Brain	41[a]	107	95	63[a]	110	86
Kidney	82[b]	81	97	66[a]	115	81[b]
10 min						
Kidney	75	95	72	62[b]	122	82

N = 4 for all groups except n = 5 for L^+P^+ in kidney. N = 9 for L^+, n = 5 for L^- and n = 4 for L^+P^+, L^+P^- and L^-P^+ in brain.

[a]Values significantly different from control, $p < 0.001$.

[b]Values significantly different from control, $p < 0.05$.

quent postweaning period. Otherwise, both 1-min levels and the labile pool disappearing rapidly after death were unaffected by dietary alterations (Table 2).

The effects of varying nutritional conditions on the 1-min postmortem levels of polyphosphoinositides in brain (17) and kidney and 10-min postmortem levels in kidney are given in Table 3. One-min postmortem levels of PtdIns4P at weaning were decreased to a similar extent in brain and kidney. These deficits continued to persist in the kidney, while there was partial reversal in the case of brain during subsequent postweaning rehabilitation. As percent of control, deficits observed in PtdIns4,5P_2 were lower than in PtdIns4P in kidney. In brain, deficits in PtdIns4P were similar to those in kidney, but deficits in PtdIns4,5P_2 were much greater. Similarly, the 10-min postmortem levels of kidney PtdIns4P were preferentially reduced at weaning, and the deficits continued to persist on subsequent rehabilitation.

A preferential decrease of PtdIns4P as compared with PtdIns4,5P_2 suggests that this lipid is not merely an intermediate in the synthesis and catabolism of PtdIns4,5P_2. A selective negative effect on PtdIns4P metabolism also has been observed in response to cAMP in rabbit kidney cortex slices (24). Separate enzymes mediating the phosphodiesteratic cleavage of PtdIns4P and PtdIns4,5P_2 have been demonstrated in kidney cortex (5). The existence of separate enzymes could be responsible for the preferential decrease of PtdIns4P as well as the different effects of nutritional deprivation on the two lipids observed in kidney as compared with brain (17) of 21-day-old animals.

Several authors have studied the influence of hormones on polyphosphoinositide metabolism. Parathyroid hormone, which regulates a variety of renal functions, rapidly increases the concentrations of PtdIns4,5P_2 and PtdIns4P in rabbit kidney cortex. This effect is abolished by pretreatment with cycloheximide, which also inhibits certain renal functions (6,7). However, the functional importance of polyphosphoinositides in parathyroid hormone action is unknown. Khanna and Reddy observed no major changes in the concentration of different phospholipids from the kidney during neonatal undernutrition (25). However, these authors did not report polyphosphoinositide levels, as their extraction procedure did not permit the isolation of these lipids. Tou et al. reported no changes in kidney polyphosphoinositide levels of rats starved for 48 hr, whereas an increase in polyphosphoinositide specific radioactivity was observed after injection of $^{32}P_i$ (3). It is not known what effect changes in polyphosphoinositide metabolism caused by altered nutritional status have on renal functions.

In sum, these studies indicate that undernutrition during the pre- and postweaning periods may have different effects on polyphosphoinositide pools in brain and kidney. The factors regulating these effects in different tissues remain to be investigated. It is not known whether the rapid disappearance of even small portions of polyphosphoinositides after death might be related to changes after hormonal stimulation which have been so widely shown and studied in other tissues. Although studies on hormonal influences implicate polyphosphoinositides in renal functions (8,26), no direct evidence is available to ascribe a specific role to these lipids in the functional characteristics of the tubular membrane. Nevertheless, the identification and study of metabolic pools may contribute to the elucidation of their function.

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Effect of Chlorphentermine on Incorporation of (^{14}C)Choline in the Rat Lung Phospholipids[1]

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The effect of chlorphentermine (CP) treatment (50 mg/kg/day, per os [po]) on the incorporation of [^{14}C]choline into rat lung phospholipid was studied. Total phospholipid content was increased 2.0-fold and 1.7-fold after seven and 14 days, respectively, compared with the pair-fed rats. The incorporation of [^{14}C]choline into phosphatidylcholine (PC) was significantly inhibited by either seven or 14 days of CP treatment. Nevertheless, the PC content was significantly increased by day 7 and stayed elevated at day 14 of CP treatment. Choline and phosphorylcholine contents were significantly decreased by the CP treatment. These results suggest that the higher accumulation of PC is due to inhibition of enzymes involved in the hydrolysis of phospholipids rather than to a stimulation of the phospholipid synthesis.
Lipids 21, 230–234 (1986).

Chronic administration of several amphiphilic cationic drugs is known to cause pulmonary phospholipidosis (1). Among such drugs, chlorphentermine (CP) is well known to have a high affinity for pulmonary tissue and to cause pulmonary phospholipidosis. Although considerable information on the mechanisms of pulmonary uptake and accumulation of CP is available (2–4), the mechanism of drug-induced phospholipidosis is not well understood. Increased phospholipid synthesis or a decreased catabolism could jointly or independently account for the excessive accumulation of phospholipids in the lung tissue. Lüllmann et al. (5) suggested that the biochemical mechanism of drug-induced lipidosis is a metabolic disturbance due to binding of phospholipids with drugs of amphiphilic character. Hostetler and Matsuzawa (6) reported a direct inhibition of lysosomal phospholipid catabolism by CP in rat liver. The effect of these drugs on the phospholipid biosynthesis in the lung tissue is not well known. The present study was designed to examine the effect of CP on the biosynthesis of phosphatidylcholine (PC), which is the major component of CP-induced phospholipidosis (7). Our findings are consistent with the interpretation that the mechanism underlying phospholipidosis is likely to be inhibition of phospholipid degradative enzymes rather than stimulation of phospholipid biosynthesis.

MATERIALS AND METHODS

Chemicals. Methyl [^{14}C]choline chloride (50.5 mCi/mmol) was obtained from New England Nuclear (Millis, Massachusetts). Chlorphentermine hydrochloride was a gift of Warner-Lambert Research Institute (Morris Plains, New Jersey).

Animals and treatment. Male Sprague-Dawley rats weighing 370–475 g (Charles River Breeding Labs., Wilmington, Massachusetts) were maintained under a 12-hr photoperiod, under standard humidity and temperature conditions in our central animal facilities. Animals were treated by stomach gavage for four, seven and 14 days with a saline solution of CP at a dose of 50 mg/kg daily, based on the initial body weight. Pair-fed controls received only saline, and their food and water consumption were restricted to match the consumption of the CP-treated animals as described previously (4).

Surgical procedure and lung incubations. Animals were decapitated 24 hr after the last drug treatment or saline administration. The lungs were rapidly removed, rinsed in ice-cold saline, blotted on slightly moistened filter paper and weighed. Two portions of right lobe were weighed and dried by heating in an oven at 90 C, until a constant weight had been obtained, in order to obtain dry weight of the lung tissue. The remainder of right lobe was used for measurement of choline. Slices ca. 0.5-mm thick were obtained from the left lobe by using a McIlwain tissue chopper (Brinkman Instruments, Westbury, New York). The lung slices (250–350 mg) were immediately placed in individual flasks containing 4 ml Krebs-Ringer bicarbonate solution and 0.6 ml of 0.1 M glucose (pH 7.4). The medium was bubbled with a 95:5 mixture of O_2/CO_2 just before use. Slices were preincubated for 5 min at 37 C in Dubnoff metabolic shaking incubator. [^{14}C]Choline chloride was added (1 μCi/incubation flask) to initiate the reactions. After 60 min of incubation, reactions were stopped by placing the identification flasks in ice-bath.

Phospholipid extraction. Immediately after the incubation period, tissue slices were removed from the medium. Lipid extraction was performed by the method of Folch et al. (9). A portion of the lipid phase was used for the determination of total phospholipid content and total radioactivity. The remainder was evaporated under nitrogen gas and dissolved in 50 μl of a 2:1 (v/v) mixture of chloroform/methanol. The duplicate samples (10 μl each) were spotted on 250 μm silica gel H thin layer plates (Analtech, Newark, Delaware) and the plates were developed in a chloroform/methanol/water (65:25:4, v/v/v) system. Phospholipid spots were visualized by iodination and the spots of lysophosphatidylcholine (LPC), sphingomyelin (Sph), PC, phosphatidylethanolamine (PE) and phosphatidylserine (PS) were scraped. One spot was used for the determination of radioactivity. Phospholipid fractions were eluted from the other spot of the duplicate run by the method of Arvidson (10). A part of each fraction was used for the determination of phosphorus. Disaturated phosphatidylcholine (DSPC) was further isolated from the remainder of the PC fraction by the use of osmium tetroxide (11). The DSPC fraction also was used for the determination of phosphorus and radioactivity.

Determination of free choline and phosphorylcholine. Portions of the right lung lobe were homogenized and analyzed for free choline and phosphorylcholine by the method of Barak and Tuma (12).

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Determination of phosphorus. A modification of the Bartlett method (13) was used for the determination of phosphorus content of each phospholipid class. Sample was evaporated under nitrogen gas and added to 0.7 ml $HClO_4$. It was heated at 155 C in an oven for three hr. Two drops of 30% H_2O_2 were added to each sample and the solution was returned to the oven for at least 1.5 hr, following which 3.98 ml water, 0.18 ml 5% ammonium molybdate and 0.2 ml Fiske-SubbaRow reagent (Sigma Chemical Co., St. Louis, Missouri) were added, mixed thoroughly and heated for seven min in a boiling water bath. The optical density was measured at 830 nm in a Gilford spectrophotometer model 260 (Oberlin, Ohio).

Radioassay. Radioactivity was determined by the addition of Aquasol (New England Nuclear Co., Boston, Massachusetts) in counting vials containing the samples and by counting in a Mark II liquid scintillation spectrometer (Searle Analytic, Des Plaines, Illinois) with automatic quench correction.

Statistics. Results are given as mean ± S.E. of at least duplicate incubations of four to five experiments. Student's t-test was used to analyze the data, and statistical significance was set at $p < 0.05$.

RESULTS

Effect of CP treatment on body weight and lung weight. Because CP is an anorexic drug, water and food consumption were found to be reduced significantly in CP-treated animals compared to nontreated animals. Therefore, pair-fed animals were maintained as the most appropriate controls in these experiments. The body weight was reduced significantly in CP-treated and pair-fed rats in comparison to nontreated groups having free access to food and water. However, there was no significant difference between CP-treated and pair-fed rats.

The four- and seven-day CP treatments had no effect on lung weight nor lung-to-body weight ratios in comparison to the pair-fed rats. The weight of the 14-day CP-treated rat lung was significantly higher than in the pair-fed rats (2.70 ± 0.29 and 1.53 ± 0.12 g, respectively). A 1.8-fold increase was observed in the lung-to-body weight ratio, from 4.33 ± 0.35 for pair-fed rats to 7.97 ± 0.61 for rats treated with CP for 14 days. The ratio of lung dry weight to lung wet weight for CP-treated rats (7 and 14 days) was increased in comparison with free-fed rats (free-fed, 0.207 ± 0.005; CP 7 days, 0.215 ± 0.001; CP 14 days, 0.230 ± 0.002).

Effect of CP treatment on food and water consumption. The food consumption of CP-treated rats was significantly decreased throughout the period of treatment. However, after four or five days there was a progressive recovery toward normal food consumption. Likewise, a similar response was noted with water consumption which was reduced in comparison to the CP-treated group.

Effect of CP on [^{14}C]choline incorporation into phospholipid. Figure 1 depicts the concentration of total radioactivity, and of label associated with LPC, Sph, PC and DSPC in lung. [^{14}C]PC was significantly decreased in the lungs of the seven- and 14-day CP-treated rats, and [^{14}C]DSPC also was decreased significantly in the lungs of the four-, seven- and 14-day CP-treated rats. After CP administration, 86%, 74% and 97% of [^{14}C]PC were composed of [^{14}C]DSPC in the lungs of the four-day, seven-day and 14-day CP-treated rats, respectively. After the restriction of food and water consumption in pair-fed groups, the ratios of [^{14}C]DSPC in [^{14}C]PC were significantly decreased from 95% at day 4 to 74% at day 7 and 57% at day 14. No significant change in the amount of [^{14}C]LPC was observed between CP-treated rats and pair-fed rats.

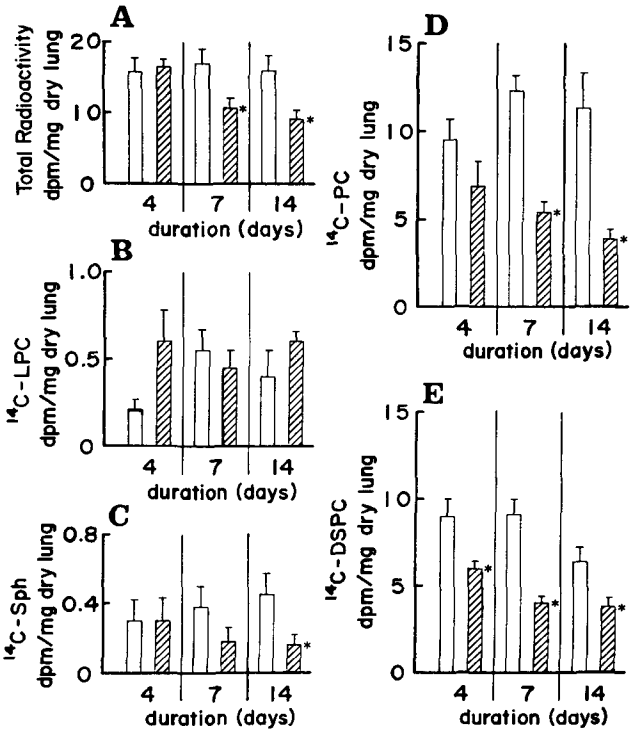

FIG. 1. Effect of CP treatment on [^{14}C]choline incorporation into phospholipid in lung. Animals were treated with CP (50 mg/kg/day, po) for four, seven and 14 days. Pair-fed (PF) groups were allowed access to food and water consumption based on CP-treated animals. Asterisk indicates that the value is significantly different from pair-fed groups ($p < 0.05$). Results are given as mean ± S.E. of at least duplicate incubations of four to five experiments. (A) Total [^{14}C]radioactivity; (B) [^{14}C]lysophosphatidylcholine; (C) [^{14}C]sphingomyelin; (D) [^{14}C]phosphatidylcholine, and (E) [^{14}C]disaturated phosphatidylcholine. Open column, PF; lined column, CP.

Effect of CP on total phospholipid, LPC, Sph, PC, DSPC, PS and PE contents in lung. Figure 2 depicts the amounts of the total phospholipid, LPC, Sph, PC and DSPC and the sum total of PS and PE in the lung as estimated by phosphorus content. The seven-day CP-treatments caused a 2.0-fold increase in lung total phospholipid. The 14-day CP-treatments caused a 1.7-fold increase in lung total phospholipid. Significant increases in the amount of PC and DSPC were observed in the lungs of seven- and 14-day CP-treated rats compared to the pair-fed rats. Significant increases in the amount of LPC were observed in the lungs of four- and 14-day CP-treated rats. Sph was also increased significantly in the lungs of four-day CP-treated rats compared to the pair-fed rats. The sum total of PS and PE was significantly increased in the lungs of four- and seven-day CP treated rats.

Effect of CP on free choline and phosphorylcholine. Table 1 depicts the amounts of the free choline and phosphorylcholine in the lung. The CP-treatments caused

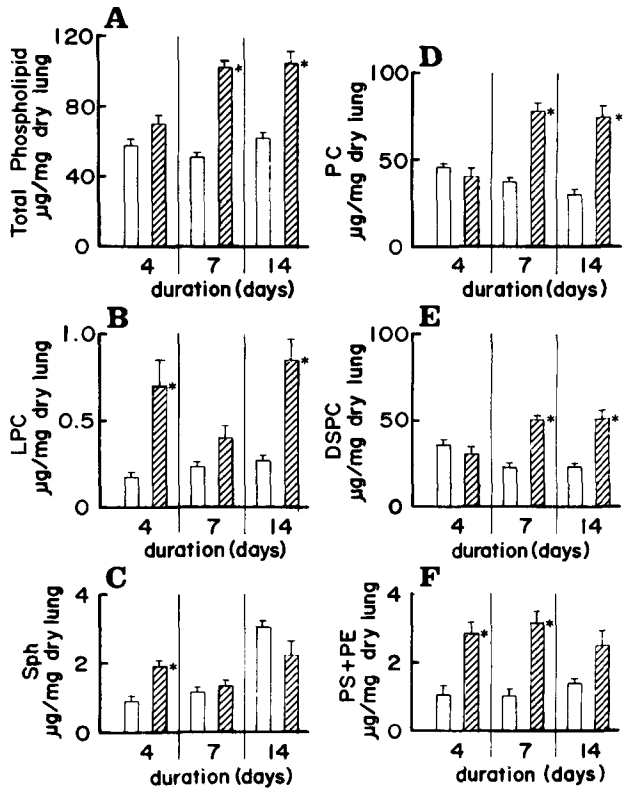

FIG. 2. Effect of CP treatment on total phospholipid, LPC, Sph, PC, DSPC, PS and PE contents in lung. Animals were treated with CP (50 mg/kg/day, po) for four, seven and 14 days. Pair-fed (PF) groups were allowed access to food and water consumption based on CP-treated animals. Asterisk indicates that the value is significantly different from pair-fed groups ($p < 0.05$). Results are given as mean ± S.E. of at least duplicate incubations of four to five experiments. (A) total phospholipid; (B) lysophosphatidylcholine; (C) sphingomyelin; (D) phosphatidylcholine; (E) disaturated phosphatidylcholine, and (F) sum total of phosphatidylethanolamine and phosphatidylserine. Open column, PF; lined column, CP.

a significant decrease of the amounts of choline and phosphorylcholine in the lungs of seven- and 14-day CP-treated rats. The significant decrease in the amounts of phosphorylcholine was also observed in the lungs of four-day CP-treated rats.

DISCUSSION

Gloster et al. (14) found that the lung-to-body weight ratios were the same in both free-fed and food-restricted rats and also observed the doubling of the lung-to-body weight ratios after CP administration. Thus, the increase in lung weight can be attributed to the action of the drug and not simply by the drug's anorexic effect (4,7). In the present study, a 1.8-fold increase was observed in the lung-to-body weight ratio between pair-fed rats and rats treated with CP for 14 days. These increases in lung weight and lung-to-body weight ratios were not due to pulmonary edema, because the ratio of lung dry weight to lung wet weight for CP-treated rats (7 and 14 days) was in fact increased in comparison with free-fed rats.

Franken et al. (15) found the huge "foam cells" in the alveoli of lungs of rats chronically treated with the anorogenic drug CP. Lüllmann-Rauch and Reil (16) studied the foam cells and found the enlarged alveolar macrophages, packed with lamellated cytoplasmic inclusions. Gloster et al. in a later study (7) demonstrated that chronic CP administration to rats (50 mg/kg for 50 days) resulted in an accumulation of all classes of phospholipids in the lung. PC accumulation in particular was most striking, with nine-fold increase in comparison with free-fed rats after this prolonged CP treatment. The phospholipid content in lung of CP-treated rats was increased to a greater extent than any of the other biochemical parameters, i.e., β-N-acetylglucosaminidase activity, protein content, acid-phosphatase activity and lactate dehydrogenase activity (17,18). The CP-induced rise in

TABLE 1

Effect of Chlorphentermine Treatment on Free Choline and Phosphocholine

Treatment[a]	Food consumption for 24 hr prior to sacrifice (g)	Choline[b] (μmol/g lung)	Phosphocholine[b] (μmol/g lung
4 days			
CP	9.7	0.115 ± 0.008	0.027 ± 0.004*
PF	—	0.093 ± 0.025	0.078 ± 0.009
7 days			
CP	15.0	0.122 ± 0.012*	0.029 ± 0.004*
PF	—	0.205 ± 0.025	0.174 ± 0.006
14 days			
CP	17.2	0.116 ± 0.005*	0.080 ± 0.007*
PF	—	0.205 ± 0.012	0.176 ± 0.008

[a]Treated with CP (50 mg/kg/day, po) for 4, 7 and 14 days. Pair-fed groups (PF) were allowed access to food and water consumption based on consumption by the CP-treated animals.

[b]Results are given as mean ± S.E. of four to five experiments.

*Significantly different from pair-fed groups ($p < 0.05$).

phospholipid components was time-dependent in lungs (19-22), while the CP-induced changes in phospholipid levels were reversible (21,23). In the present study, total phospholipid content was increased two-fold from 52.1 ± 2.3 mg/g in the lungs of the pair-fed rats to 103.0 ± 3.6 mg/g in the seven-day CP-treated rats and 1.7-fold from 62.3 ± 3.0 mg/g in the lungs of pair-fed rats to 105.0 ± 7.2 mg/g in the 14-day CP-treated rats. A shorter period of treatment was chosen for our study to obviate any other biochemical or physiological changes that might occur after prolonged drug treatment.

[^{14}C]Choline incorporation into PC was decreased significantly after CP pretreatment. Two pathways for the incorporation of [^{14}C]choline into [^{14}C]PC are known, one via the CDP-choline pathway (24) and the other via the *N*-methylation of the ethanolamine base of phosphatidyl ethanolamine (PE) (25-29). The latter is not possible in the healthy lung, because *N*-methyl-transferase, which is the enzyme for *N*-methylation, has a high activity with saturated substrates (30), and the saturated PE is not detectable in lung (31). However, the effect of CP treatment on the molecular species of PE is not known. Gardiner and Lee (32) observed a significant increase of [^{14}C]labeled hemicholinium in the lipid fraction and proposed that hemicholinium could have stimulated either CDP-choline pathway of PC synthesis, enhanced the base-exchange reaction of [^{14}C]choline for phospholipid bases or blocked the incorporation of choline into something else. In the present study, the significant decrease in [^{14}C]PC suggests that CP could have inhibited the CDP-choline pathway of PC synthesis. An alternate explanation is the possibility that CP interferes with the uptake of choline under in vitro conditions. This is unlikely, however, for two reasons. We did not find significant differences in [^{14}C]choline incorporation between seven- and 14-day CP treatments. Pulmonary CP accumulation is known to increase with the duration of CP treatment. Also, in preliminary experiments inclusion of CP in the incubations did not influence the uptake of [^{14}C]choline by lung slices (data not shown). Stimulation of the incorporation of choline into something else is also a possibility, although the product of such a diversion is not known.

The CP treatment caused a significant decrease in the lung contents of choline and phosphorylcholine. This finding suggests that the increased PC level is not due to an expansion of the choline pool. Furthermore, the decrease in choline pool may not be attributed to increased substrate utilization, for we did not observe increased phospholipids after seven days of CP treatment, when the choline pool was decreased.

Drug-induced phospholipidosis was reported to be species-dependent and tissue-dependent, and this metabolic phenomenon has been associated with inhibition of lysosomal phospholipase (33). A significant increase in DSPC content was observed in the lungs of the seven- and 14-day CP-treated rats. The microsomal phospholipase A_2 is known to be less active toward DSPC than toward PC containing an unsaturated fatty acid at the 2-position (34), and this might account for the increased DSPC observed in our studies. Our results show that the LPC content was increased in the lungs of the four- and 14-day CP-treated rats. This finding suggests that lysophospholipase also might be inhibited with CP treatment.

Since the lung contents of PC and DSPC were significantly increased in vivo after seven days of CP treatment, in spite of an apparently reduced synthesis in in vitro studies, it appears that the degradation of PC and DSPC is significantly inhibited, more than offsetting the depressed synthesis. Karabelnik and Zbinden (35) demonstrated that CP inhibited the incorporation of [^{14}C] palmitate into phospholipid fraction in vivo and concluded that phospholipid storage after CP treatment is mainly due to decreased degradation of phospholipids. Supporting this suggestion are observations of Lüllmann et al. (36) that CP is tightly bound to tissue components, and NMR studies of Seydel and Wassermann (37,38) showing a strong binding ability between cationic amphiphilic drugs and certain polar lipids. If the development of phospholipidosis is due to an impairment in phospholipid catabolism, it would follow that the higher the level of CP present in the tissue, the greater the accumulation of phospholipids (39). Our results are consistent with and supportive of these proposals, although a direct demonstration of inhibition of enzyme involved in phospholipid synthesis would be necessary to propose a more precise site of action for CP.

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Partial Specific Volume and Preferential Hydration of Low Density Lipoprotein Subfractions

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We have determined the partial specific volume ($\bar{v}$) for five low density lipoprotein (LDL) subfractions (n = 5–7) and evaluated preferential hydration (n = 2) for LDL subfraction 3 in normolipoproteinemic subjects in order to characterize these highly atherogenic components of the human plasma lipoprotein spectra. Values for $\bar{v}$ at 1 g were determined by sixth place density measurements of the solvent and lipoprotein solutions and carbon, hydrogen and nitrogen (CHN) absolute mass of the lipoprotein concentrations. Mean values for $\bar{v}$ were 0.9757 ± 0.0019, 0.9701 ± 0.0007, 0.9674 ± 0.0016, 0.9616 ± 0.0016 and 0.9550 ± 0.0025 ml/g for subfractions 1, 2, 3, 4 and 5, respectively. However, molecular densities (σ) obtained from ϱ (rho) = $1/\bar{v}$ for respective LDL subfractions were 1.0249, 1.0308, 1.0337, 1.0399 and 1.0471 g/ml, respectively. The preferential hydration of lipoprotein subfraction 3 (n = 2) in NaCl/H_2O solutions was 2.9–4.8 wt percent, whereas values were much lower (0.3–0.6 wt percent) in NaCl/NaBr/H_2O solvent system. Unhydrated densities for LDL subfraction 3 (n = 2) at 1 g (sixth-place density meter) were 1.0287 and 1.0269 g/ml, whereas at 200,000 × g (used in D_2O flotation ηF° vs ϱ determinations) both values were 1.0308 g/ml, indicating that these similar LDL fractions have 23 and 53% higher compressibility than the solvent at 200,000 × g force. It was observed that the linearity of ηF° vs ϱ may not be valid for solvents NaCl/NaBr/H_2O of density as high as 1.4744 g/ml. Thus, flotation velocity data using extreme salt concentrations (1.4744 g/ml and higher) may be viewed with caution.
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Partial specific volume ($\bar{v}$) and preferential hydration of LDL subfractions need to be determined to characterize these atherogenic components of the human plasma lipoprotein spectra. Molecular weights of LDL subfractions have been determined by sedimentation equilibrium (1), using an estimated $\bar{v}$, as given by $\bar{v} = 1/\varrho$, where ϱ is the corresponding background gradient density of isolation. However, for accurate molecular weight determinations of LDL subfractions by sedimentation equilibrium, valid determination of $\bar{v}$ is essential. Also, preferential hydration of LDL has not been thoroughly investigated, although values of no preferential hydration have been reported (2). We have determined $\bar{v}$ for five LDL subfractions (n = 5–7) and evaluated preferential hydration (n = 2) for LDL subfraction 3. Subfraction 3 is at the hinge point (ϱ = 1.0357 g/ml) of the LDL equilibrium gradient used for subfractionation (3).

EXPERIMENTAL PROCEDURES

Partial specific volume. Apparent $\bar{v}$, ml/g, of LDL subfractions is determined using the equation given by Schachman (4).

$$\bar{v} = 1/\varrho_0 - 1/x(\varrho - \varrho_0)/\varrho_0$$

Solvent and lipoprotein solution densities ϱ_0 and ϱ, respectively, are determined using a DMA-60 Mettler/Paar sixth-place density meter (Mettler Instrument Corp., Princeton, New Jersey). The mass of LDL in solution (x, g/ml) is determined by a modified CHN analyzer (Hewlett-Packard Co., Palo Alto, California). Each LDL subfraction along with an aliquot of its corresponding equilibrium gradient background salt solution is dialyzed together against a 1.0063 g/ml NaCl solution (10 mg/dl EDTA) in the same cylinder in which CO_2 has been evacuated and displaced by N_2. The dialyzed density gradient background gives an accurate ϱ_0 which is an approach to equilibrium of the final dialysate. Solvent and solution densities as measured are accurate to ±0.000001 g/ml, where temperature of the density measuring cell is stabilized to 20 ± 0.001 C. CHN analysis determines lipoprotein mass (x, g/ml) within ±0.5%, using acetanilide as the standard (peak heights vs mass of C or N are linear within the 250 μg of mass used). Unhydrated mass of a lipoprotein solution in micro quantities (250 μg) can be determined very accurately (±0.5%) by CHN elemental analysis without interference of the inorganic salts present. Using the 24-hr dialysate from the cylinder after three dialysate changes, the background salt gradient and lipoprotein sample are in equilibrium to the dialysate only to the fourth decimal place. An error of one part in the fourth decimal place in solvent density would result in a lipoprotein $\bar{v}$ error of two parts at the second decimal place. Thus, the approach to equilibrium dialysis of the lipoprotein solution must be quantified by the difference in density of the dialyzed background density and dialysate. These limitations include constant chemical potentials (μ) of the solvent on each side of the dialysis bag (5). Here

$$(\partial\varrho/\partial c)\mu = \frac{\varrho - \varrho_0}{c} \cong (1 - \bar{v}\varrho_0)$$

This experimentally derived quantity can be directly substituted into the sedimentation equilibrium equation as follows to yield the unhydrated molecular weight:

$$\frac{\partial \ln c}{\partial r^2} = M_u \left(\frac{\partial \varrho}{\partial c}\right)\frac{\omega^2}{\mu} \Big/ 2RT$$

Preferential hydration. Preferential hydration of LDL subfraction 3 is determined from unhydrated and hydrated densities obtained from an ηF° vs ϱ plot of lipoprotein floation velocity data in D_2O and H_2O/salt solutions, respectively. LDL subfraction 3, along with its corresponding gradient background density 1.0357 g/ml, is dialyzed against NaCl/H_2O or NaCl/D_2O solutions of densities 1.0631, 1.0900, 1.1130 or 1.1955 g/ml. In 1.0631 and 1.0900 g/ml, the solution consists of an appropriate mixture of NaCl/H_2O of density 1.0063 g/ml and an NaCl/D_2O solution of 1.1130 g/ml. Also, this subfraction is dialyzed against NaCl/H_2O density 1.0063 g/ml with added NaBr to obtain 1.0631, 1.2030 or 1.4744 g/ml. This keeps the 0.196 *m* NaCl concentration constant. Flotation velocities of the dialyzed subfraction 3 samples are

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determined using dialyzed 1.0357 g/ml salt solution against the above solutions as a background in the Beckman Model E analytical ultracentrifuge. This procedure is given in detail elsewhere (6). From $\eta F°$ vs ϱ plots, ϱ_0 intercepts give the unhydrated (in D_2O systems) and hydrated (in H_2O/salt systems) densities. The ϱ_0 intercepts are obtained at $\eta F° = 0$, where η = viscosity of dialyzed solvent, F° = flotation velocity at zero concentration and ϱ_0 is the density of the solvent (dialyzed background). The 1-g LDL density is obtained from $1/\bar{v} = \varrho$ by sixth-place density measurements. Molecular weight of LDL subfraction 3 is obtained by the sedimentation equilibrium procedure given in detail elsewhere (1) and by flotation velocity (6). Preferential hydration (Γ') of LDL subfraction 3 is calculated from (7) (see appendix) using the equation

$$\Gamma' = \frac{\bar{v}_{U\text{-}LDL} - \bar{v}_{H\text{-}LDL}}{\bar{v}_{H\text{-}LDL} - \bar{v}_{H_2O}}$$

where partial specific volumes of unhydrated LDL (U-LDL) are obtained by flotation velocity ϱ intercept in H_2O/salt and $\bar{v}_{H_2O} = 1/\varrho_{H_2O}$.

RESULTS

Apparent partial specific volumes of five low density lipoprotein (LDL) subfractions calculated from sixth-place densities and CHN lipoprotein mass are given in Table 1. Mean values for $\bar{v}$ are 0.9757 ± 0.0019, 0.9701 ± 0.0007, 0.9674 ± 0.0016, 0.9616 ± 0.0016 and 0.9550 ± 0.0025 ml/g for subfractions 1, 2, 3, 4 and 5, respectively. Thus, these densities obtained from $\sigma = 1/\bar{v}$ for respective LDL subfractions are 1.0249, 1.0308, 1.0337, 1.0399 and 1.0471 g/ml, respectively.

Table 2 gives the flotation velocity data of LDL subfraction 3. In Figures 1A and 1B are shown $\eta F°$ vs ϱ_{26} plots for two subjects (one male and one female, respec-

TABLE 1

Partial Specific Volume ($\bar{v}$) of LDL Subfractions[a]

Subject	Sex	Age	Subfractions 1	2	3	4	5
6622	M	35	.9697	.9690	.9673	.9673	.9612
6769	M	52	.9759 (2.75)	.9722 (3.92)	.9641 (8.36)	.9624 (17.72)	.9575 (7.92)
7029	M	52	.9817 (2.00)	.9697 (3.27)	.9640 (8.09)	.9602 (13.53)	.9582 (3.96)
7222	M		.9763 (2.03)	.9712 (3.51)	.9658 (10.29)	.9609 (8.57)	.9499 (2.07)
7384	M	37	.9748 (2.20)	.9684 (5.68)	.9638 (11.87)	.9574 (5.27)	.9484 (1.96)
7563	M	37	— (2.10)	— (8.33)	.9721 (7.06)	— (3.20)	— (2.10)
7684	F	65	—	—	.9747	—	—
Mean ± SEM			.9757 ± .0019	.9701 ± .0007	.9674 ± .0016	.9616 ± .0016	.9550 ± .0025
Mean density			1.0249	1.0308	1.0337	1.0399	1.0471

[a]Concentrations in mg/ml are given in parentheses.

TABLE 2

Flotation Velocity Data, LDL Subfraction 3

Sample	Dialysate	Solvent ϱ_{26}[a]	Solvent η_{26}[a]	S_f^{o}[b] or F°[c]	$\eta F°$	ϱ Intercept
7563 Male	$NaCl/H_2O$	1.0609	1.020	6.854[c]	6.991	
		1.1102	1.1922	15.096	17.997	1.0298
		1.1923	1.6864	21.593	36.414	
	$NaCl/D_2O/H_2O$	1.0606	.9978	6.167[c]	6.153	
	$NaCl/D_2O$	1.119	1.0948	15.090	16.521	1.0308
		1.1944	1.3157	25.341	33.341	
	$NaCl/NaBr/H_2O$	1.0610	.9181	7.147[c]	6.562	
		1.1998	1.0944	33.334	36.481	1.0306
7684 Female	$NaCl/H_2O$	1.0610	1.0202	6.499[c]	6.630	
		1.0876	1.1039	11.334	12.512	1.0292
		1.1104	1.1931	14.278	17.035	
	$NaCl/D_2O/H_2O$	1.0606	.9978	6.095[c]	6.082	
		1.0865	1.0631	11.045	11.742	1.0308
	$NaCl/D_2O$	1.1119	1.0948	15.253	16.699	
	$NaCl/NaBr/H_2O$	1.0610	.9181	6.895[c]	6.330	
		1.1998	1.0947	32.399	35.358	1.0307
		1.4697	1.7413	44.127	76.838	

[a]All densities (g/ml) and viscosities are given at 26 C.

[b]S_f^{o} values are corrected to infinite dilution and to standard conditions of 1.744 m NaCl at 26 C (1.0630 g/ml, η = 1.026 cp).

[c]$F° = F°(1 - Kc)$, where c is the concentration of lipoprotein in mg/100 ml, and $K = 0.89 \times 10^{-4}$ (mg/100 ml)$^{-1}$.

tively). Unhydrated density of LDL subfraction 3 in one male (7563) and one female (7684) is the same, 1.0308 g/ml, whereas hydrated densities in NaCl/H_2O are 1.0292 and 1.0298 g/ml, respectively, resulting in preferential hydration of lipoprotein in NaCl/H_2O solution of 2.9 and 4.8 wt percent, respectively. Hydrated densities in NaCl/NaBr/H_2O solution are 1.0306 and 1.0307 g/ml, giving preferential hydration in this solvent system of 0.6 and 0.3 wt percent, respectively.

Molecular weights of LDL subfraction 3 in these two subjects were 3.39 and 3.92 × 10^6 daltons by sedimentation equilibrium, using their respective $\bar{v}$ of 0.9721 and 0.9747 ml/g, whereas molecular weights by flotation velocity, assuming Stokes' spheres, were 2.37 and 2.36 × 10^6 daltons. The shape factor f/f_o obtained by (sedimentation equilibrium mol wt/flotation velocity mol wt)$^{2/3}$ is 1.27 and 1.40 for subjects 7563 and 7684, respectively.

DISCUSSION

Partial specific volume ($\bar{v}$) is crucial for determining true molecular weight of LDL subfractions. Standard error of measurement (SEM) (Table 1) represents a small technical error but largely a biological variability within subjects over time and between individuals. Higher SEM in subfractions 1 and 5 are due to lower plasma lipoprotein concentrations in these two LDL subfractions, as they are at either end of the equilibrium gradient. Variability in $\bar{v}$ within each subfraction is due to the differences in lipoprotein profile between and within subjects over time. Higher $\bar{v}$ for LDL subfraction 3 of sample 7563 is due in part to a concentration shift in lipoprotein profile in this individual from subfraction 3 to subfraction 2.

Partial specific volumes ($\bar{v}$) of 0.965 from total LDL are reported by Crossley et al. (8) and Nelson et al. (9) using lipoprotein concentration (c) obtained by drying to constant weight. Similarly, Fless and Scanu (10) and Fless et al. (11) reported $\bar{v}$ for total LDL in rhesus monkeys as 0.960 and 0.969. Jeffrey et al. (12) obtained $\bar{v}$ of 0.963 ± 0.004 for total LDL, using a specific refractive increment of 0.00154 Δn/g/100 ml to obtain lipoprotein concentration. In order to obtain accurate solvent density, dialysis of corresponding salt gradient background density solution is required. Preferential hydration of LDL subfraction 3 in NaCl/H_2O solution ranged from 2.9 to 4.8 wt percent, whereas values were much lower in 0.196 *m* NaCl/NaBr/H_2O solutions (0.3 to 0.6 wt percent). Fisher et al. (2) reported no preferential hydration of total LDL in KBr/H_2O solution, whereas Schumaker et al. (personal communication) indicated 10% preferential hydration of LDL using NaCl/H_2O and NaBr/H_2O as solvents. Unhydrated densities obtained at 1 g from the $\bar{v}$ of these two subjects were 1.0287 and 1.0260 g/ml, whereas unhydrated densities at 200,000 × g for these two subjects were larger and the same value, 1.0308 g/ml. This difference is due to 23% and 53% higher lipoprotein compressibility of LDL than the solvent in these subjects, respectively, at the pressure involved with 200,000 × g force used in flotation velocity determinations. Lipoprotein subfraction density (σ) does not increase proportionally to pressure during equilibrium gradient centrifugation, indicating that different subfractions may have different compressibilities. Since the LDL lipid core consists of cholesteryl ester and triglyceride, preferential hydration involves surface phospholipid and/or protein components. The important role of apolipoprotein in determining overall lipoprotein structure and metabolism is well established. Preferential hydration of H_2O or binding of other small molecules may be important in determining degree of atherogenicity of LDL subfractions. In the case of subject 7684, flotation velocity values are also determined in a NaCl/NaBr/H_2O solution of density 1.4744 g/ml. It is shown in Figure 1B that the linearity of ηF° vs ϱ may not be valid for very high salt concen-

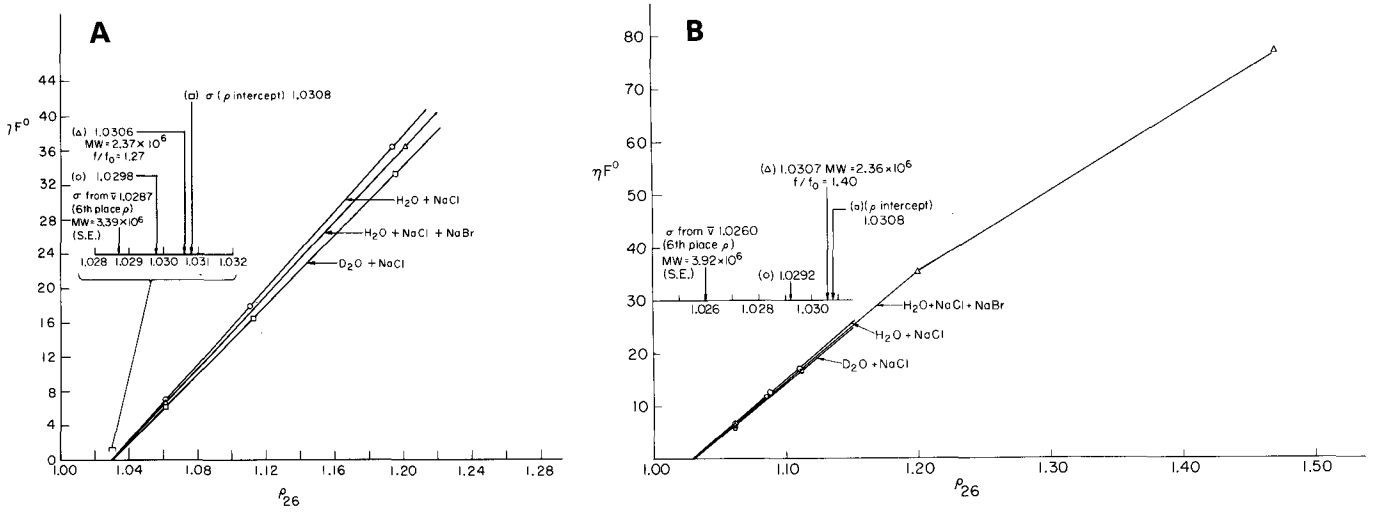

FIG. 1. (A) Low density lipoprotein subfraction 3, male subject 7563. ηF° vs ϱ plot. □, D_2O/NaCl; ○, H_2O/NaCl; △, H_2O/NaCl/NaBr. Inset: ϱ intercepts (□) 1.0308, (○) 1.0298 and (△) 1.0306 g/ml. Density ϱ from $\bar{v}$ = 1.0287 g/ml obtained from sixth-place solvent and solution densities and CHN elemental mass analysis. Molecular weights by flotation velocity (2.37 × 10^6) and by sedimentation equilibrium (3.39 × 10^6) daltons. Shape factor f/f_0 (sedimentation equilibrium mol wt/flotation velocity mol wt)$^{2/3}$ = 1.27. (B) Low density lipoprotein subfraction 3, female subject 7684. ηF° vs ϱ plot. □, D_2O/NaCl; ○, H_2O/NaCl; △, H_2O/NaCl/NaBr. Inset: ϱ intercepts (□) 1.0308, (○) 1.0292 and (△) 1.0307 g/ml. Density ϱ = 1.0260 g/ml. Flotation velocity MW = 2.36 × 10^6 and sedimentation equilibrium MW = 3.92 × 10^6 daltons. f/f_0 = 1.40.

tration solvents; thus, data reported in the literature (13) using extreme salt concentrations (1.4744 g/ml or higher g/ml) may be viewed with caution.

ACKNOWLEDGMENTS

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APPENDIX

The thermodynamic equations describing the equilibria in a three-component system in a centrifugal field are (6)

$$M_1 (1 - \bar{v}_1\varrho)\, \omega^2 r dr = \left(\frac{\partial \mu_1}{\partial m_1}\right)_{m_3} dm_1 + \left(\frac{\partial \mu_1}{\partial m_3}\right)_{m_1} dm_3 \quad [1]$$

$$M_3 (1 - \bar{v}_3\varrho)\, \omega^2 r dr = \left(\frac{\partial \mu_3}{\partial m_3}\right)_{m_1} dm_3 + \left(\frac{\partial \mu_3}{\partial m_1}\right)_{m_3} dm_1 \quad [2]$$

ϱ = density of the solution
ω = angular velocity
Subscripts 1 and 3 refer to solute and macromolecules, respectively.
M = molecular weight
$\bar{v}$ = partial specific volume
μ = chemical potential
m = molality

These differential equations are valid at constant temperature and pressure.

The solvation parameter $\Gamma \equiv -(\partial \mu_1/\partial m_3)_{m_1}/(\partial \mu_1/\partial m_1)_{m_3}$ represents the net solvation of the polymer in moles solute per mole polymer and is equal to $(\partial m_1/\partial m_3)_\mu$.

Γ is the number of moles of solute 1 which must accompany one mole of macromolecules at a constant chemical potential.

Substituting Γ in equations 1 and 2

$$\left(\frac{\partial \mu_1}{\partial m_3}\right)_{m_1} = \left(\frac{\partial \mu_3}{\partial m_1}\right)_{m_3}$$

$$M_1 (1 - \bar{v}\varrho)\, \omega^2 r dr = \left(\frac{\partial \mu_1}{\partial m_1}\right)_{m_3} dm_1 - \Gamma\left(\frac{\partial \mu_1}{\partial m_1}\right)_{m_3} dm_3 \quad [3]$$

$$M_3 [1 - \bar{v}_3\varrho)\, \omega^2 r dr = \left(\frac{\partial \mu_3}{\partial m_3}\right)_{m_1} dm_3 - \Gamma\left(\frac{\partial \mu_1}{\partial m_1}\right)_{m_3} dm_1 \quad [4]$$

$$[(M_3 + M_1\Gamma) - (M_3\bar{v}_3 + \Gamma M_1\bar{v}_1)\varrho]\, \omega^2 r dr = \left[\left(\frac{\partial \mu_3}{\partial m_3}\right)_{m_1} - \Gamma^2\left(\frac{\partial \mu_1}{\partial m_1}\right)_{m_3}\right] dm_3 \quad [5]$$

Solvation parameters may be defined on weight basis $\Gamma' = \Gamma(M_1/M_3)$.

$$M_3 (1 + \Gamma')\left[1 - \left(\frac{\bar{v}_3 + \Gamma'\bar{v}_1}{1 + \Gamma'}\right)\varrho\right] \omega^2 r dr = \left(\frac{\partial \mu_3}{\partial m_3}\right)_{m_1} \left[1 - \frac{(\partial \mu_1/\partial m_3)^2_{m_1}}{(\partial \mu_1/\partial m_1)_{m_3}\,(\partial \mu_3/\partial m_3)_{m_1}}\right] dm_3 \quad [6]$$

At maximum polymer concentration $dm_3/dr = 0$ with this position defining band center $\varrho = \varrho_0$.

$$1 - \frac{\bar{v}_3 + \Gamma'\bar{v}_1}{1 + \Gamma'}\varrho_0 = 0$$

$$\frac{1}{\varrho_0} = \frac{\bar{v}_3 + \Gamma'\bar{v}_1}{1 + \Gamma'} = \bar{v}_H \text{ (hydrated } \bar{v} \text{ of macromolecules)}$$

$$\bar{v}_H = \frac{\bar{v}_3 + \Gamma'\bar{v}_1}{1 + \Gamma'}$$

$$\Gamma' = \frac{\bar{v}_3 + \bar{v}_H}{\bar{v}_H - \bar{v}_1}$$

$$\Gamma' = \frac{\bar{v}_{LDL} - \bar{v}_{H\text{-}LDL}}{\bar{v}_{H\text{-}LDL} - \bar{v}_{H_2O}}$$

METHODS

Complete Separation of Phospholipids from Human Heart Combining Two HPLC Methods

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The separation of phospholipid classes from human heart was achieved in two steps by high performance liquid chromatography (HPLC) using a silica column with an ultraviolet spectromonitor at 206 nm. A complete partitioning of phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylinositols (PI), phosphatidylserines (PS), cardiolipins (CL), lysophosphatidylcholines (LPC) and sphingomyelins (Sph) was obtained for further analysis.
Lipids 21, 239-240 (1986).

The separation of phospholipid classes traditionally uses thin layer or column chromatography (1). Many HPLC methods (2) have been developed that offer advantages over other chromatographic methods. However, the separations were not easy because of the complexity and diversity of phospholipid structures as well as their limits of detection (absorption in the 203-210 nm region). Many applications of separation came from the techniques of Hax et al. (3) and Geurst Van Kessel et al. (4), which allowed the separation of all phospholipid classes except PC and Sph, or from the work of Jungalwala et al. (5), which allowed the separation of PC and Sph but not other classes.

Our aim was to separate all of the phospholipid classes of human or rat heart (CL, PC, PE, PI, PS, LPC and Sph). Moreover, we wished to obtain these fractions as pure as possible and in sufficient quantity (milligrams) for further analysis or for preparation of membrane models.

The preliminary assays with the existing methods (6-9) were less satisfactory than the combined methods of Hax et al. (3) and Jungalwala et al. (5). Particularly mobile phases containing acidic solvents which can alter plasmalogens (7,10) were avoided. The present paper shows the results of a complete separation of phospholipid classes from human heart.

EXPERIMENTAL PROCEDURES

Materials. The HPLC was a Varian model Vista 54 with a Pye Unicam detector, model PU4020. The fraction collector was a 401 Gilson model.

The HPLC columns were packed in our laboratory according to the procedure of Coq et al. (11). Organic solvents were of HPLC grade and used as such after degassing. Distilled water was purified on an RP-8 column (E. Merck, Darmstadt, West Germany). Standards of phospholipids were obtained from Sigma Chemical Co. (St. Louis, Missouri).

Methods. Samples of human heart (about 1 g) were obtained from the Surgical Clinic at the Dijon University Hospital. They were removed in the course of an open-heart surgery and stored immediately in a chloroform/methanol mixture (2:1, v/v). The total lipids were extracted according to the method of Folch et al. (12) and the phospholipids were separated from the nonphosphorus lipids in silica cartridges (13). The phospholipids were dissolved in 2-propanol/hexane (8:6, v/v). The analyses were done at room temperature.

The first separation followed the procedure of Hax et al. (3). A column with a similar length (25 cm) was used but with a bigger internal diameter (7.5 mm) packed with Lichrosorb Si60, 5 μm (Merck). This modification compelled us to modify the gradient. The elution mixtures of 2-propanol/hexane/water varied from 54:41:5 (v/v/v) to 52:39:9 (v/v/v) in 10 min instead of 5 min plateau and 20 min gradient. The flow rate was only double (2 ml/min) in order to avoid excessive head pressure (100 atm).

The second separation was a modification of the procedure of Jungalwala et al. (5). We used a shorter (25 cm) column of larger internal diameter (4.8 mm) packed with Lichrosorb Si60, 10 μm (Merck).

The quantity injected (3 or 4 mg) and the ratio of the PC and Sph compelled us to modify the mixture of acetonitrile/methanol/water to 71:21:8 (v/v/v) instead of 65:21:14 (v/v/v). The flow rate was tripled (3 ml/min). The detector in the two analyses was set at 206 nm with a sensitivity of 1.28 absorbance unit full scale (AUFS). The collected fractions were evaporated to dryness at 42 C in a rotary vacuum apparatus and kept in chloroform/methanol (2:1, v/v) until analyzed.

RESULTS AND DISCUSSION

Figure 1 shows the results obtained after the first separation. The CL, PE, PI, PS and LPC were well separated. PC and Sph were collected together. Ethanolamine and choline plasmalogens are eluted with PE and PC respectively. Peaks were identified by comparing their retention times to those of phospholipids standards. The purity of the collected fractions was tested by thin layer chromatography (TLC) (14). As shown by Hax et al. (3) and Geurst Van Kessel et al. (4), a part of Sph was in the PC peak. This first analysis lasted 60 min (45 min for complete elution and 15 min for column equilibration). The maximum injected quantity was 7 mg of phospholipids, according to the column characteristics. We verified with a phosphorus assay (15) that no phospholipids remained on the column. The column was used for more than 200 runs without loss of reproducibility and allowed us to automate the collecting of the different fractions.

Figure 2 shows the separation of PC and Sph. These two classes were well separated (TLC assay) and identified with standards. There was no retention of

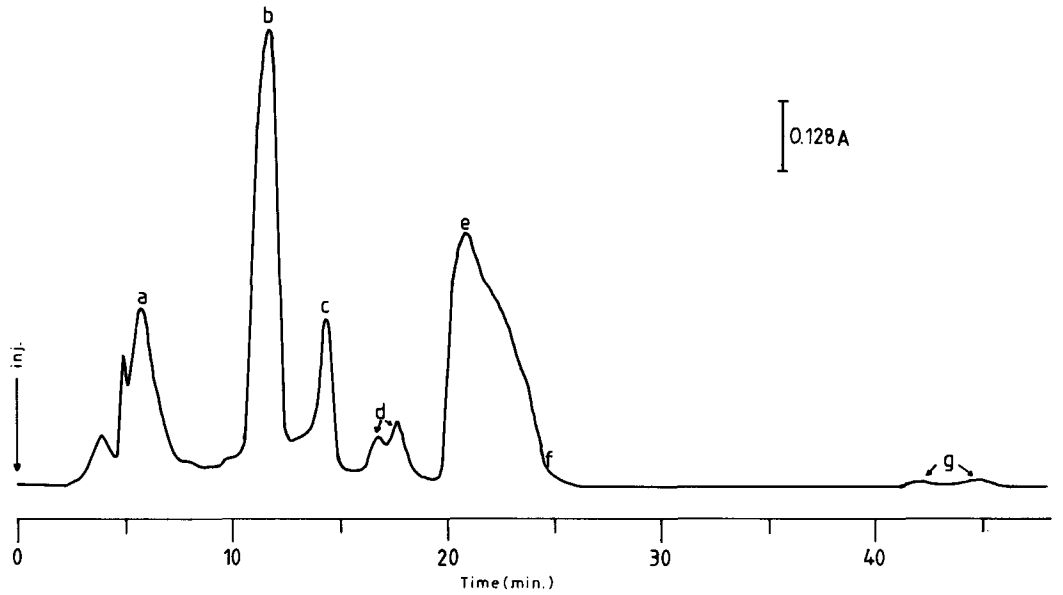

FIG. 1. Separation of human heart phospholipids. Solvent: 2-propanol/hexane/water (54:41:5 to 52:32:9, v/v/v in 10 min); flow rate: 2 ml/min; column: Lichrosorb Si60, 5 μm; UV detection at 206 nm; sensitivity: 1.28 absorbance unit full scale (AUFS). a: Cardiolipin, b: phosphatidylethanolamine, c: phosphatidylinositol, d: phosphatidylserine, e: phosphatidylcholine, f: sphingomyelin, g: lysophosphatidylcholine.

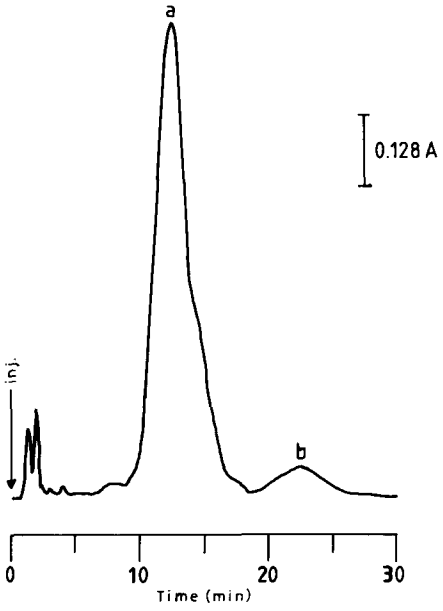

FIG. 2. Separation of the phosphatidylcholine (a) and sphingomyelin (b) fraction from human heart phospholipids. Solvent: acetonitrile/methanol/water (71:21:8, v/v/v); flow rate: 3 ml/min; column: Lichrosorb Si60, 10 μm; UV detection at 206 nm; sensitivity: 1.28 absorbance unit full scale (AUFS).

phospholipids on the column. The analysis took 30 min.

We could similarly separate phospholipids from rat heart, kidney and testes. For rat liver phospholipids, it was necessary to modify the relative proportions of acetonitrile/methanol/water in the second step to 72:22:6 (v/v/v) instead of 69:21:8 (v/v/v) for a complete separation of PC and Sph.

The quantity injected (7 mg) produced sufficiently pure classes and quantity of minor classes like PS, PI, Sph and LPC. With the automated collections of fractions, the time of analysis was 90 min for a complete separation, but the evaporating time was rather long, partly because of the water contained in the mobile phase but mainly because of the number of fractions.

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Practical Liquid Chromatographic Separation of the Phenols in Technical Cashew Nutshell Liquid from *Anacardium occidentale*[1]

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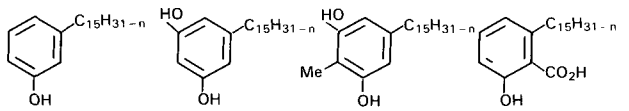
The practical separation of cardanol from technical cashew nutshell liquid or its distillate has been effected by liquid chromatography on Silica Gel H 60 (TLC type) with solute/absorbent in the range 1:5–1:6. Concentrates of the monoene, diene and triene constituents of cardanol have been prepared by argentation liquid chromatography on Silica Gel H 60 with dry incorporation of 15% silver nitrate. The present use of pressurized columns was made prior to the advent of flash chromatography.
Lipids 21, **241–246 (1986).**

Cardanol (I) the principal component of technical cashew nutshell liquid (CNSL) together with cardol (II) and 2-methylcardol (III) is formed industrially (2,3) by the hot decarboxylation of the anacardic acid (IV) in natural CNSL in the cashew nut *(Anacardium occidentale).*



(I) (II) (III) (IV)

The production of the cashew nut industry, which has grown steadily in the last two decades, has been projected (4) to be in excess of 10^6 ton/yr by the early 1990s and theoretically should yield 25% of by-product technical CNSL though processing losses result in smaller yields. While the established use of technical CNSL in friction dusts involves no purification stage, increasing interest in chemical uses has led to various distillation procedures (5–7) for obtaining cardanol. Cardol also has a potential chemical utility due to its greater side chain unsaturation and reactivity in the aromatic ring than cardanol, but because it is not recoverable by these methods due to its higher boiling point and greater sensitivity than cardanol to thermal deterioration, it is best obtained by column chromatography.

In the present work cardanol and cardol have been separated by this technique with thin layer chromatographic Silica Gel H type 60 at much higher solute/adsorbent ratios than previously (8,9) with the additional objectives of effecting solvent recovery and column reuse.

The conditions we have found valuable are similar to those described in the technique of flash chromatography (10), although our experiments with the pressurized technique were done prior to that description. The important factors in preparative liquid chromatography of sample load and resolution (11), of large samples of solute (12) and theoretical aspects (13) have not been examined in the field of phenolic lipid separation.

Argentation methods are necessary (14) for resolving the monoene, diene and triene constituents of the component phenols as fractional crystallization procedures are unduly slow (15), although for some applications (16) complete resolution of the constituents is not necessary. Argentation columns have been prepared previously by slurrying aqueous (17) or aqueous methanolic solution of silver nitrate with Silica Gel G (18) and by the use of silver nitrate dissolved in acetonitrile (19). We have examined the novel method of dry incorporation of finely powdered silver nitrate into Silica Gel H 60 and much higher solute/adsorbent ratios for the separation of the constituents of cardanol.

All fractions obtained in chromatographic separations have been monitored by thin layer chromatography (TLC) followed by gas liquid chromatography (GLC) examination (20) of the combined fractions. Fractions from argentation columns were analyzed subsequently by high performance liquid chromatography (HPLC) (21). Quantitative analysis has not been used previously (8,22,23).

EXPERIMENTAL PROCEDURES

Materials. Technical CNSL was obtained from British Coco Mills (Hull, United Kingdom), Tar Residuals Ltd. (London, S.E.I., United Kingdom) and Minnesota 3M Research Ltd. (Harlow, United Kingdom) Natural CNSL was extracted as described (9) as were the recovered cardol, 2-methylcardol and cardanol after removal of anacardic acid used for the separation in Table 2. Technical CNSL was molecularly distilled in a single stage wiped-wall still to remove polymeric materials. Vacuum-distilled CNSL was isolated as described (5).

Silica Gel H (TLC grade, type 60, article 7736, E. Merck, Darmstadt, West Germany) or Silica Gel (article 9385) and/or Silica Gel MFC was used. Silica Gel G would be equally effective.

Chromatography. Preparative column chromatography for the experiments in Table 1 was carried out with a wide bore (id 6.5 cm × 50 cm length) glass column of known volume, equipped with a sintered disc (porosity 2), a stopcock with a PTFE key at the lower end and a tightly fitting rubber bung at the upper end having a glass entry tube for pumped solvent or for attachment of a hand-operated double rubber bulb, air pressurized (scent bottle type), which was invaluable in the absence of a compressed air supply.

The column was packed by slurrying the silica gel with a known volume of light petroleum and consolidation by light tapping during filling. From the volume of solvent collected when the solvent and adsorbent levels were equal, the dead space was found. Three closely fitting circles of filter paper were placed on the adsorbent to ensure even passage of the solute solution (in light

[1]This publication is Part 26 in the series of "Long Chain Phenols." Reference 1 is Part 25.

petroleum 60–80 C). A hand-actuated air pressure ensured a practical rate of development and elution; in the absence of this, a small reciprocating pump (Hyflo model B, Metcalf Bros., London, United Kingdom) was useful for stepwise elution, although it was practicable to remove the bung and add the new solvent when the adsorbent was almost exposed.

Light petroleum (60–80 C) with the progressive addition of diethyl ether was preferred to light petroleum/ethyl acetate to assist solvent recovery by distillation. Chloroform/ethyl acetate (Table 3) was an alternative solvent with which TLC experience could be related to that of column chromatography. All fractions were monitored by analytical TLC on Silica Gel G type 60 (commercial plates) with the solvent, light petroleum (40–60 C) diethyl ether (70:30, v/v) and visualization of bands with 0.1% ethanolic rhodamine 6G or 50% aqueous sulphuric acid followed by charring at 150 C. For fraction volumes of 20 ml or less, a fraction collector was used of the turntable type with 84 tubes (Central Ignition Co., London, N.I., United Kingdom). Fractions were combined appropriately into six groups and concentrated and the phenolic material was weighed.

The comparative separations with Silica Gel MFC were performed with glass columns packed as before and usable without pressurization (i.e., 2.5 cm × 65 cm length) equipped with a PTFE stopcock.

Dry incorporation argentation columns (Table 3) were prepared from Silica Gel H (type 60, article 7736) by mixing with dry, finely ground silver nitrate of particle size distribution as indicated in Table 3, followed by a brief activation of the mixture at 110 C and then packed as before. Control wet incorporation columns were prepared using a concentrated aqueous solution of silver nitrate with the Silica Gel H and by drying, grinding and activation of the mixture in subdued light at 110 C. Columns were protected from the light by wrapping with foil and were used with pressurization. The solvent light petroleum (60–80 C) diethylether was preferred to light petroleum/ethyl acetate. Fractions eluted with light petroleum/ether were free of silver nitrate; it was removed from methanolic eluates by evaporation and aqueous washing of the ethereal extract, which was then dried and weighed. All fractions were monitored by analytical TLC as described (5) with reference samples of the cardanol constituents.

GLC on the fractions from argentation columns was conducted at 190 C with 4% (w/w) polyethyleneglycol adipate on silanized 60–100 mesh chromosorb W and apparatus as described (20). Fractions from column separations on Silica Gel 4 type 60 were analyzed on 3% SE 30 as described (20).

HPLC of fractions from argentation columns was effected more recently by the reversed phase procedure on 5 μm ODS Spherisorb with equipment and the gradient elution program as described (21).

The following unsaturated constituents had the retentions (min) indicated, (15:3)-cardol (2.26), (15:3)-cardanol (3.37), (15:2)-cardanol (4.07), (15:1-cardanol (7.63) and polymer (14.16).

RESULTS AND DISCUSSION

Adsorption chromatography of cashew phenols. Following earlier experiments in which Davison grade silica gel 60–120 mesh had been used with an adsorbent/solute ratio of 50:1, it seemed feasible to effect practical separations with TLC type silica gel having considerably greater surface area and uniformity of particle size. Table 1 summarizes two experiments of the series in which two grades

TABLE 1

Adsorption Chromatography of Technical CNSL on Silica Gel H TLC Type

Technical CNSL (g)	Silica Gel H (g)	Combined fraction	Wt (g)	%	Solvent[a]	Volume (ml)	Component phenol
Experiment 1 (25.6)	Article 7736; 4th reuse of column (125)	1	17.68	69.1	P; P/E (12:1); P/E (10:1)	650; 650; 650	Cardanol pure
		2	0.90	3.5	P/E (7:1)	650	Cardanol/2-methylcardol
		3	0.98	3.9	P/E (3:1)	650	Cardanol/2-methylcardol
		4	0.80	3.1	P/E (1:1)	650	2-methylcardol/cardol
		5	2.84	11.1	P/E (1:1)	650	Cardol (pure)
		6	2.40	9.3	M	650	Polymer
Experiment 2 (50.0)	Article 9385 (250)	1 (1–80)[c]	34.42	61.0	P; P/E (12:1); P/E (10:1) P/E (7:1)	1000; 500; 500; 500	Cardanol (98.5%)[b]
		2 (82–89)	1.04	3.7	P/E (3:1)	500	Cardanol (87.5%), 2-methylcardol
		3 (90–97)	2.34	4.2	P/E (3:1); P/E (1:1)	500; 500	Cardanol (61.5%), 2-methylcardol (36.4%)
		4 (98–106)	4.17	7.4	P/E (1:1)	500	2-methylcardol (52.3%), cardol (40.8%)
		5 (107–122)	9.19	16.3	P/E (1:1)	650	Cardol (98.8%)
		6 (128–141)	5.24	9.3	M	2000	Polymer

[a]LP, light petroleum (60–80); E, diethylether; M, methanol.

[b]GLC analyses of trimethylsilylether.

[c]Figures in brackets show the ranges of subfractions of eluant collected.

of Silica Gel (type 60) were used. The vertical columns show the combined phenolic fractions, the weight and percent of the bulked fractions, the compositional assignment of the fraction and the relevant solvent and its volume for that part of the chromatogram. In a preliminary experiment with semipurified technical CNSL, Silica Gel H type 60 (article 7736) was used, after which the column then was reused four times with the same solvent system. For the first three runs, 30.20 g, 34.97 g and 35.60 g, respectively, of semipurified CNSL were used; in the fourth, 25.60 g of technical CNSL was used, as shown in the table. The amount of solute was increased in the first three because the resolution was sustained in the series. Visual examination alone of the final array of fractions clearly indicated the demarcation between the main component phenols. Figure 1 shows a typical chromatogram by analytical TLC of alternate samples in which a total of 141 fractions was collected. Figure 2 gives the results from combining fractions. It was convenient to combine fractions into six groups in the two experiments, the first representing pure cardanol and the fourth or fifth being pure cardol, while 2-methylcardol containing some cardanol or cardol was present in intermediate fractions. Polymeric material was eluted with methanol and tenaciously held colored material (in the case of technical CNSL) with a small volume of acetic acid, following the removal of which the column could be reused for other separations. That complete separations of the two main component phenols, cardanol and cardol, were achieved was confirmed by GLC (20) as shown for experiment 2 (Table 1). For six experiments, the recoveries of cardanol were 77.5, 77.5, 81.0 and 76.0% from single stage molecularly distilled CNSL and 69.1 and 61.0% from technical CNSL; recoveries of cardol were 17.7, 14.2, 8.6, 10.2, 11.9 and 16.3%.

A number of purely comparative separations of technical CNSL were carried out on Silica Gel MFC. In the three experiments at the adsorbent/solute ratios of 45:1, 30:1 and 16:1, the recoveries of cardanol were 71.8, 66.4 and 71.07 and of cardol were 16.7, 12.0 and 17.0%, respectively. In the first two experiments the main component phenols were resolved, as shown by analytical TLC and GLC while separations were inadequate in the final experiment. Generally, therefore, Silica Gel MFC is only effective compared with Silica Gel H (type 60) if used in a 10-fold greater proportion. In a final experiment with a 3:1 mixture of Silica Gel H (type 60) and MFC grade and an adsorbent/solute ratio of 3.2:1, the quality of the recovered cardanol (66.9%) and cardol (5.79%) was diminished and the presence of a number of intermediate mixed fraction rendered the separation much less effective.

Natural CNSL is a useful source of the component phenols, cardol and 2-methylcardol, after removal of anacardic acid. Table 2 shows a separation on Silica Gel G (type 60) with an adsorbent/solute ratio of 25:1, in which cardol (61.2%), 2-methylcardol (4.9%) and cardanol (25.8%) were recovered. Both analytical TLC and a plot of fraction weight/fraction number or volume eluant indicated three distinct stages in the separation. Comparison with an earlier separation on Silica Gel MFC type (adsorbent/solute, 40:1) confirmed the remarkable effectiveness of Silica Gel G type 60.

Plots of both fraction weight/fraction number and fraction weight/volume of eluant for the experiment given in Table 1 with Silica Gel H type 60 showed that the separations were optimal. Below an adsorbent/solute ratio of 5:1 with Silica Gel H type 60, it appears likely that the elu-

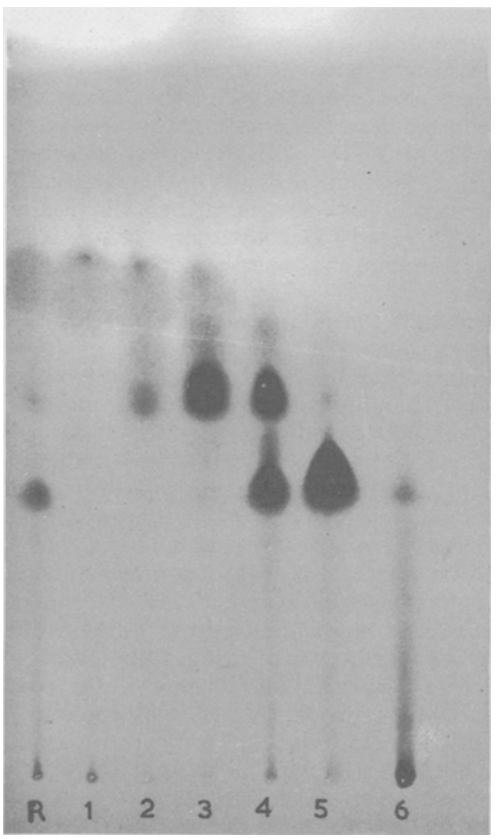


FIG. 1. Analytical TLC on combined fractions from experiment 2, Table 1. R (ref. technical CNSL), alternate fractions examined to fraction 121, then every fourth fraction to fraction 141, A frn. 10, B frn. 30, C frn. 50, D frn. 70, E frn. 90 (X frn. 81), F frn. 110, G frn. 130.

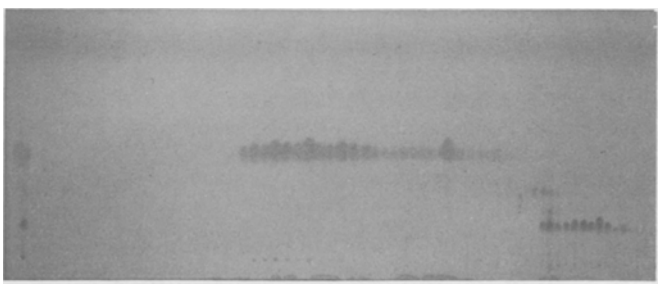


FIG. 2. Analytical TLC on fractions 1–141 from experiment 2, Table 1, on Silica Gel G type 60. Fraction 81 was a fraction collected after the column had been left overnight in the middle of a separation. It shows a trace of cardol and 2-methylcardol to be present. R (ref. technical CNSL), combined phenolic fractions 1, 2, 3, 4, 5 and 6.

TABLE 2

Adsorption Chromatography of Natural CNSL Phenols (After Removal of Anacardic Acid) on Silica Gel G (TLC Type)

Natural CNSL (g)	Silica Gel G (g)	Combined main fractions	Wt (g)	%	Solvent[a]	Volume (ml)	Component phenol
9.8	250	1	0.07	0.71	C	1000	Minor components
		2	2.54	25.8	C/EA (50:1)	1200	Cardanol (pure)
		3	0.10	1.02	C/EA (50:1)	400	Cardanol, 2-methylcardol
		4	0.48	4.90	C/EA (19:1)	800	2-Methylcardol (pure)
		5	0.08	0.82	C/EA (9:1)	500	2-Methylcardol, cardol
		6	6.02	61.2	C/EA (9:1)	2000	Cardol (pure)

[a]C, chloroform; EA, ethyl acetate.

tion program would have to be modified to achieve an equivalent resolution. It is of interest that the volumes of eluant/g technical CNSL in two experiments with Silica Gel MFC and in experiment 2 (Table 1) were ca. 500 ml, 200 ml and 100 ml, respectively, and the use of Silica Gel H type already results in an economy of solvent.

The range of the particle size and its distribution appear in our experience to be particularly significant, although the influence of solute load on resolution (11) and of large samples of solute (12) and the effect of tamping compared to natural settlement of the adsorbent slurry (25) are important. Silica Gel H (type 60, article 7736) has probably more than 20 times the surface area/g compared with the MFC type if the particle size in the middle of the ranges of 0.01-0.04 mm and 0.075 mm, respectively, is compared. Silica Gel (article 9385), by contrast, with a particle size range of 0.040-0.063 mm may be intermediate in surface area/g but probably is more uniform. Silica Gel H or G, type 60 has a considerable potential use for the column separation of various synthetic or natural product mixtures.

Argentation column chromatography of cardanol. For synthetic purposes pure constituents of phenolic lipids are required, whereas in other work, concentrates of an excess of one constituent are adequate (16). Cardanol methyl ether has been separated on alumina into its 15:1, 15:2 and 15:3 constituents (26). These cannot be easily demethylated (27) to the corresponding cardanol compounds, although the trimethylsilyl ether (28) probably could be used because it is readily reconverted to cardanol, itself not resolvable on such a column. Argentation column chromatography (17) appeared to be the method of choice with Silica Gel type 60 as the adsorbent with higher solute/adsorbent ratios than previously used (8). To avoid the lengthy procedure involved with aqueous solutions of silver nitrate and Silica Gel G, we adopted for column work the novel expedient of dry incorporation of the finely divided salt. Table 3 summarizes two experiments of the series on the separation of cardanol with a pressurized column packed with Silica Gel H type 60 containing 15% (w/w) silver nitrate, 10% having proved insufficient. The initial experiments were carried out with dry incorporation and adsorbent/solute ratios of 16:1, 20:1 and 10:1, giving total recoveries of 55.0, 76.7 and 77.2%, respectively, of main constituents (monoene, diene and triene), with the remaining material representing mixed fractions. The percent in the first experiment was low, probably due to its being a preliminary run. For comparative purposes only, two control experiments were conducted on columns prepared by wet incorporation of silver nitrate with adsorbent/solute ratios of 28:1 and 18:1. The total recoveries of cardanol constituents were 85.0 and 75.1%, the last giving a comparable separation to experiment 1 in Table 3. All fractions were monitored by analytical TLC with visualization by the acidic charring method and then were combined to give the main fractions described in the table. Plots of fraction weight/fraction number or of fraction weight/volume of eluant for the experiments showed that an adsorbent/solute ratio of 25:1 is probably optimal and that below this, unresolved intermediate fractions begin to increase. Experiment 1 was carried out with the lowest adsorbent/solute ratio in the series and, although both analytical TLC and GLC had indicated good separations, the lack of resolution of the 15:0- and 15:1-constituents led us to use HPLC analysis some time after the fractions had been collected. Fractions 3, 7 and 9 (Table 4) contain concentrates of the 15:1-, 15:2- and 15:3-constituents, respectively.

By comparison, at the much greater adsorbent/solute ratio of 900:1 on Silica Gel (60-120), which is larger in particle size than Silica Gel MFC containing 100% silver nitrate, complete resolution of cardanol with virtually 100% recovery of solute was effected (8). The resolution $2d/(w_1 + w_2)$ can be readily found to be 5 from the information given; both the adsorbent/solute proportion and the percent of silver nitrate could have been considerably lowered. The present described experiments had to be concluded before the full scope of the dry incorporation technique could be examined in the higher adsorbent/solute ratio range between 25:1 and 40:1. Similar separations of other cashew phenols have been effected at higher adsorbent/solute ratios by the wet method. Methyl anacardate, the methyl ester of (IV) (2.5 g), was readily resolved into the 15:0, 15:1, 15:2 and 15:3 constituents on Silica Gel H type 60 (170 g) impregnated with silver nitrate (340 g) by the slurry method. Cardol (II) contains more than 65% of the 15:3 constituent and on Silica Gel H type 60 (200 g) containing 15% silver nitrate, with an adsorbent/solute ratio of 48:1, it very readily was obtained pure.

TABLE 3

Argentation Adsorption Chromatographic Separation of Mixed Cardanol on Silica Gel H TLC Type Containing 15% Dry Incorporated Silver Nitrate[a]

Cardanol (g)	Silica Gel H (g)	Combined main fractions	Wt (g)	%	Cardanol constituents	Solvent[b]		Volume (ml)
12.5	250	1	0.6	6.0	15:0, 15:1	P; P/E (20:1)		100; 500
		2	1.22	12.2	15:1	P; (15:1)		300
		3	1.36	13.6	15:1	P/E (12:1)		300
		4	0.52	5.2	15:1	P/E (10:1)		400
		5	0.75	7.5	15:1, 15:2	P/E (8:1)		300
		6	0.91	9.1	15:2, 15:1	P/E (5:1)		1000
		7	1.28	12.8	15:2	P/E (2:1)		400
		8	0.52	5.2	15:2, 15:3	P/E (2:1)		100
		9	2.34	23.4	15:3	P/E (2:1)		100
10	100	1 (33-64)[c]	6.48	42.30	Monoene	P; P/E (20:1) for fractions	(1-12)	100; 500
		2 (65-82)	2.27	14.82	Monoene, diene	P/E (15:1)	(13-24)	300
		3 (83-104)	2.04	13.32	Diene, monoene	P/E (12:1)	(25-46)	300
		4 (105-122)	0.99	6.46	Triene, diene	P/E (10:1)	(47-70)	400
		5 (123-140)	1.00	6.50	Triene	P/E (8:1)	(71-90)	300
						P/E (5:1)	(91-146)	1000
		6 (141-166)	2.54	16.60	Triene	P/E (2:1)	(147-166)	600

[a]Particle size sieve distribution: 94.6% passed through 500 μm, 87.1% through 355 μm, 68.1% through 210 μm and 9.4% through 53 μm.
[b]P, light petroleum (60-80); E, diethylether.
[c]Figures in parentheses show the ranges of subfractions of eluant collected.

TABLE 4

Composition (%) by HPLC Analysis of Fractions from Argentation Adsorption Chromatography of Cardanol[a]

Fractions	Wt (g)	%	% Composition of cardanol constituents			
			15:3 (Triene)	15:2 (Diene)	15:1 (Monoene)	15:0 (Saturated)
1	0.60	6.0	—	—	—	—
2	1.22	12.2	2.95	0.62	81.35	
3	1.36	13.6	2.29	4.26	85.19	8.26
4	0.52	5.2	4.27	4.90	89.31	1.52
5	0.75	7.5	5.83	13.54	80.03	0.60
6	0.91	9.1	18.99	45.78	35.22	0.0
7	1.28	12.8	3.06	82.21	13.17	1.55
8	0.52	5.2	32.03	65.00	2.77	—
9	2.34	23.4	96.54	1.99	1.47	—

[a]The cardanol had the composition 15:3-cardanol (43.43%), 15:2-cardanol (18.20%), 15:1-cardanol (25.11%), 15:0-cardanol (3.01%), cardol (1.15%), polymer (7.32%), and was used for the separation on 100 g Silica Gel G (Table 3).

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Simultaneous Analysis of Low Plasma Levels of Deuterium-Labeled Saturated and Unsaturated Fatty Acids as *t*-Butyldimethylsilyl Esters

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A sensitive and accurate method for detection and quantitation of deuterated fatty acids in the presence of large amounts of unlabeled fatty acids is described using mass fragmentography in combination with the preparation of tertiarybutyldimethylsilyl esters (*t*-BDMS). The method has been applied to determination of deuterated stearic, oleic, elaidic and linoleic acids in human plasma lipoproteins following duodenal perfusion with a micellar mixture of acids. Over a concentration range of 10–1000 ng/ml, the average coefficient of variation for the linoleate was 3% and for the oleate (elaidate) ester was 2%.
***Lipids* 21, 247–251 (1986).**

The analysis of fatty acid composition of human body fluids has proven to be an important aspect of the investigation of metabolic abnormalities (1,2). Ideally, the metabolism of individual fatty acids should be studied simultaneously to control for biological variability and analytical differences. The use of fatty acids with radioactive carbon or tritium is limited to dual label experiments and, more importantly, their use in human subjects is precluded because of radiation hazards. Emken et al. (3,4) have overcome these problems by labeling the long chain fatty acids with the stable isotope deuterium. When each fatty acid is labeled with a definite number of deuterium atoms per molecule, each exogenous acid can be identified by its characteristic molecular weight using gas chromatography with mass spectrometry (GC-MS). However, the fatty acid methyl esters generally used for this analysis yield very weak signals for the parent or any other characteristic ion (4) and large quantities of deuterium-labeled fatty acids must be administered to human subjects, which is inefficient and expensive. Likewise, the trimethylsilyl esters of fatty acids yield only marginally larger proportions of the higher mass fragments than the methyl esters in the electron impact mode of MS (5). However, Phillipou et al. (6) have demonstrated that the electron impact ionization of the *t*-BDMS esters of fatty acids display prominent $(M\text{-}C_4H_9)^+$ ions and that the proportion of the total ionization carried by this fragment for the saturated and mono-, di- and triunsaturated acid derivatives permits their detection at the subnanogram level by mass fragmentography.

The present study confirms the qualitative findings of Phillipou et al. (6) and extends them to the quantitation of unlabeled and deuterium-labeled fatty acids in human plasma following duodenal perfusion with micellar solutions of appropriate precursors.

MATERIALS AND METHODS

Standards and substrates. Individual C_{16} and C_{18} fatty acids commonly found in natural fats were purchased from Applied Science Laboratories (State College, Pennsylvania) as were standard mixtures of these acids or their methyl esters. Deuterated linoleate (74.0% 17, 17, 18, 18-d_4), stearate (87.26% 9, 10, 13, 13, 14, 14-d_6), oleate (82.24% 14, 14, 15, 15, 17, 18-d_6) and elaidate (86.81% 13, 13, 14, 14-d_4) were prepared and identified as previously described (3). These compounds are further characterized in the Results section.

Calibration. For calibration of the GC-MS response, the deuterated acids were combined in duplicate sets of 10, 20, 40, 75 and 100 μg of d_4-linoleate and 100 μg of d_6-stearate, d_6-oleate or d_4-elaidate and were subjected to GC-MS analysis in the 20 to 200 nanogram range of mass per sample injection. The response was calibrated and correction factors were calculated assuming that parallel analyses of the corresponding mixtures of fatty acids as the methyl esters by gas liquid chromatography (GLC) gave the true weight response. The validity of this assumption was confirmed by quantitative analysis of appropriate standard mixtures of fatty acids. The linearity of the response of the mass spectrometer was determined by diluting known amounts of the mixed deuterated fatty acids with increasing amounts of total lipid extracts of representative plasma samples, and the relative recovery and linearity of response was determined using gas chromatographic analysis response as the reference standard.

Preparation of derivatives. The fatty acids were converted to methyl esters (7) by reaction with boron trifluoride in methanol for GLC (one-half of the sample) and into the *t*-BDMS esters (8) for combined GC-MS (the other half of the sample). For the latter purpose the fatty acid samples were dried under nitrogen, dissolved in 500 μl of N,N-dimethylformamide and derivatized by adding 500 μl of the silylating mixture containing 1 mM *t*-butyldimethylchlorosilane and 2.5 mM imidazole dissolved in 1 ml dimethylformamide (Applied Science Laboratories). The samples were heated at 80 C for 90 min. After cooling, the reaction mixture was diluted with 5 ml of water and extracted three times with petroleum ether, and the extracts were taken to dryness and redissolved in hexane for injection into the GC-MS system.

GLC and GC-MS analyses. GLC was performed on a Hewlett-Packard model 5840 instrument equipped with a 25 m fused silica SP-2100 (methyl silicone fluid) capillary column. The column oven temperature was programmed from 150–200 C at 5 C/min with the injector and flame ionization detector ports at 250 C. Nitrogen was the carrier gas at 5 ml/min.

GC-MS was carried out on a Hewlett-Packard 5985B automated gas chromatograph/quadrupole mass spectrometer system equipped with a 25 m SP-2100 fused silica capillary column. The carrier gas was helium at 5 psi head pressure. The column oven temperature was programmed from 100 to 270 C at 6 C/min. Transfer lines and the ion source were at 250 C. All spectra were determined in the electron impact mode at an electron energy of

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70 eV. Mass chromatograms were obtained by a scanning search in the mass range m/z 200–400 at one scan/sec for the $(M-57)^+$ ion of linoleate (m/z 337, d_0 and m/z 341, d_4), stearate (m/z 341, d_0 and m/z 347, d_6), oleate (m/z 339, d_0 and m/z 345, d_6) and elaidate (m/z 339, d_0 and m/z 343, d_4).

Method of duodenal perfusion. A micellar solution was prepared of 10.6 mM d_4-linoleate, 10.6 mM d_4-elaidate, 9.5 mM d_6-oleate and 4.3 mM d_6-stearate in 45 mM sodium tauracholate in 1.5 l of Kreb's-Ringer phosphate buffer. This mixture was perfused at 7.5 ml/min for four hr into the duodenum of a 24-year-old male with no gastrointestinal disease after an overnight fast. Plasma samples were taken at 0, 1, 2, 4, 8, 12 and 24 hr. The plasma samples were separated into the major lipoprotein classes by gradient ultracentrifugation (9) and the lipids were extracted by the method of Folch et al. (10). The chylomicron triacylglycerols were isolated by thin-layer chromatography (11) and the fatty acids were prepared by saponification and acidification (12).

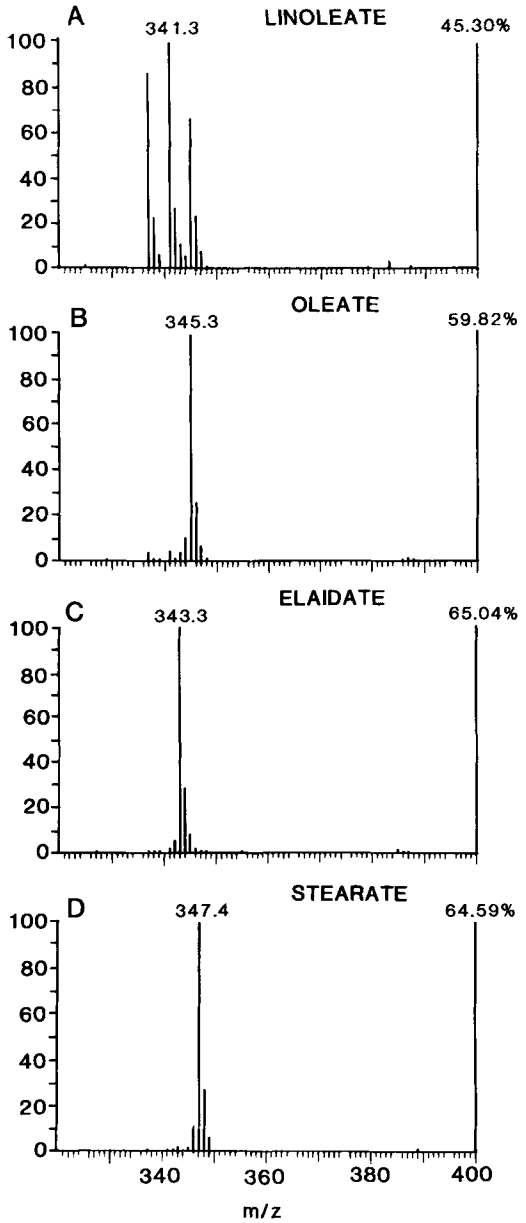

FIG. 1. Mass spectra of the *t*-BDMS esters of deuterated fatty acids in the m/z 300–400 range. A, linoleate; B, oleate; C, elaidate; D, stearate. The $(M-57)^+$ used for identification and its percentage of total ion yield are indicated.

Characterization of the deuterated fatty acids. Figure 1 gives the mass spectra of the *t*-BDMS esters of the deuterated fatty acids in the m/z 300–400 range. The intensities of the major ions are tabulated in Table 1. It is seen that the *t*-BDMS esters yield very prominent $(M-57)^+$ ions for all the fatty acids, which account for 50–60% of the total ion intensity, as previously reported by Phillipou et al. (6) for the unlabeled fatty acids. This facilitates greatly the detection of these acids at low levels of concentration and represents an average increase of 10- to 100-fold in the sensitivity over that realized for the corresponding fatty acid methyl esters. From the mass spectra, it is possible to recognize that the various fatty acids contain different amounts of the deuterated species, which can be readily estimated from the $(M-57)^+$ ions. Table 2 gives the distribution of deuterium in the labeled

TABLE 1

Major Mass Abundance[a] of Mass Spectra of Standard Deuterated Fatty Acids as the *t*-BDMS Esters in the m/z 200–400 Range

Mass number $(M-57)^+$	% Linoleate $18:2d_4$	% Oleate $18:1d_6$	% Elaidate $18:1d_4$	% Stearate $18:0d_6$
337.3	15.33	2.98		
338.3	4.60	0.79		
339.3	1.89			
340.3	0.8			
341.3	45.30	2.94	1.72	
342.3	12.76	0.98	4.33	
343.3	4.09	2.87	65.04	1.36
344.4	1.29	6.86	19.34	
345.3	8.82	59.82	6.26	1.68
346.3	2.97	16.31	1.55	7.68
347.4	1.18	4.69	0.88	64.95
348.4		0.90		18.49
349.4				4.93
383.4	1.42			
385.5			0.88	
387.4		0.87		

[a]Data is corrected for natural abundance.

TABLE 2

Distribution of Deuterium in Synthetic Fatty Acids[a]

Number of deuterium	% Stearate $18:0d_6$	% Oleate $18:1d_6$	% Elaidate $18:1d_4$	% Linoleate $18:2d_4$
0	0.000	0.989	2.223	22.667
1	0.000	0.386	1.075	0.662
2	0.107	1.033	2.316	1.198
3	0.102	0.769	4.674	1.150
4	0.749	3.399	86.806	74.070
5	7.698	7.158	2.278	0.048
6	87.261	82.240	0.000	0.036
7	3.509	3.096	0.628	0.170
8	0.221	0.639		
9	0.308	0.289		
10	0.045			

[a]Data is corrected for natural abundance.

fatty acids and illustrates the content of major deuterated species in each acid. Thus, linoleic acid contained 74.07% 18:2d_4, stearate 87.26% 18:0d_6, oleate 82.24% 18:1d_6 and elaidate 86.81% 18:1d_4. In addition, each fatty acid contains significant amounts of d_0 and d_1, d_2, d_3 and some d_4 and d_5 species.

Calibration of the MS response. Mass chromatograms obtained from the (M-57)$^+$ ion of unlabeled linoleate (m/z 337) and stearate (m/z 341) mixtures and for the total ion current in the 200 to 400 mass range are illustrated (Fig. 2). On the basis of relative peak areas, it was possible to calculate ion yield for esters of linoleic and stearic acid esters and to relate them to each other and to the mass proportions for the same mixtures of acids following GLC analysis of the methyl esters. Comparison of relative responses of *t*-BDMS esters of fatty acids in the mass spectrometer in relation to mass responses in the flame ionization detector indicate that ester of linoleic acid is recovered in nearly correct weight ratio to the stearate acid internal standard (Table 3). In the GC-MS system, however, the *t*-BDMS ester of the linoleic acid compared to stearate gives only about 50% recovery (response factor of 2.13 ± 0.22) over the tested concentration range. Using similar mass chromatograms, average response factors were obtained for the other unsaturated fatty acid and linoleate. The average relative response factors for linoleate/oleate and linoleate/elaidate peaks were similar but significantly lower than those for the linoleate/stearate pair (1.82 ± 0.43 and 1.84 ± .13, respectively). These correction factors were applicable over the concentration range of 10 to 100 μg. It follows that oleate and elaidate gave comparable ion yields in the mass spectrometer but were slightly lower than those given by stearate, yielding an oleate (elaidate) stearate correction factor of 1.13 ± 0.1. Reproducibility of analyses was excellent over the entire range of working concentrations as indicated by the coefficients of variance, which range from 0 to 2.83%.

Fatty acids give linear response in the concentration range 10–100 μg, which has been extrapolated to the origin (Fig. 3).

Relative recoveries of the deuterated fatty acids following addition to plasma lipid extracts also are illustrated (Table 4 and Fig. 4). Recoveries are expressed in relation to GC-MS/GC and gas chromatographic analysis. All deuterated acids are recovered in the proportion in which they are added, and no serious interferences were detected for any of them at this level of concentration. In addition, the added fatty acids gave linear quantitative responses over the range of 5–150 μg of added fatty acid per ml of plasma.

Application. Figure 5 illustrates a mass chromatogram obtained for the M-57)$^+$ ions of the various deuterated fatty acid esters in a sample of chylomicrons recovered from a subject following intraduodenal infusion of a micellar mixture of the fatty acids in the molar ratio given

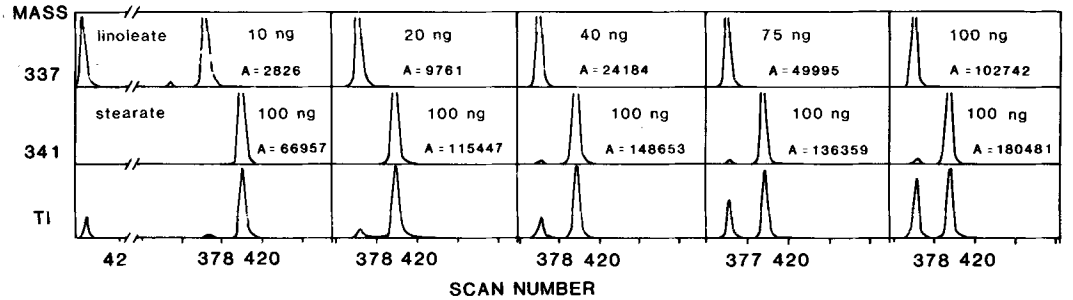

FIG. 2. Mass chromatograms for the (M-57)$^+$ of the *t*-BDMS esters of linoleate (m/z 337) and stearate (m/z 341) mixtures and the total ion current in the 200–400 mass range. A, area.

TABLE 3

Response of Fatty Acid *t*-BDMS Esters in the GC/MS System Relative to Recoveries by GLC[a]

16 Absolute weight (μg)	18:2d_4/18:0d_6			18:2d_4/18:1d_6			18:2d_4/18:1d_4		
	GLC	GC/MS	CF	GLC	GC/MS	CF[c]	GLC	GC/MS	CF
10[b]	9.42[b]	4.24[b]	2.22	8.51[b]	4.59[b]	1.85	11.40[b]	5.63[b]	2.02
20	17.25	8.25	2.09	19.10	10.37	1.84	21.15	10.94	1.93
40	39.63	16.44	2.41	39.29	21.15	1.86	40.93	23.02	1.77
80	77.57	36.82	2.10	66.33	37.05	1.79	85.50	47.56	1.79
100	104.56	57.45	1.82	91.70	51.95	1.76	106.97	62.81	1.70
Mean ± S.E.			2.13 ± 0.22			1.82 ± 0.43			1.84± 0.13

CF, correction factor; GLC, gas liquid chromatography; GC/MS, gas chromatography/mass spectrometry.

[a]Relative recoveries are based on a constant concentration of stearate.

[b]Values are expressed in μg.

[c]Correction factors were calculated by dividing the amount determined by GLC by the amount determined by GC-MS.

in the Methods section. The lowest pattern in Figure 5 represents the profile of the total fatty acid ester as detected by the total ion current of the mass spectrometer. A total of eight long chain fatty acids, four exogenous and four endogenous, was identified by the anticipated characteristic ions. In addition to the natural (m/z 339) and deuterium labeled (d_6) oleate (m/z 345) peak, small isolated satellite peaks of oleate are present at m/z 341 ($d_0 + P_2$), and at m/z 347 ($d_6 + P_2$). In addition, 1.033% and 3.096% of the labeled oleate were d_2 and d_7 species, respectively. A small isotope satellite peak (m/z 343) also is present for the unlabeled occurring stearate. Relative percentages of $d_x/d_0 + d_x \times 100$ of each labeled fatty acid incorporated in the chylomicron triacylglycerols is indicated (Table 5). Repeat analysis of the same sample established that 45.9% linoleate, 45.93% oleate, 83.93% elaidate and 38.05% stearate in chylomicron triacylglycerols originated from the perfusate. After application of appropriate response factors and knowledge of the percentage of each labeled fatty acid fed, and with several time points during the course of absorption and incorporation, the relative recovery of linoleate to stearate, oleate to stearate, oleate to elaidate, and oleate (elaidate) to stearate can be determined and compared in normal individuals and in subjects with abnormalities of lipid absorption.

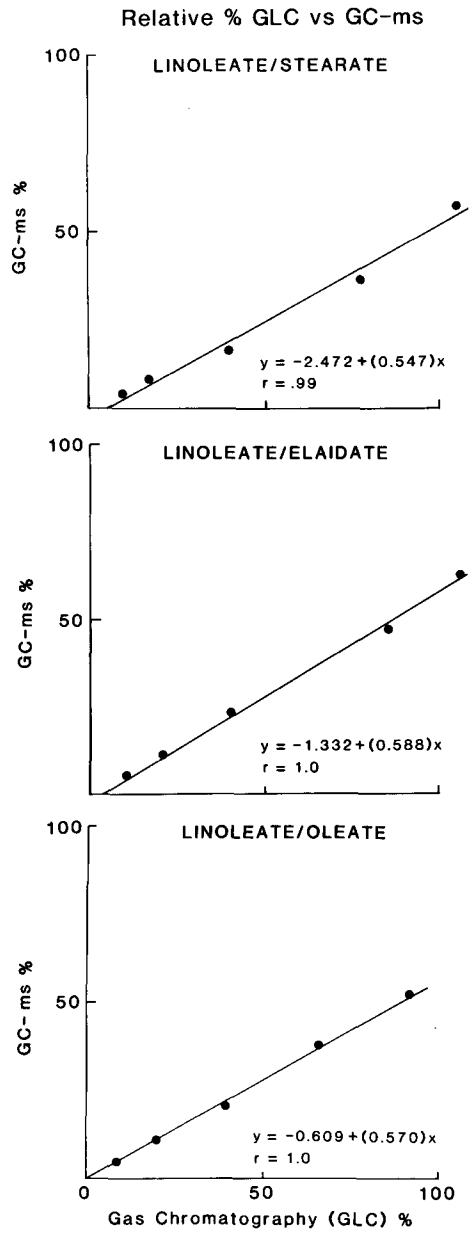

FIG. 3. Relative percent of linoleate/elaidate, linoleate/stearate and linoleate/oleate in standard mixtures (10–100 μg). Comparison of recoveries (%) by GC-MS vs GLC.

DISCUSSION

The present study confirms the usefulness of the *t*-BDMS esters for the GC-MS analysis of long chain fatty acids in general and linoleic acid in particular. Use of this derivative is particularly advantageous for analysis of small amounts of fatty acids or low enrichments of stable isotope-labeled species in a large pool of unlabeled molecules. On the basis of quantitation of the fatty acids as their *t*-BDMS esters, it may be estimated that *t*-BDMS esters, compared to previous work with methyl esters, permit work at sensitivities greater by one or two orders of magnitude. Therefore, the method should be of special interest to studies with stable isotope-labeled fatty acids in infants who can provide only small samples of blood. There appear to have been no previous quantitative analyses of the *t*-BDMS esters of fatty acids and no attempts at calibration of the GC-MS response, although

TABLE 4

Recovery of Labeled Fatty Acids Added to Plasma GC-MS[a] vs GLC Analysis

18:2		18:1 (c)		18:1 *(t)*		18:0	
GC-MS[b]	GLC[b]	GC-MS	GLC	GC-MS	GLC	GC-MS	GLC
89.5	95.8	127.1	123.4	128.5	134.6	130.3	132.2
51.8	54.9	90.7	84.4	95.8	96.2	89.9	88.1
39.5	41.6	76.7	67.5	72.1	71.8	80.9	76.9
19.7	16.1	22.46	32.9	41.2	38.5	41.0	38.5
5.8	8.2	15.46	16.5	20.0	19.2	17.9	17.6
		7.1	8.2	10.0	9.6	7.9	8.8
		4.1	4.1	3.6	4.8	4.3	4.4

[a]GC-MS, % of total fatty acid labeled × total fatty acid determined by GC analysis.

[b]Values illustrated are in μg.

TABLE 5

Relative Percentage of Deuterium-Labeled Fatty Acids Incorporated into the Chylomicron Triglycerides 4 Hr after Beginning the Perfusion

	$d_x/d_0 + d_x \times 100$			
	18:2	18:1 (c)	18:1 *(t)*	18:0
	Relation % of each fatty acid labeled			
	45.89	46.02	83.76	38.56
Repeat	46.09	45.84	84.09	37.54
Mean	45.99	45.93	83.93	38.05
Difference	0.20	0.18	0.33	1.02

d_x = Labeled fatty acid, d_0 = unlabeled fatty acid.

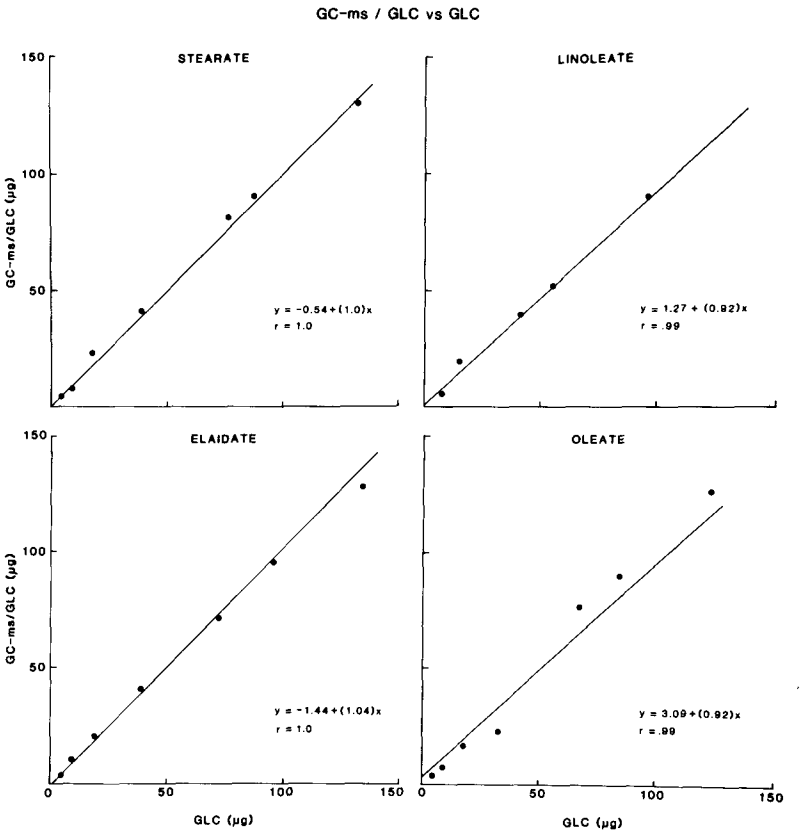

FIG. 4. Recovery of deuterium-labeled fatty acids stearate, elaidate, oleate (5–150 µg) and linoleate (10–100 µg) added to 1 mg of plasma. Comparison of recovery by GC-MS/GLC vs GLC.

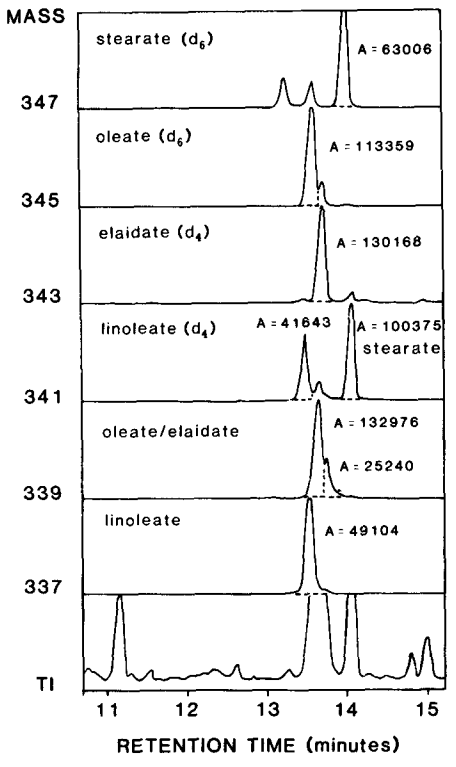

FIG. 5. Mass chromatograms of various deuterated fatty acids to *t*-BDMS esters of a sample of chylomicron triacylglycerides. TI, total ion current; A, area; (*d*+), deuterium label mass.

the general usefulness of *t*-BDMS esters in analysis of fatty and urinary acids has been demonstrated (6,8).

The present study reports a method for the accurate assessment of the deuterated fatty acid ratios in the presence of large amounts of unlabeled fatty acids. The method also is capable of handling mass peak interferences caused by imperfect isotopic labeling and of providing a measure of the quality of the result.

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COMMUNICATIONS

The Hydrolysis of Very Low Density Lipoproteins and Chylomicrons of Intestinal Origin by Lipoprotein Lipase in Ruminants

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The hydrolysis by lipoprotein lipase of a very low density lipoprotein/chylomicron fraction, obtained from the intestinal lymph of sheep, has been studied in vitro. Rapid hydrolysis of triacylglycerols, with an accumulation of free fatty acids, was observed. After an initial lag period, phosphatidylcholine also was hydrolyzed. No specificity for particular fatty acids in the triacylglycerols (or phosphatidylcholines) was observed.
Lipids 21, 252–253 (1986).

In a comparison of triacylglycerol structures of very low density lipoproteins (VLDL)/chylomicrons of intestinal lymph and the corresponding lipoprotein fraction from sheep plasma, a great deal of similarity was observed, as would be expected if these lipids have a common origin (1). However, there was one important difference, i.e., the content of linoleic acid in the primary positions (but not in position *sn*-2) of plasma triacylglycerols was much less than that in lymph triacylglycerols. It appeared that this essential fatty acid was being selectively removed and conserved for some specific function in the animal. As a consequence of biohydrogenation in the rumen, essential fatty acids are in relatively short supply in the tissues of ruminant animals, and tend to be directed away from nonessential functions (reviewed by Noble [2]). Some selective hydrolysis of plasma triacylglycerols could potentially occur in the liver or peripheral tissues, leaving triacylglycerol molecules in the plasma with a higher proportion of saturated fatty acids. In the latter instance, the key enzyme would be lipoprotein lipase. Relatively little is known of the molecular specificity of this enzyme, especially in ruminant animals. Two recent studies of the substrate specificity of human lipoprotein lipase reached apparently contradictory conclusions. In one (3), no specificty for particular molecular species of triacylglycerols was observed, while in a second (4), a marked preference for triacylglycerols containing short chain or unsaturated fatty acids was found. Accordingly, we have examined the specificity of lipoprotein lipase in the hydrolysis in vitro of the triacylglycerols and other lipids from the VLDL/chylomicrons of sheep intestinal lymph.

EXPERIMENTAL METHODS

Lipoprotein lipase was partially purified from bovine milk as described by Kinnunen (5); it had a specific activity of 15.6 units/mg protein for triacylglycerol hydrolysis under the assay conditions of Nilsson-Ehle & Schotz (6). The ratio of triacylglycerol hydrolase activity to phospholipase activity, determined with defined phospholipid emulsions, was the same as that reported for more highly purified preparations of lipoprotein lipase. Intestinal lymph was obtained by catheterization of four mature wethers of the Clun Forest breed of sheep as described previously (7). VLDL/chylomicrons (the two form a continuum in composition in ruminants and any subdivision tends to be artificial [7,8]) were isolated by ultracentrifugal flotation according to the method of Lindgren et al. (9). Immediately before use, the enzyme preparation and the VLDL/chylomicrons were dialyzed against tris-HCL buffer (20 mM, pH 8.1) containing 0.16 M sodium chloride. In an experiment designed to study the time course of the reaction, the initial incubation medium contained VLDL/chylomicrons (10 ml; 4.83 mg/ml of lipid, i.e., equivalent to the concentration in lymph originally), albumin (fatty acid-free, 81 mg), heat-inactivated bovine serum (1 ml), lipoprotein lipase preparation (0.06 ml; 0.94 units) and the above buffer in a final volume of 15 ml. The mixture was maintained at 37 C in a shaking water bath. At the appropriate time intervals, an aliquot (1.5 ml) was withdrawn, and the lipids were extracted and separated by thin layer chromatography for quantification by gas chromatography of the methyl ester derivatives of the fatty acids with an internal standard; the methods have been described in detail elsewhere (7). All experiments were performed four times, i.e., with a lipoprotein preparation from different animals.

RESULTS AND DISCUSSION

In a preliminary experiment (data not shown), it was established that the extent of hydrolysis of the triacylglycerols after 60 min was linearly related to the concentration of enzyme present, over the range of 0.025 to 0.3 ml of the enzyme preparation. Bovine milk lipoprotein lipase was used in this study because it is readily obtainable; it is known to share immunological identity with the corresponding enzyme from the peripheral tissues of many other species (10). The results of an experiment to determine the time course of the hydrolysis of the lipid constituents of the lipoprotein fraction are shown in Figure 1. The triacylglycerols were hydrolyzed rapidly, and there was an accumulation of free (unesterified) fatty acids in the medium. The intermediate monoacylglycerols tended to accumulate, and diacylglycerols also were formed (concentrations were too close to those of the lysophosphatidylcholine for both to be depicted in the figure). The reaction was not linear with respect to time, except perhaps in the early stages. No hydrolysis of cholesterol esters was detected (also not shown in the figure). No hydrolysis of the phosphatidylcholine was seen during the first 20 min at least, but later the concentration of this component tended to drop and

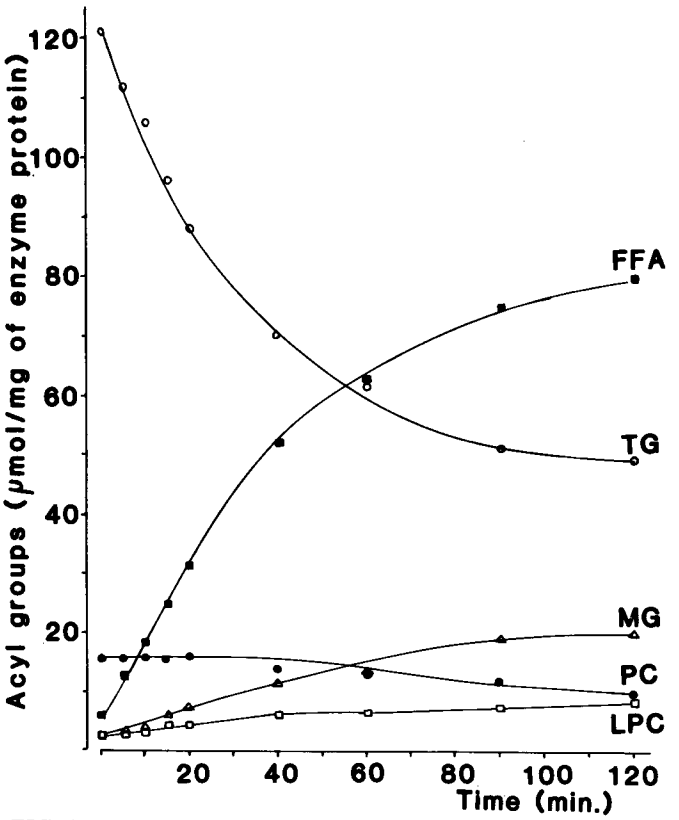

FIG. 1. Time course of hydrolysis of the acyl groups (μmol acyl groups/mg enzyme protein) of individual lipids of the VLDL/chylomicrons of sheep intestinal lymph by lipoprotein lipase isolated from bovine milk. Results represent the mean of four separate experiments. ○, Triacylglycerols (TG); ■, free fatty acids (FFA); △, monoacylglycerols (MG); ●, phosphatidylcholine (PC); □, lysophosphatidylcholine (LPC).

there was a compensatory rise in the concentration of lysophosphatidylcholine. It is possible that the phosphatidylcholine in the VLDL/chylomicron particles becomes accessible to the lipase only after some of the triacylglycerol has been hydrolyzed. Hydrolysis of triacylglycerols and phosphatidylcholine, with accumulation of the intermediates, and not of cholesterol esters was observed in an analogous study of the reaction of human lipoprotein lipase with human VLDL (3).

The fatty acid compositions of each of the lipid classes also were determined. In particular, no change in the fatty acid composition of the residual triacylglycerols or phosphatidylcholines was observed as the reaction proceeded. The compositions of these lipids at the start and the end of the incubation period are listed in Table 1 to illustrate this point. On the other hand, a somewhat higher proportion of unsaturated fatty acids was found in the lysophosphatidylcholine as time progressed, presumably as a consequence of hydrolysis of the fatty acids from position 1, which tend to be saturated. The results, therefore, support the conclusion of Wang et al. (3), reached from experiments like those reported here in which the natural lipoprotein substrate for lipoprotein lipase was used, that

TABLE 1

Fatty Acid Compositions (Wt % of Total) of Triacylglycerols and Phosphatidylcholines in Intestinal Lipoproteins Initially and After Reaction with Lipoprotein Lipase for Two Hr

Fatty acids	Triacylglycerols		Phosphatidylcholine	
	0 Hr	2 Hr	0 Hr	2 Hr
16:0	29.4 ± 1.72	30.6 ± 1.95	21.9 ± 1.68	20.9 ± 1.93
18:0	18.3 ± 1.91	19.8 ± 1.48	19.0 ± 1.04	19.7 ± 2.03
18:1	25.2 ± 1.24	25.4 ± 1.53	18.3 ± 1.33	17.8 ± 1.49
18:2	8.2 ± 0.86	7.6 ± 0.67	26.4 ± 1.79	24.5 ± 2.63
20:4	—	—	4.7 ± 0.35	4.6 ± 0.66

Means ± S.D. of four experiments.

lipoprotein lipase has little specificity for the hydrolysis of particular fatty acid bonds in the primary positions. Wang et al. (4) obtained somewhat different results, but used emulsions of pure synthetic triacylglycerols; these may not have been in the optimum physical form for interaction with the enzyme.

From the results of this work, it does not appear that lipoprotein lipase per se is responsible for the selective removal of linoleic acid from the triacylglycerols of sheep intestinal lymph that is observed in vivo. There appears to be a much greater probability that selective hydrolysis and resynthesis/export of triacylglycerols occur in the liver, which might explain the phenomenon. The structural similarities in the triacylglycerols in plasma and intestinal lymph of the sheep then may simply reflect similarities in the specificities of the acyltransferases in the liver and intestines, respectively.

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The Peroxidizing Effect of α-Tocopherol on Autoxidation of Methyl Linoleate in Bulk Phase

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In order to understand the effect of α-tocopherol on the autoxidation mechanism of edible oil under storage conditions, methyl linoleate was allowed to autoxidize at 50 C in bulk phase without any radical initiator. The reaction was monitored by determining the production of four isomeric hydroperoxides (13-*cis,trans;* 13-*trans,trans;* 9-*cis,trans;* 9-*trans,trans*) by high performance liquid chromatographic analysis after reduction. In the absence of α-tocopherol, the rate of autoxidation depended on the sample size, and the duration of the induction period was affected by the initial level of hydroperoxides. However, the distribution of c-t and t-t hydroperoxide isomers remained constant during the propagation period regardless of the sample size. The addition of α-tocopherol at 0.1 and 1.0% caused a linear increase in the amount of hydroperoxides and elevated the distribution of the c-t isomers. The rate of hydroperoxidation appeared to be governed by the initial concentration of α-tocopherol rather than the sample size or the initial hydroperoxide level. This peroxidizing effect of α-tocopherol was suppressed by the presence of ascorbyl palmitate. A mechanism in which chromanoxy radical participates is proposed for the effect of α-tocopherol on lipid autoxidation in bulk phase. It is therefore suggested that α-tocopherol at high concentrations influences the mechanism of autoxidation of edible oil.
Lipids 21, 255–260 (1986).

There has been much concern regarding lipid oxidation in foods. Autoxidation of unsaturated fatty acids undoubtedly plays an important role in nutritional damage, discoloration and appearance of toxic substances and off-flavors in edible oils and fats.

At present, structures of primary autoxidation products from unsaturated fatty acids and their secondary oxidation products have been clarified by means of gas chromatography/mass spectrometry, high performance liquid chromatography (HPLC) and other instrumental analysis (1). Four isomeric monohydroperoxides—13-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic (13-c-t), 13-hydroperoxy-9-*trans*,11-*trans*-octadecadienoic (13-t-t), 9-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic (9-c-t) and 9-hydroperoxy-10-*trans*,12-*trans*-octadecadienoic (9-t-t) acids are the major products formed from autoxidation of linoleic acid (2). The hydroperoxy radicals are known to undergo rearrangement to isomers in which the hydroperoxy group is relocated and stereochemistry of a double bond is changed (3).

Porter et al. (4,5) have proposed an autoxidation mechanism of linoleic acid involving β-scission of peroxy radicals and have demonstrated that the distribution of c-t and t-t hydroperoxide isomers was dependent on the temperature of the medium and the ability of cosubstrate to donate a hydrogen atom to linoleate peroxy radicals. Furthermore, they have studied the effect of α-tocopherol on the distribution of c-t and t-t hydroperoxide isomers formed by autoxidation of dilinoleoylglycerophosphatidylcholine (4,6). Peers et al. (7,8) suggested that in the presence of α-tocopherol at higher concentrations, c-t hydroperoxides are selectively formed from unsaturated lipid due to the high hydrogen donating activity.

Tocopherols are generally present in edible oils and are viewed as natural antioxidants (9). However, it has long been suggested that α-tocopherol can act simultaneously as a prooxidant and an antioxidant in edible oil (10). The work of Porter and his colleagues (4–6) dealt with oxidation initiated by azo compounds in dilute solutions. Little is known about the effect of the α-tocopherol on the autoxidation mechanism of unsaturated fatty acid without an external free radical initiator in bulk phase, although a study of this condition would contribute to a better understanding of the oxidative deterioration of edible oils.

We investigated the hydroperoxidation caused by α-tocopherol at high concentrations during autoxidation of methyl linoleate in bulk phase without any external initiator. The results presented here should be useful for the evaluation of the effects of α-tocopherol on the oxidative deterioration of edible oil under storage conditions.

MATERIALS AND METHODS

Materials. Methyl linoleate (99%) supplied by Sigma Chemical Co. (St. Louis, Missouri) was further purified by column chromatography with Florisil (100/200 mesh) to remove any peroxides (11). DL-α-tocopherol was obtained from Nakarai Chem. Co. (Kyoto, Japan) and fractionated by HPLC before use to avoid the contamination of its oxidation products. Details of the HPLC conditions are given below. Butyl hydroxytoluene (BHT) and methyl stearate were obtained from Nakarai Chem. Co. and used without further purification. Ascorbyl palmitate was obtained from Daiichi Pure Chemicals Co. Ltd (Tokyo, Japan). Solvents used for chromatography were of HPLC grade.

HPLC. HPLC was performed with a Shimadzu LC-4A model and Shimadzu SPD-2A variable wavelength UV detector. A Zorbax SIL column (4.6 × 250 mm, 6 μm in particle size, Dupont) was used with elution of 1.0% isopropanol in hexane. Flow rate was maintained at 2.0 ml/min and the effluent was monitored at 235 nm and 290 nm.

Oxidation procedure. Methyl linoleate, either with or without additives, was placed in a glass vial (15 mm in diameter) and allowed to autoxidize at 50 C in the dark. To assure that the additives and methyl linoleate were well mixed, they were dissolved in hexane and then evaporated under nitrogen. At regular intervals, aliquots of the sample were withdrawn and dissolved in methanol. The methanol solution was then reacted with $NaBH_4$ to reduce the four monohydroperoxide isomers (2). The reduced sample was taken up in hexane solution and injected into the HPLC. Each isomer of the corresponding hydroxy derivatives was identified by comparing the retention time with the results of Chan et al. (2). Proportions of c-t to t-t isomers were calculated from the peak

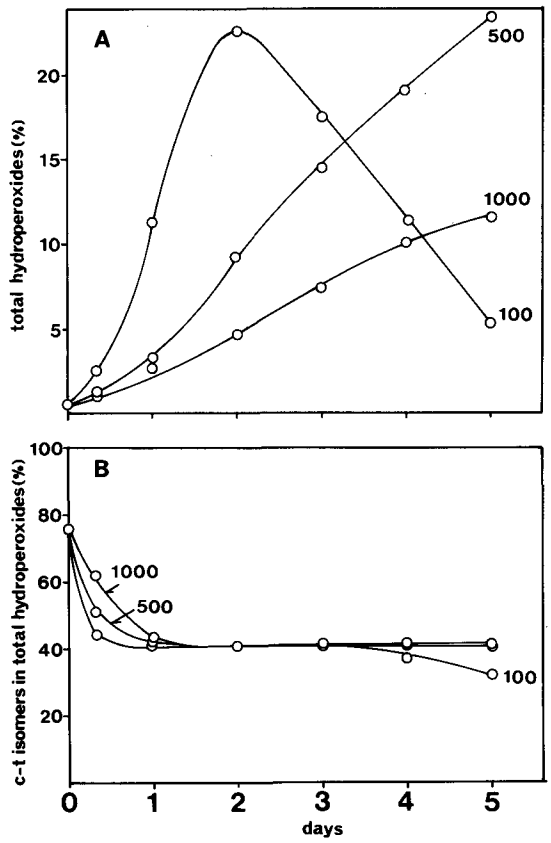

FIG. 1. Effect of sample size on the autoxidation of methyl linoleate. (A) Formation of total hydroperoxides; (B) ratio of c-t isomers to total hydroperoxides. Initial level of hydroperoxides was 0.45%. Numbers indicate the sample size (mg) of methyl linoleate in a glass vial.

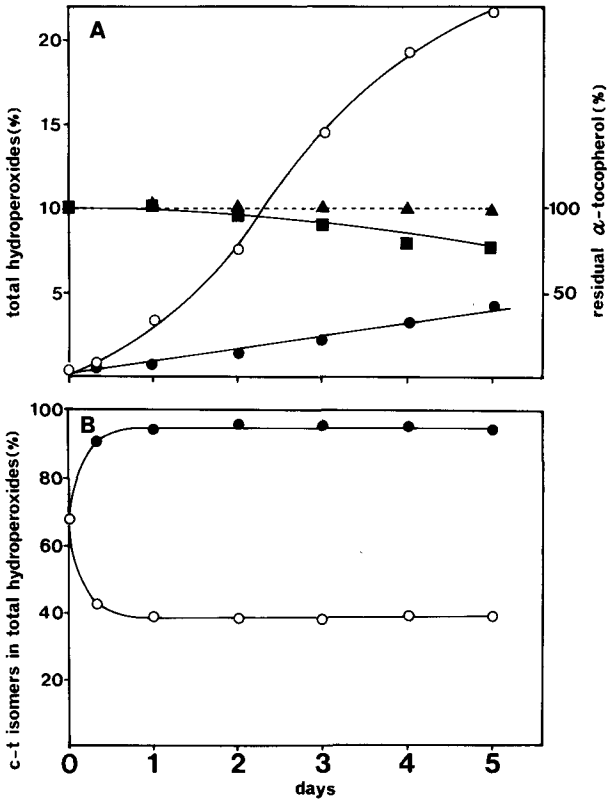

FIG. 2. Formation of hydroperoxides and loss of α-tocopherol during methyl linoleate autoxidation. (A) Formation of total hydroperoxides; (B) ratio of c-t isomers to total hydroperoxides. Initial level of hydroperoxides was 0.13%. Methyl linoleate (500 mg) was autoxidized with 1.0% α-tocopherol (●) and without α-tocopherol (○). Methyl stearate (500 mg) containing 1.0% α-tocopherol was incubated under the same conditions. Residual amounts of α-tocopherol in methyl linoleate (■) and in methyl stearate (▲) are shown.

areas by assuming that molar absorbances were the same at 235 nm (12). α-Tocopherol was determined by monitoring the elution at 290 nm.

RESULTS

Figure 1 shows the results of autoxidation of methyl linoleate with different sample sizes in a period of five days. The amount of hydroperoxides before autoxidation (initial level of hydroperoxides) was estimated to be 0.45% of methyl linoleate. Hydroperoxides were formed starting from the initial stage in each sample without an observable induction period. The rate of formation was inversely proportional to the sample size. The ratio of c-t isomers to the total hydroperoxides decreased during the first day of autoxidation, but thereafter this ratio remained constant regardless of the sample size. Both the total amount of hydroperoxides and the proportion of c-t isomers decreased at the later stage of autoxidation when 100 mg of methyl linoleate was used.

Methyl linoleate having an initial value of 0.13% hydroperoxides also showed no observable induction period (Fig. 2). The ratio of c-t isomers to the total hydroperoxides reached a constant level after 1 day of autoxidation as in the case of Figure 1. Methyl linoleate containing 1.0% α-tocopherol, on the other hand, showed a linear increase in total hydroperoxides with incubation time, and c-t isomers were formed predominantly in total hydroperoxides throughout the incubation period. Regression treatment of this curve showed that the slope was 0.91. α-Tocopherol in methyl linoleate decreased very slowly and mostly still remained after oxidation for five days. No decrease was observed in the level of α-tocopherol when it was mixed with methyl stearate.

Figure 3 shows the autoxidation curves of methyl linoleate with and without 1.0% α-tocopherol under the same conditions as those in Figure 2, except that the initial level of hydroperoxides was low (0.04%). Similar results were obtained for the ratio of c-t isomers to total hydroperoxides. However, methyl linoleate without α-tocopherol gave an induction period prior to subsequent rapid oxidation. Addition of 1.0% α-tocopherol caused a linear increase in total hydroperoxides with a slope of 0.80.

The influence of 1.0% α-tocopherol on the early stage of autoxidation was shown by using methyl linoleate at different initial levels of hydroperoxides (Fig. 4). Methyl linoleate containing 0.42% hydroperoxides initially (I) showed a rapid increase in total hydroperoxides without an induction period. The ratio of c-t isomers reached a constant value after incubation for one day. The percentages of total hydroperoxides after one day were determined to be as follows: 15.6% (1.3 days), 23.3% (1.9 days), 22.3% (2.3 days) and 19.3% (2.9 days). However, methyl linoleate

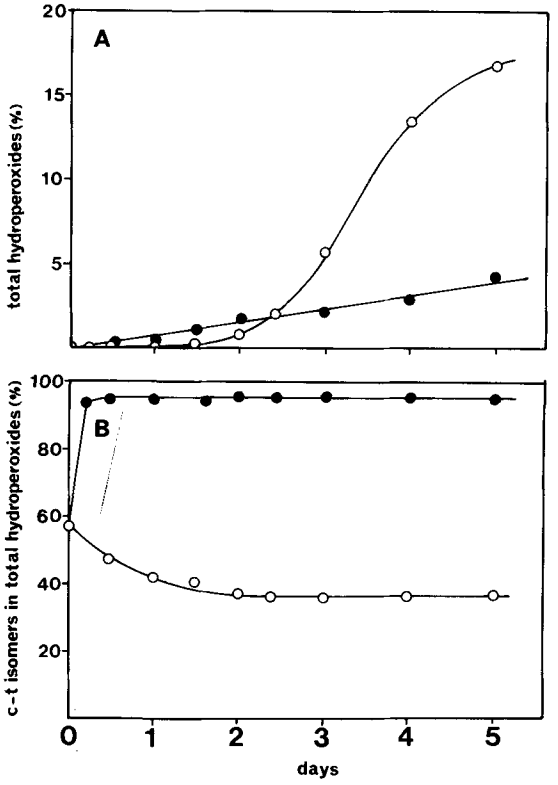

FIG. 3. Effect of α-tocopherol on the autoxidation of methyl linoleate containing lower level of hydroperoxides. (A) Formation of hydroperoxides; (B) ratio of c-t isomers to total hydroperoxides. Initial level of hydroperoxides was 0.04%. Methyl linoleate (500 mg) was autoxidized with 1.0% α-tocopherol (●) and without α-tocopherol (○).

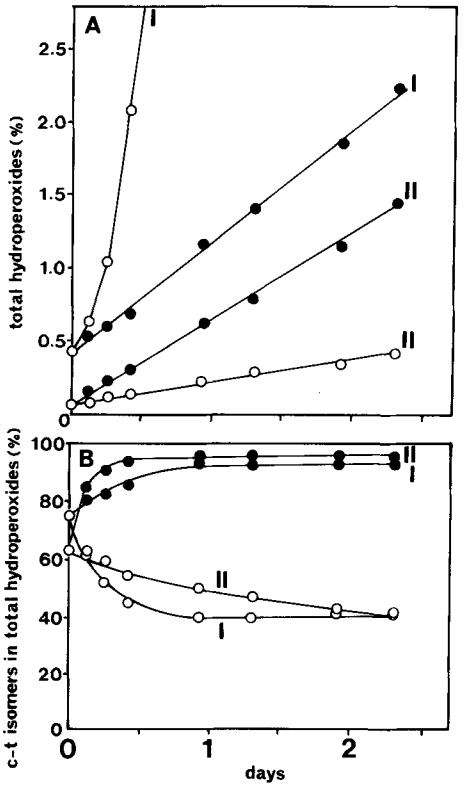

FIG. 4. Effect of initial level of hydroperoxides on the formation of hydroperoxides at the early stage of autoxidation in the presence of α-tocopherol. (A) Formation of total hydroperoxides; (B) ratio of c-t isomers to total hydroperoxides. Methyl linoleate (100 mg) was autoxidized with 1.0% of α-tocopherol (●) and without α-tocopherol (○). Initial levels of hydroperoxides were 0.42% (I) and 0.05% (II), respectively.

containing 0.05% hydroperoxides (II) displayed a different reaction curve in which oxidation was very slow and the ratio of the c-t isomers also decreased gradually. Apparently the induction period continued throughout the incubation time. On the other hand, total hydroperoxides accumulated linearly in both cases with slopes of 0.77 (I) and 0.63 (II). The level of c-t isomers was higher than 90% in each preparation containing α-tocopherol.

The effect of different concentrations of α-tocopherol (1.0, 0.1 and 0.01%) on autoxidation of methyl linoleate was compared with that of a synthetic antioxidant, BHT (Fig. 5). Methyl linoleate used in these experiments had an initial hydroperoxide value of 0.13%. No induction period was observed in methyl linoleate without α-tocopherol. Addition of 0.01% α-tocopherol gave a biphasic autoxidation curve and the induction period reached four days. During this period, the ratio of the c-t isomers was slightly higher than that without α-tocopherol. Methyl linoleate containing 0.1% α-tocopherol accumulated a considerable amount of hydroperoxides, although the amount was lower than that found in the methyl linoleate containing 1.0% α-tocopherol. α-Tocopherol at 0.1% also raised the ratio of the c-t isomers. The slopes of the lines in the accumulation of hydroperoxides were determined to be 0.67 (1.0% tocopherol) and 0.18 (0.1% tocopherol). On the other hand, BHT almost completely inhibited the formation of hydroperoxides throughout the incubation time. This inhibitory effect was observed to be independent of BHT concentration. The amounts of hydroperoxides 10 days after the start of autoxidation in the presence of BHT were 0.10% (1.0% BHT), 0.14% (0.1% BHT) and 0.38% (0.01% BHT). The ratio of c-t hydroperoxides to the total hydroperoxides in the presence of BHT was not as high as that of α-tocopherol at the same concentration of the antioxidants.

Figure 6 shows the influence of ascorbyl palmitate (0.1%) on the autoxidation in bulk phase when 0.1% α-tocopherol is present. Before autoxidation, the initial level of hydroperoxides was 0.01% and the slope of the reaction curve of methyl linoleate containing α-tocopherol was 0.18. Addition of ascorbyl palmitate containing α-tocopherol to this system suppressed the formation of hydroperoxides almost completely for up to four days. During this period, c-t hydroperoxides were produced predominantly as when α-tocopherol alone was used.

DISCUSSION

We have dealt with autoxidation of methyl linoleate in bulk phase at moderate temperature without the addition of an external radical initiator. These mild conditions may be regarded as a typical model for autoxidation of edible oils. The fact that the rate of hydroperoxidation was affected by the sample size supports the idea that the surface area of the exposed oil is an important factor in determining the autoxidation rate of edible oil, as indicated by Kwon et al. (13). On the other hand, the extent of the induction period was affected by the initial level of

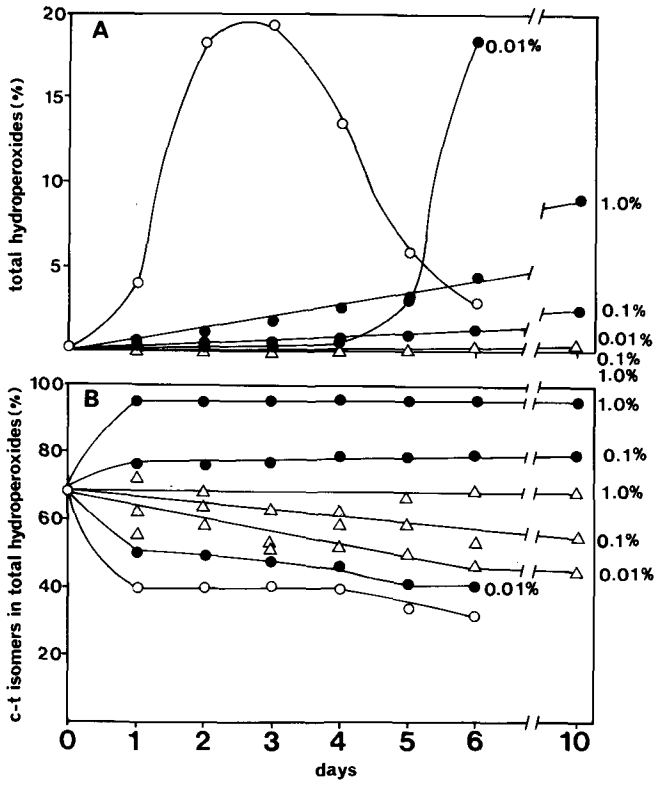

FIG. 5. Effect of α-tocopherol and BHT on methyl linoleate autoxidation. (A) Formation of total hydroperoxides; (B) c-t isomers in total hydroperoxides. Methyl linoleate (100 mg) was autoxidized with α-tocopherol (1.0%, 0.1% and 0.01%) (●), BHT (1.0%, 0.1% and 0.01%) (△) and without additives (○). Initial level of hydroperoxides was 0.13%.

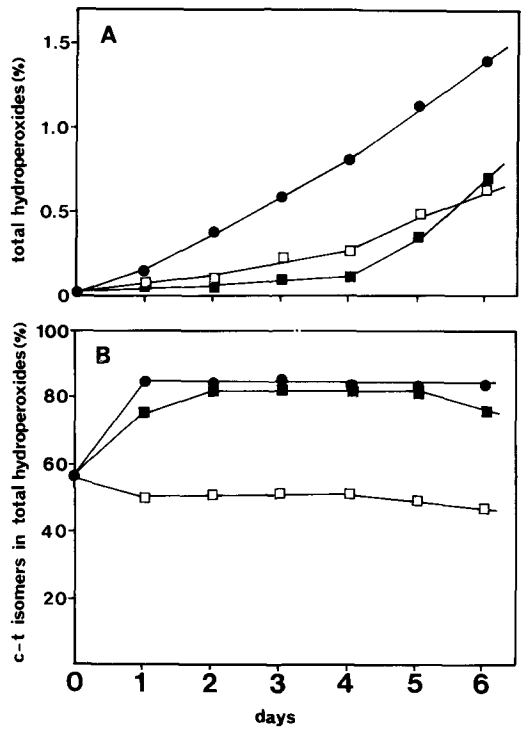

FIG. 6. Effect of ascorbyl palmitate on the peroxidizing activity of α-tocopherol in methyl linoleate autoxidation. (A) Formation of total hydroperoxides; (B) ratio of c-t isomers to the total hydroperoxides. Methyl linoleate (100 mg) was autoxidized with 0.1% α-tocopherol (●), 0.1% ascorbyl palmitate (□) and 0.1% α-tocopherol and 0.1% ascorbyl palmitate (■). Initial level of hydroperoxides was 0.01%.

hydroperoxides. A similar result was obtained by Hicks and Gebicki (14), who showed that thermal autoxidation of linoleic acid membrane was preceded by a lag period when the level of preformed hydroperoxides was low. Without an external radical initiator, initial hydroperoxides might contribute to the initiation reaction by producing free radicals via homolysis by heat or the presence of trace metals. The induction period may precede the propagation period when the level of free radicals at the initial stage is insufficient to induce a rapid rate of oxidation.

It is well known that in the propagation period conjugated diene hydroperoxides (LOOH) are formed from linoleic acid (LH) by a free radical chain reaction as shown (15,16):

$$L\cdot + O_2 \rightarrow LOO\cdot \quad [1]$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot \quad [2]$$

Porter et al. (4,5) investigated the hydroperoxidation of linoleic acid in a solution using an azo compound as the initiator, and they have proposed a mechanism for the formation of c-t and t-t hydroperoxides via reversible oxygen addition to the intermediate pentadienyl radical, resulting in c-t and t-t peroxy radical. Furthermore, they demonstrated that the ratio of c-t to t-t hydroperoxide isomers depended on the ability of the medium to donate a hydrogen atom, assuming steady state conditions. Our results clearly showed that the proportion of c-t isomers in total hydroperoxides was constant throughout the rapid oxidation. Therefore, the mechanism proposed by Porter et al. (4,5) seems applicable to the autoxidation of linoleic acid moiety in edible oil during the propagation period. At a later stage of autoxidation, the decomposition reaction of hydroperoxides occurs predominantly. The ratio of c-t isomers then may decrease because of the difference in the stability of the two geometrical isomers (17).

Tocopherols (TocH) are known to act as antioxidants by donating a hydrogen atom to chain-propagating free radicals (18,19):

$$LOO\cdot + TocH \rightarrow LOOH + Toc\cdot \quad [3]$$

$$LOO\cdot + Toc\cdot \rightarrow \text{inactive products} \quad [4]$$

Tocopherols can terminate a chain reaction by donating hydrogen atoms to peroxy radicals (LOO·); this results in the formation of hydroperoxides and chromanoxy radicals (Toc·) [3]. Tocopherols are consumed by the reaction of chromanoxy radicals with other peroxy radicals [4] or with each other, and dimers are formed. By determining the rate constant of reaction [3], Burton and Ingold (18) concluded that α-tocopherol is the most effective chain-breaking antioxidant among the tocopherols and other phenolic antioxidants known.

It has been shown that tocopherols in edible oils exert an antioxidant effect by delaying rapid oxidation (20–22), i.e., the induction period is lengthened by α-tocopherol. The length depends on the concentration of α-tocopherol,

and α-tocopherol is consumed during this period irrespective of oxidation conditions (19,23-25). The propagation period was retarded by 0.01% α-tocopherol (Fig. 5). The transition from the induction period to the propagation period (that is, from slow oxidation to rapid oxidation) is observed in methyl linoleate containing 0.1% and 1.0% α-tocopherol after longer incubation times. α-Tocopherol at these levels, however, enhanced the formation of c-t hydroperoxides significantly. It was reported that the length of the induction period caused by α-tocopherol was affected by the initial level of hydroperoxides (14). However, slopes of the reaction curve obtained from methyl linoleate containing α-tocopherol did not vary appreciably with the sample size or the initial level of hydroperoxides. The main factor responsible for the rate of hydroperoxidation seems to be the concentration of α-tocopherol rather than the sample size and the initial hydroperoxide level. Previous studies on the kinetics of autoxidation (18,19) demonstrated that the rate of oxidation during the induction period was inversely proportional to the concentration of α-tocopherol. However, higher levels of tocopherol in methyl linoleate increased the rate of hydroperoxidation during the induction period (Fig. 5). Therefore, an alternative pathway which accumulates exclusively c-t isomers takes place in autoxidation of methyl linoleate in bulk phase when α-tocopherol is present at high concentrations.

Cillard et al. (26-28) demonstrated that α-tocopherol at high concentrations acts as a pro-oxidant during linoleic acid oxidation in an aqueous medium. Peers et al. (7,8) observed that α-tocopherol at higher concentration in bulk phase autoxidation of polyunsaturated fatty acids and phospholipids did not exert an antioxidant effect but allowed oxidation to proceed smoothly, and c-t conjugated diene hydroperoxides were produced predominantly. However, the mechanism for this prooxidant effect of α-tocopherol has not been proposed. From the above results, we propose that chromanoxy radicals participate in this prooxidant effect. Thus:

$$\text{Toc}^{\cdot} + \text{LH} \rightarrow \text{TocH} + \text{L}^{\cdot} \qquad [5]$$

This chain transfer reaction [5] seems necessary to explain the alternative pathway by α-tocopherol at high level. Mahony and Ferris (29) suggested that the chain transfer reaction can take place during the retardation in autoxidation of hydrocarbon by high concentrations of phenol. This reaction may happen competitively with reaction [4] or with dimer formation, when α-tocopherol concentration in the methyl linoleate is high. Since α-tocopherol is an efficient hydrogen donor, as mentioned above, the formation of c-t hydroperoxide precedes the elimination of oxygen molecule from the peroxy radical and results in c-t hydroperoxides.

Our hypothesis proposes that α-tocopherol suppresses the autocatalytic oxidation by inhibiting the reaction of reversible oxygen attack on the pentadiene radical that is probably essential for autocatalytic oxidation in a steady state (4,5). Chromanoxy radicals may also abstract a hydrogen atom from an active methylene group of unoxidized methyl linoleate. Thus, hydroperoxidation would be initiated but tocopherol would return to its original form. A cyclic reaction via chromanoxy radical of this type would result in the continuous formation of c-t hydroperoxides.

The observation that there is no decrease of α-tocopherol in saturated fatty acids (Fig. 2) indicates that most of the α-tocopherol must be consumed by reaction with peroxy radicals during linoleate oxidation. If c-t hydroperoxide isomers were formed exclusively by donation of a hydrogen atom from α-tocopherol, it would be almost consumed after the accumulation of equimolar hydroperoxides. However, Figure 2 shows that α-tocopherol remained at a high level after the c-t hydroperoxides had accumulated beyond the equivalent level (0.68% of methyl linoleate). It is known that α-tocopherol at higher levels decreases very slowly during the autoxidation of edible oil (22). The slow decrease in α-tocopherol at the 1.0% level may be explained, at least partly, by the regeneration of α-tocopherol from the chromanoxy radical.

Packer et al. (30) demonstrated directly that ascorbic acid can regenerate α-tocopherol by donating a hydrogen atom to the chromanoxy radical. Therefore, the observation that ascorbyl palmitate suppressed the hydroperoxidation (Fig. 6) strongly suggests the participation of the chromanoxy radical in this peroxidizing effect of α-tocopherol. Ascorbyl palmitate may remove the chromanoxy radical from the cyclic reaction of α-tocopherol-induced hydroperoxidation. Furthermore, the reverse of reaction [3]:

$$\text{LOOH} + \text{Toc}^{\cdot} \rightarrow \text{LOO}^{\cdot} + \text{TocH} \qquad [6]$$

which would produce the peroxy radical, may also participate in the regeneration of α-tocopherol and α-tocopherol-induced hydroperoxidation. Further studies are required to ascertain the mechanism of the peroxidizing effect of α-tocopherol.

BHT is known to have a much lower hydrogen-donating activity (18) than tocopherol, and that is reflected by the level of c-t hydroperoxide formation in the presence of BHT (Fig. 5). It also shows no peroxidizing effect. Accordingly, it may be concluded that the effectiveness of antioxidants at high concentrations cannot be judged by hydrogen-donating ability alone, and that contribution to the peroxidizing effect should be taken into account when autoxidation occurs in bulk phase. α-Tocopherol may require a suitable hydrogen donor not only to generate the synergistic effect for the antioxidant property, but also to prevent the peroxidizing effect in the early stage of oxidation of edible oil.

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Enzymic Oxidation of Linolenic Acid to 1,Z-5-Octadien-3-ol, Z-2,Z-5-Octadien-1-ol and 10-Oxo-E-8-decenoic Acid by a Protein Fraction from Mushrooms *(Psalliota bispora)*

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The pathway for the oxidative cleavage of linolenic acid was investigated using a protein fraction from mushrooms *(Psalliota bispora)* as the enzyme source. Incubation of the protein fraction with linolenic acid resulted in 1,Z-5-octadien-3(R)-ol and Z-2,Z-5-octadien-1-ol (in a 3:2 ratio) and 10-oxo-E-8-decenoic acid (10-ODA). Experiments with molecular oxygen-18 indicated that the oxygen in the hydroxy group of both octadienols originates from the gaseous phase and not from water. The protein fraction was incubated with the individual 9-, 10-, 12-, 13-, 15- and 16-hydroperoxide isomers of linolenic acid. Only the 10-hydroperoxy-E-8,Z-12,Z-15-octadecatrienoic acid (10-HPOT) served as substrate, and was cleaved into the two octadienols and the 10-ODA. This result suggests that in the oxidation of linolenic acid by the mushroom fraction, the 10-HPOT is an intermediate cleaved by a hydroperoxide lyase into the octadienols and the 10-ODA. Model experiments in which the methyl esters of both 10-hydroperoxy-E-8,Z-12-octadecadienoic acid (10-HPOD) and 10-HPOT were treated with the Lewis acid BF_3 yielded only various C_9 compounds. It was therefore concluded that the reactions of the mushroom hydroperoxide lyase cannot be explained by the heterolytic rearrangement mechanism, which was proposed for the corresponding plant enzyme. β-Scission of the 10-HPOD and the 10-HPOT explains the reactions of the mushroom hydroperoxide lyase and is discussed in detail.
***Lipids 21,* 261–266 (1986).**

The unsaturated alcohol 1-octen-3-ol occurs in many mushroom (1–5) and several mold (6–8) species. 1-Octen-3(R)-ol, which contributes significantly to the characteristic flavor of the edible mushroom *Psalliota bispora,* is formed from linoleic acid (9–12). We recently isolated from mushrooms a protein fraction, which cleaves oxidatively linoleic acid into 1-octen-3(R)-ol and 10-oxo-E-8-decenoic acid (13). The oxygen atoms from molecular oxygen are incorporated into the hydroxy group of the alcohol and the carbonyl group of the oxo acid (14). A reaction intermediate is 10(S)-hydroperoxy-E-8,Z-12-octadecadienoic acid which is cleaved by a hydroperoxide lyase into 1-octen-3(R)-ol and the oxo acid (15).

In addition to 1-octen-3-ol, Tressl et al. (11) identified 1,Z-5-octadien-3-ol and Z-2,Z-5-octadien-1-ol as components of the volatile fraction obtained from mushrooms. They ascribed linolenic acid as precursor of both octadienols.

1,Z-5-octadien-3-ol has also been identified in the red seaweed *Chondrococcus hornemanni* (16), in eight Australian crustaceans (17) and in the emerald shiner *Notropis atherinoides* (18). The absolute configuration of the 1,Z-5-octadien-3-ol found in the red seaweed was established to be the S-configuration (16). The formation of 1,Z-5-octadien-3-ol, which contributes to the fresh fish aroma of emerald shiners, was almost completely inhibited by immediate exposure to inhibitors of cyclooxygenase and lipoxygenase (18). It was therefore postulated that the octadienol is formed by a breakdown of prostaglandin H_3 which originates from an enzymic oxidation of an ω-3 eicosapentaenoic acid (18). The purpose of our work was to elucidate the pathway which leads from linolenic acid to both octadienols in mushrooms.

EXPERIMENTAL PROCEDURES

Materials. Mushrooms *(Psalliota bispora)* were purchased from local markets; the cultivars were unidentified. Other materials used were α-linolenic acid, methyl linoleate and methyl linolenate, all at least 99% pure (Sigma, Munich, Federal Republic of Germany); E-2,Z-6-nonadienal and E-2-nonenal (Atlanta, Heidelberg, Federal Republic of Germany); Tween 80 (Schuchardt, Munich, Federal Republic of Germany); gaseous oxygen containing 90% $^{18}O_2$ (Ventron GmbH, Karlsruhe, Federal Republic of Germany); 5 μm silicic acid for HPLC (Hypersil; Shandon, Karlsruhe, Federal Republic of Germany); Florisil (60–100 mesh; Serva, Heidelberg, Federal Republic of Germany); BF_3-etherate containing 45% (w/w) BF_3 (Merck, Darmstadt, Federal Republic of Germany).

The 9-, 10-, 12-, 13-, 15- and 16-hydroxystearic acid phenacyl esters were a gift from F. Haslbeck (DFA, Garching, Federal Republic of Germany) and the corresponding methyl esters a gift from C. Sondermann (DFA, Garching, Federal Republic of Germany).

The mushroom protein fraction, 10-HPOD and 10-oxo-E-8-decenoic acid were prepared as described (13,14). The emulsified linolenic acid substrate was prepared as described for linoleic acid (14). Oct-1-en-5-yn-3-ol was synthesized according to Whitfield et al. (17), then hydrogenated to 1,Z-5-octadien-3-ol with borohydride-reduced nickel as catalyst (19) and purified by gas chromatography (GC). Its IR and ^{1}H-NMR data agreed with those published by Whitfield et al. (17) for 1,Z-5-octadien-3-ol. 1,Z-5-octadien-3-one was prepared by oxidation of 1,Z-5-octadien-3-ol with pyridinium chlorochromate (20); the mass spectrometry (MS) agreed with that published by Swoboda and Peers (21). Methyl 9-oxononanoate was obtained by reductive ozonolysis of methyl oleate (22).

Preparation of hydroperoxide isomers. Methyl linoleate, methyl linolenate and linolenic acid were photooxidized with *meso*-tetraphenylporphine in benzene as described for linoleic acid (13). The monohydroperoxide fraction was isolated by thin layer chromatography (TLC) (13), except that oxalic acid was omitted for the separation of the methyl esters. The isomers were separated by high performance liquid chromatography (HPLC). To obtain enough material, multiple samples (each ca. 0.25 mg) were applied to a stainless steel column (4.6 × 500 mm) packed with 5 μm silicic acid. Elution of the esters was performed with hexane/ethanol (997:3, v/v) and of the acids with hexane/2-propanol/acetic acid (992:7:1, v/v/v) with a flow rate

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of 4.2 ml/min. The effluent was monitored with a UV detector operating at 206 nm. The elution sequences are presented in Table 1. Each hydroperoxide isomer was collected individually, freed from the solvent mixture (13) and then dissolved either in anhydrous diethyl ether (methyl esters) or methanol (acids). The 10-hydroperoxide isomers of methyl linoleate, methyl linolenate and linolenic acid were completely freed from other isomers by rechromatography with the solvent systems described above. The concentration of the hydroperoxide isomers was determined by the ferrous thiocyanate test (13). The substrates containing the hydroperoxide isomers of linolenic acid were prepared as described for the 10-HPOD (13).

For identification, an aliquot of each isomer was hydrogenated to the corresponding isomeric hydroxystearic acid or methyl hydroxystearate, respectively. The hydroxystearic acid isomer was then converted to the corresponding phenacyl ester derivative (23,24). Cochromatography (HPLC) was performed with methyl hydroxystearate standards (25) and phenacyl ester standards, respectively (23). In addition to the comparison of the chromatographic properties, the identification of the positional isomers was based on the agreement of the electron impact (EI) mass spectra with those of the reference substances.

TABLE 1

Relative Retention Volumes of Hydroperoxide Isomers Separated by HPLC[a]

Carbon positions of the OOH-group	Relative retention volume of hydroperoxides from[b]		
	Methyl linoleate	Methyl linolenate	Linolenic acid
9	1.31	1.31	1.49
10	1.23	1.23	1.27
12	1.06	1.06[c]	1.07
13	1.00	1.00	1.00
15	—	1.16	1.22
16	—	1.04[c]	1.11

[a]See *Preparation of hydroperoxide isomers* (Results).
[b]The 13-hydroperoxide isomer is used as reference.
[c]The isomers are only partially separated.

Products from linolenic acid. The reaction mixture contained 450 μmol linolenic acid, 24 μl Tween 80, the mushroom protein fraction (250 mg protein) and 0.1 M phosphate buffer, pH 6.5, in a total volume of 150 ml. After 30 min incubation at room temperature, the reaction mixture was acidified to pH 3 with diluted HCl and extracted twice with 100 ml of diethyl ether. After concentration and methylation of the acids with diazomethane (26), the reaction mixture was separated by TLC (Silica Gel HF_{264}, hexane/diethyl ether [3:2, v/v]).

Products from the 10-HPOT. The incubation and the analysis of the products were performed as described for the analysis of the products from the 10-HPOD (13), now substituting the 10-HPOD by 10-HPOT.

Incubation with $^{18}O_2$. The composition of the reaction systems is listed in Table 2. The incubation of the reaction mixture (20 min, 20 C) was carried out as previously (14). It was begun by flushing the substrate with gaseous oxygen having an $^{18}O_2$ to $^{16}O_2$ ratio of 9:1 (v/v) followed by injection of the mushroom protein fraction (10 ml) through a septum of the reaction vessel. The incubation was stopped by addition of NaOH to a pH value of 9.0. The mixture was shaken with 10 ml pentane and centrifuged ($10^4 \times$ g; 10 min; 2 C). The pentane extract was concentrated to 0.2 ml by distilling off the solvent. In one-half of the extract both octadienols were identified by GC/MS and their concentrations were determined by GC. The second half of the pentane extract was treated with a stream of nitrogen to remove the solvent. The residue was then dissolved in 0.5 ml methylene chloride and a suspension of both 30 μmol pyridinium chlorochromate and 6 μmol sodium acetate in 1 ml methylene chloride was added (20). The supernatant was decanted and the insoluble residue was extracted twice with 3 ml diethyl ether. The combined diethyl ether solutions were filtered through a Florisil column (0.8 × 10 cm), concentrated to 0.2 ml in a stream of nitrogen and analyzed by GC/MS.

Reaction of the 10-hydroperoxides with BF_3-ether. The reaction was performed as described by Gardner and Plattner (27). The methyl esters of the 10-HPOD and the 10-HPOT (8 μmol each) were individually treated with 3.5 ml of 0.5% (v/v) BF_3-etherate in anhydrous diethyl ether. After 5 min at 25 C each reaction system was diluted with 3.5 ml water. The ether layers were washed three times with water and then dried over sodium

TABLE 2

Incorporation of Oxygen-18 into Octadienols[a]

Carbonyl compound[b]	Oxygen isotope in the reaction system	Relative intensity of the ion (%)[c]		Incorporation of ^{18}O into the corresponding octadienol (%)
		m/z 125	m/z 127	
1,5-Octadien-3-one	100% $^{16}O_2$	100	1	1
1,5-Octadien-3-one	90% $^{18}O_2$, 10% $^{16}O_2$	20	100	83.5
2,5-Octadienal	100% $^{16}O_2$	100	1	1
2,5-Octadienal	90% $^{18}O_2$, 10% $^{16}O_2$	30	100	77.0

[a]The reaction system contained 180 μmol linolenic acid, 50 μl Tween 80, mushroom protein fraction (325 mg protein), 25 ml 0.1 M phosphate buffer, pH 6.5, and gaseous oxygen in a total volume of 125 ml.
[b]For CI mass spectra analysis the octadienols were oxidized to the corresponding carbonyl compounds.
[c]The mass spectra were recorded after chemical ionization.

sulfate. The hydroperoxide concentration was determined in aliquots of each ether solution with the ferrous thiocyanate test (13). The structures of the volatile compounds formed were established by GC/MS. The quantitative determination of the volatiles in the ether extracts was done by capillary GC, using methyl stearate as internal standard (27).

Absolute configuration of 1,Z-5-octadien-3-ol. The method of Van Os et al. (28) was followed. Ca. 1 mg of 1,Z-5-octadien-3-ol was acetylated and then the double bonds were cleaved by oxidative ozonolysis. The acetylated malic acid obtained was esterified to the (−)-2(R)-butyl ester. The ratio of the resulting diastereomers was determined by capillary GC (HRGC).

Quantitation of the octadienols. An aliquot of the pentane extract containing the products after incubation was spiked with a measured quantity of 3-octanol and then analyzed by GC on the free fatty acid phase (FFAP) column (see below).

Instrumental analysis. GC: The FFAP column (3 m × 2 mm stainless steel column packed with 10% FFAP on Chromosorb W, 80–100 mesh) was used isothermally (110 C) for purification of both octadienols. Helium was used as the carrier gas (30 ml/min). The GC column effluent was split in a ratio of 1:9 to an FID and a cooling trap (U-shaped glass capillary cooled with liquid nitrogen). For analysis of the products resulting from the incubation experiments with the mushroom protein fraction, the temperature was programmed from 80 C to 230 C at 4 C/min.

Capillary GC was performed with a 30 m × 0.3 mm glass capillary coated with SE 54. The following temperature program was used (except for the (−)-2(R)-butyl esters): after 5 min at 50 C the temperature of the GC oven was increased to 200 C at a rate of 4 C/min. For the analysis of the (−)-2(R)-butyl ester of acetylated malic acid, the program was as follows: after 10 min at 120 C, the temperature was increased to 200 C at a rate of 2 C/min. The FID detector temperature was 250 C and the flow rate of the helium carrier gas was 1.9 ml/min.

GC-MS analyses were performed using an MS 112 (Varian, Bremen, Federal Republic of Germany) in tandem with a 25 m fused silica capillary (CP Sil 5 B; Chrompack, Middelburg, Holland). The samples were applied by the "on column injection technique" at 35 C and the temperature of the column was raised by 20 C/min to 100 C and then by 4 C/min to 200 C and finally held at 200 C until the end of the analysis.

EI mass spectra were generated at 70 eV and chemical ionization (CI) spectra were obtained at 250 eV with isobutane as reagent gas. The proton magnetic resonance (^{1}H-NMR) spectra were recorded with a Bruker WP-80 operating at 80 MHz with the sample dissolved in $CDCl_3$. The infrared (IR) spectra were obtained with a Perkin-Elmer Model 299 B using 2 mm KBr pellets.

RESULTS

Products from linolenic acid. After incubation of linolenic acid with the protein fraction from mushrooms, the acid product mixture was converted to methyl esters and separated by TLC. In addition to unreacted linolenic acid (R_f 0.95), three compounds were isolated: I (R_f 0.41), II (R_f 0.49) and III (R_f 0.60). Compounds II and III were further purified by GC on the FFAP column. By comparison of the GC retention time and the EI and CI mass spectra with those of reference substances, compound I was identified as methyl 10-ODA. Compound II showed a retention index (definition according to [29]) of 1700 on the FFAP column and the following EI mass spectra: 108 (12%), 93 (23%), 79 (95%), 67 (48%), 55 (55%), 41 (100%). The fragments of this mass spectra are in agreement with those published by Tressl et al. (11) for Z-2,Z-5-octadien-1-ol. Analysis of compound II by IR and ^{1}H-NMR supported this structure as follows: IR 3450 (OH), 3010, 1640 (CH = CH) cm^{-1} and no absorption at 975 cm^{-1}; ^{1}H-NMR ∂5.2 to 5.65 (m, H2, H3, H5, H6), 4.2 (d, J 5.3 Hz, H1), 2.81 (t, J 6.6 Hz, H4), 2.05 (quintet, J 7.0 Hz, H7), 1.17 (s, OH), 0.93 (t, J 7.2 Hz, H8).

By comparison of the GC retention index (1493 on the FFAP column), the EI and CI mass spectra and the ^{1}H-NMR with the corresponding data of a reference substance, compound III was identified as 1,Z-5-octadien-3-ol. For the determination of the absolute configuration at the chiral carbon 3, the dienol was converted into the (−)-2(R)-butyl ester of acetylated malic acid. The R-enantiomer predominated, as found by HRGC (Fig. 1). This was confirmed by cochromatography with the acetylated (−)-2(R)-butyl esters of racemic malic acid and also of (S)-malic acid. From the peak areas of the separated diastereomers (Fig. 1) the proportion of the enantiomers of 1,Z-5-octadien-3-ol was calculated: 95% R, 5% S. This ratio was also found when a mushroom homogenate was incubated with linolenic acid (data not shown).

Origin of the oxygen in the hydroxy groups. After incubation of linolenic acid with the mushroom protein fraction in the presence of $^{18}O_2$, two major peaks appeared in the GC (Fig. 2). Based on their retention time and their EI mass spectra, these were identified as 1,Z-5-octadien-3-ol (peak 2) and Z-2,Z-5-octadien-1-ol (peak 3). The quantitative analysis indicated that 40 μmol 1,Z-5-octadien-3-ol and 26 μmol Z-2,Z-5-octadien-1-ol were formed during incubation. This 3:2 ratio between the octadienols was also

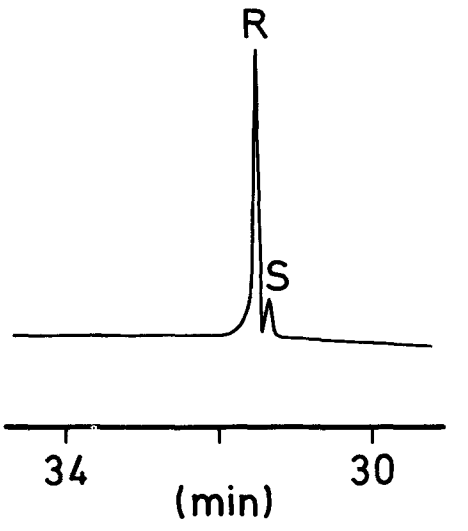

FIG. 1. Partial GC chromatogram of (−)-2(R)-butylester of acetylated malic acid derived from the 1,Z-5-octadien-3-ol isolated after incubation of the mushroom protein fraction with linolenic acid.

found when the incubation was performed in the presence of molecular $^{16}O_2$ (data not shown). In addition to the octadienols, a small quantity of 1-octen-3-ol appeared in the GC (peak 1 in Fig. 2). A control experiment (data not shown), in which the linolenic acid was omitted, demonstrated that the 1-octen-3-ol originated from the mushroom protein fraction.

The CI mass spectra of both octadienols were virtually identical. They showed a shift of the protonated molecular ion from m/z 127 to m/z 129 when the oxygen-18 was incorporated into the HO-group. This result is exemplified for 1,Z-5-octadien-3-ol in Figure 3. However, due to the rapid loss of water (appearance of the ion at m/e 109 in Fig. 3), the intensities of the protonated molecular ions were too low for an exact calculation of the oxygen-18 content. Therefore, the octadienols formed in the presence of $^{16}O_2$ as well as $^{18}O_2$ were oxidized to the corresponding carbonyl compounds and analyzed by GC/MS. The fast eluting peak (retention index 985 on the SE 54 capillary column) was identified by comparison with reference substances (GC and MS) as 1,Z-5-octadien-3-one. The next peak, eluting with a retention index of 1059, was identified as Z-2,Z-5-octadienal because its major EI mass spectra ions (m/z: 81, 124, 59, 67) agreed with those reported by Tressl et al. (11).

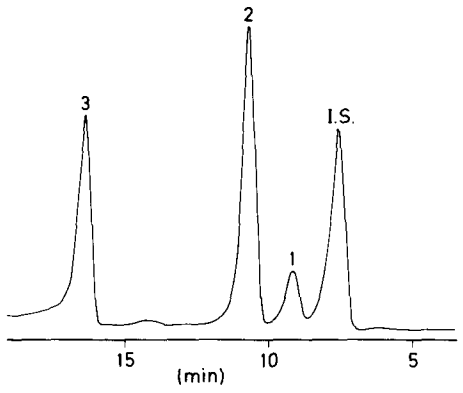

FIG. 2. GC chromatogram (FFAP column) of a pentane extract after incubation of linolenic acid with the mushroom protein fraction in the presence of gaseous $^{18}O_2$. The following compounds were identified in Results: peak 1, 1-octen-3-ol; peak 2, 1,Z-5-octadien-3-ol; peak 3, Z-2,Z-5-octadien-1-ol; I.S., internal standard (3-octanol).

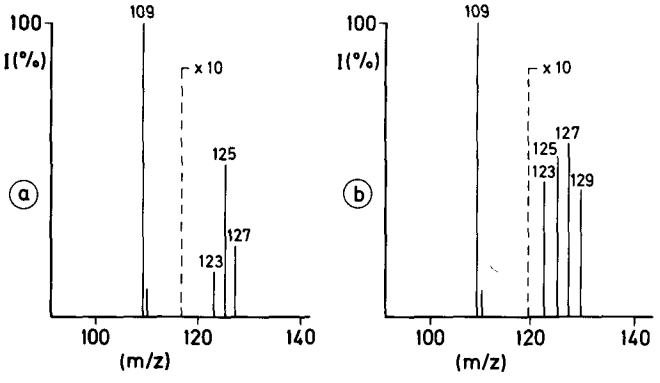

FIG. 3. CI mass spectra of 1,Z-5-octadien-3-ol formed in the presence of gaseous $^{16}O_2$ (spectrum a) and in the presence of 90% $^{18}O_2$ gas (spectrum b).

The CI mass spectra of both carbonyl compounds showed that the protonated molecular ion at m/z 125 was the predominant peak in both spectra. However, the ion m/z 105 (98%), which presumably results from the loss of water ($M^+ + 1 - 18$), appeared only in the CI mass spectra of the Z-2,Z-5-octadienal. The data, from the CI mass spectra summarized in Table 2, indicate that the protonated molecular ion at m/z 125 shifted to m/z 127, when the carbonyl group of the aldehyde and of the ketone contained an ^{18}O-atom. The data in Table 2 showed an incorporation of about 80% of oxygen-18 into both carbonyl compounds when the incubation was conducted in the presence of gaseous $^{18}O_2$. Since the carbonyl compounds are derivatives of the octadienols, we may conclude that the oxygen atom in the OH-group of both 1,Z-5-octadien-3-ol and Z-2,Z-5-octadien-1-ol originates from gaseous oxygen and not from water.

Breakdown of linolenic acid hydroperoxides. Photooxidation of linolenic acid or of its methyl ester in the presence of a sensitizer which produces singlet oxygen yields racemic mixtures of six hydroperoxide isomers (24,30–33): 9-hydroperoxy-E-10,Z-12,Z-15-octadecatrienoic acid (9-HPOT), 10-hydroperoxy-E-8,Z-12,Z-15-octadecatrienoic acid (10-HPOT), 12-hydroperoxy-Z-9,E-13,Z-15-octadecatrienoic acid (12-HPOT), 13-hydroperoxy-Z-9,E-11,Z-15-octadecatrienoic acid (13-HPOT), 15-hydroperoxy-Z-9-Z-12,E-16-octadecatrienoic acid (15-HPOT) and 16-hydroperoxy-Z-9,Z-12,E-14-octadecatrienoic acid (16-HPOT) or the corresponding methyl esters, respectively.

We separated the hydroperoxides obtained from a photosensitized oxidation of linolenic acid by HPLC and identified the elution sequence as detailed in Table 1. The position and configuration of the double bonds in the hydroperoxide isomers are postulated from the "ene"-mechanism of the reaction of singlet oxygen with olefins (34,35). Oxygen is thus inserted at either carbon of a double bond. This double bond is shifted to yield an allylic hydroperoxide in the (E) configuration. In the case of unsaturated fatty acids this high degree of stereoselectivity has been established for the hydroperoxide isomers formed during photosensitized oxidation of methyl linoleate (36).

Each of the six hydroperoxide isomers of linolenic acid was incubated with the mushroom protein fraction. As shown in Table 3, a significant decrease in the peroxide

TABLE 3

Substrate Specificity of the Mushroom Hydroperoxide Lyase

Substrate[a]	Decrease in absorbance ΔA_{490} nm (1–10 min)
9-HPOT	0.010
10-HPOT	0.130
12-HPOT	0.010
13-HPOT	0.010
15-HPOT	0.008
16-HPOT	0.009

[a]The reaction system contained 0.6 μmol of the monohydroperoxide isomer, 0.2 μl Tween 80, mushroom protein fraction (3.5 mg protein), 0.1 M phosphate buffer, pH 6.5, in a total volume of 3 ml. During incubation at 22 C, aliquots of 0.2 ml were pipetted into 2 ml methanol and the breakdown of the hydroperoxide group was measured by the ferrous thiocyanate test (13). At the beginning of the reaction, the absorbance of each hydroperoxide substrate assayed by the ferrous thiocyanate test amounted to A_{490}:0.215.

concentration was only found when 10-HPOT was used as substrate. The products formed from the breakdown of 10-HPOT by the mushroom protein fraction were identified as 1,Z-5-octadien-3-ol and Z-2,Z-5-octadien-1-ol in a 3:2 ratio and 10-oxo-E-8-decenoic acid. As before, the identities of these products were assessed by cochromatography with authentic standards on GC and by MS (data not shown).

Heterolytic cleavage of 10-HPOD and 10-HPOT. Gardner and Plattner (27) have shown that the heterolytic cleavage of hydroperoxide isomers of linoleic acid with the Lewis acid BF_3 as catalyst leads to products identical to those formed by the enzyme hydroperoxide lyase of plant origin. Since the results reported here and recently (13) suggest that a hydroperoxide lyase is involved in the cleavage of both 10-HPOT and 10-HPOD, the heterolytic cleavage of both hydroperoxide isomers was investigated.

After 5-min treatment of the methyl ester of 10-HPOD with BF_3-etherate, 95% of the initial hydroperoxide was depleted. Two major peaks appeared in the GC of the volatile products. The fast eluting peak (retention index 1163) showed the following EI mass spectra: 140 (M^+, 6%), 111 (10%), 96 (30%), 83 (48%), 70 (75%), 55 (85%), 41 (100%), which is in agreement with that reported for 2(E)-nonenal (28). This structure was confirmed by cochromatography with an authentic sample of 2(E)-nonenal. However, the configuration of the double bond in the 2-nonenal isolated from BF_3-treated 10-HPOD was not further investigated. The second peak, eluting with a retention index of 1493, was identified as methyl 9-oxononanoate by cochromatography with the reference substance and on the basis of the EI mass spectra, which showed the following major ions: m/z 158 (6.5%, M^+-CO), 155 (11.3%, M^+-CH_3O), 143 (19.5%), 111 (33%), 87 (59%), 83 (42%), 74 (100%), 55 (91%). 1-Octen-3-ol and 10-ODA were not found among the products from BF_3 catalysis of 10-HPOD.

The breakdown of the methyl ester of the 10-HPOT also was almost complete after the first 5 min of the reaction.

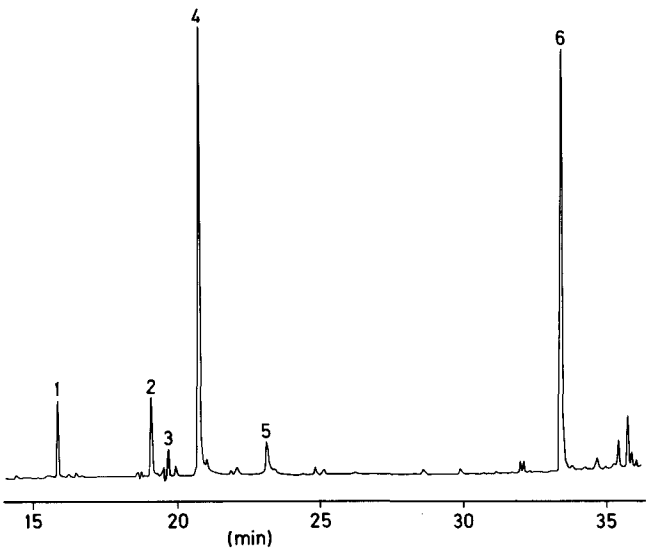

FIG. 4. Capillary GC of products from BF_3-diethyl ether-treated methyl 10-hydroperoxy-E-8,Z-12,Z-15-octadecatrienoate.

The GC (Fig. 4) revealed that two major and several minor compounds were formed. The first eluting major compound showed the following EI mass spectra: 138 (15%), 95 (15%), 84 (100%), 83 (26%), 79 (15%), 55 (28%), 43 (22%). This product and the minor compounds 1-3 in Figure 4 could not be identified, although the MS and GC data indicated that they could be neither octadienol nor 10-ODA. The CI mass spectra of these four compounds showed a protonated molecular ion at m/z 139. This result and the cucumber-like smell which was recognized at a GC-sniffing port (37) during elution of each of the compounds 1-4 support the speculation that they are isomeric nonadienals. The minor component 5 (Fig. 4) which eluted with a retention index of 1156 (SE 54) was identified as 2,6-nonadienal, after comparison of its GC retention value and MS data with those of a reference substance. The major compound 6, eluting with a retention index of 1493, showed the MS data reported above for methyl 9-oxononanoate. This structure was confirmed by cochromatography with the reference substance.

DISCUSSION

The results indicate that mushrooms contain enzymes which oxidatively cleave linolenic acid to yield 1,Z-5-octadien-3-ol, Z-2,Z-5-octadien-1-ol and 10-ODA. The oxygen in the HO group of both octadienols originates from gaseous oxygen and not from water. The 1,Z-5-octadien-3-ol has the same R-configuration at the chiral center as the 1-octen-3-ol produced by mushrooms (15) but contrasts to that reported for the corresponding octadienol of the red seaweed *Chondrococcus hornemanni* (16).

The experiments with different hydroperoxide isomers of linolenic acid as substrates show that only the 10-HPOT is cleaved by the mushroom protein fraction with formation of both octadienols and 10-ODA. This result, which corresponds to the cleavage of 10-HPOD into 1-octen-3(R)-ol and 10-ODA (13,15), allows the following conclusions: (i) 10-HPOT is the intermediate in the reaction pathway from linolenic acid to the octadienols and 10-ODA and (ii) a hydroperoxide lyase catalyzes the cleavage of the 10-HPOT. The production of two octadienols from linolenic acid as well as from 10-HPOT might be explained by the occurrence of two hydroperoxide lyases in the mushroom protein fraction.

Model experiments of the acid-catalyzed cleavage of the 9- and the 13-hydroperoxide isomers of linoleate have shown that the products obtained agree with those formed by hydroperoxide lyases of plant origin from these substrates (27). The reaction of plant hydroperoxide lyases then can be explained by a heterolytic mechanism together with a carbon-to-oxygen rearrangement of the hydroperoxide molecule (27). In a corresponding model experiment, the methyl ester of the 10-HPOD was treated with the Lewis acid BF_3. The products identified, 2-nonenal and methyl 9-oxononanoate, are in accordance with the proposed heterolytic mechanism, as their formation can be explained with an acid-catalyzed rearrangement of the 10-HPOD molecule. However, the mushroom hydroperoxide lyase produces 1-octen-3-ol and 10-ODA which are not found among the products from the BF_3-catalysis. Therefore this heterolytic mechanism cannot explain the reaction of the mushroom hydroperoxide lyase. This conclusion is confirmed by the products

obtained in a corresponding model experiment with the methyl ester of the 10-HPOT: the octadienols and the 10-ODA were absent but methyl 9-oxononanoate and another C_9-compound of unknown structure were found as major products.

The β-scission of the 10-hydroperoxide isomers is an alternative mechanism which might explain the formation of C_8-alcohols and 10-ODA by the mushroom hydroperoxide lyase. as proposed in Figure 5, a β-scission of the 10-HPOT leads (a) to 1,Z-5-octadien-3-ol and 10-ODA, and (b) to Z-2,Z-5-octadien-1-ol and 10-ODA. Homolytic mechanisms are shown in Figure 5 but the cleavages (a) and (b) might also proceed via ionic intermediates. The β-scissions proposed also explain the finding that the oxygen in the OH-group of the octadienols originate from gaseous oxygen (via the HOO-group of the 10-HPOT) and not from water.

The proposed β-scission mechanism also explains the formation of 1-octen-3-ol and 10-ODA from 10-HPOD. This is supported by the studies of Frankel et al. (38) who analyzed the volatiles from thermally decomposed hydroperoxides which originated from a photosensitized oxidation of methyl linoleate. They found that the hydroperoxide isomers decompose among others into 1-octen-3-ol and 10-ODA. The formation of these two products was explained by a β-scission of the 10-HPOD which was one component of the hydroperoxide mixture used (38).

ACKNOWLEDGMENT

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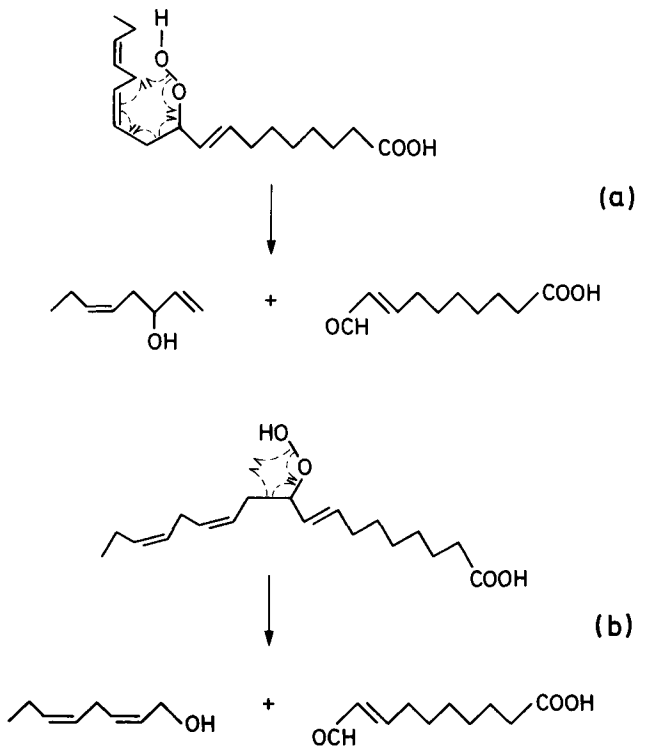

FIG. 5. Hypothetical pathways for the cleavage of 10-hydroperoxy-E-8,Z-12,Z-15-octadecatrienoic acid by a hydroperoxide lyase from mushrooms. Formation (a) of 1,Z-5-octadien-3-ol and 10-oxo-E-8-decenoic acid (10-ODA); (b) of Z-2,Z-5-octadien-1-ol and 10-ODA.

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METHODS

Separation of Underivatized Gangliosides by Ion Exchange High Performance Liquid Chromatography

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A procedure is described which separates neutral glycolipids from gangliosides, and which separates the gangliosides into classes, based on their number of sialic acid residues. In addition to separation into mono-, di- and trisialoganglioside classes, there is purification of individual disialoganglioside species. The procedure uses a commercially available $-NH_2$ high performance liquid chromatography (HPLC) column, which normally is used as an adsorption column. In this method the column is modified by protonation, to pH 5.4, so that it exhibits ion exchange as well as adsorption properties. Sensitive, nondestructive detection of eluent peaks is accomplished by monitoring continuously at 210 nm, a wavelength near which underivatized glycosphingolipids have absorbance maxima and which also will clearly detect most possible contaminants. An entire run, including re-equilibration of the column, takes two hours.
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For a systematic fractionation of the glycosphingolipids present in an extract of an animal tissue, a preliminary separation into groups of different polarity is essential (2). Chromatography on diethylaminoethyl (DEAE)-Sephadex (3) to separate neutral and acidic glycolipids has been used commonly. Separation into fractions of increasing polarity by elution from a silica column is a useful alternative (4). The neutral glycolipids of each fraction have been most efficiently separated into individual molecular species by HPLC on silica columns, without derivatization (5,6). Separation of gangliosides into classes based on number of sialic acid residues has been achieved using DEAE-Sephadex (7), DEAE silica gel liquid chromatography (8) and spherosil-DEAE-Dextran (9). Gangliosides also have been purified by HPLC either after derivatization (10) or without derivatization on silica columns with aqueous acid (11), on DEAE-controlled pore glass with an aqueous salt gradient (12) or on silica with isopropanol/hexane/water mixtures (13).

In the present study, a commercially available radial-pak NH_2 column is used. The column is charged to a pH of 5.4 with acetate buffer, so that it has anion exchange properties. A gradient of aqueous salt into methanol is used to displace the bound gangliosides, after an initial 15 min isocratic methanol elution. Neutral glycolipids are eluted from the column by methanol, and gangliosides are eluted by the increasing salt gradient. Gangliosides separate into classes based on the number of sialic acids, and within classes some further separation is achieved, especially among disialogangliosides. The procedure has been applied to bovine pineal tissue, from which GD_3 was purified as the major ganglioside. The procedure is highly reproducible.

The ganglioside nomenclature is according to the system of Svennerholm (1). GM_3, NeuAcα2 → 3Galβ1 → 4Glc-Cer; GM_1, Galβ1 → 3GalNAcβ1 → 4Gal(3 ← 2αNeuAc)β1 → 4Glc-Cer; GD_{1a}, NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4Gal(3 ← 2αNeuAc)β1 → 4Glc-Cer; GD_{1b}, Galβ1 → 3GalNAcβ1 → 4Gal(3 ← 2αNeuAc8 → 2αNeuAc)β1 → 4Glc-Cer. GT_1 is used to represent a mixture of trisialogangliosides which have the same structure of the uncharged portion as GM_1 described above. Globoside, GalNAcβ1 → 3Gal α1 → 4Galβ1 → 4Glc-Cer.

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MATERIALS AND EXPERIMENTAL METHODS

Commercially purified preparations of sphingolipids were purchased from Supelco Inc. (Bellefonte, Pennsylvania). Fresh frozen bovine pineals were purchased from Pel-Freeze Biologicals Inc. (Rogers, Arkansas). Commercial solvents used were HPLC grade, purchased from MCB. Reagents were all analytical grade. Water was glass-distilled. Silica was Mallinckrodt SilicAR CC-7, 100–200 mesh, dried overnight at 100 C before use.

HPLC was performed with Waters Associates model 6000A pumps equipped with a Model 720 system controller, and column eluent was continuously monitored by a Perkin-Elmer LC-55 spectrophotometer with an 8 μl flow-through cell, attached to a Hewlett-Packard 3390A reporting integrator. A Waters radial-pak cartridge with micro bondapak NH_2, 10 μm particle size packing, was used.

The column was initially charged by equilibration to pH 5.4 with 0.05 M acetate buffer. Equilibration was considered complete when the pH of the eluent was the same as that of the equilibrating buffer. The initial charging of the column normally required 60 min. Subsequent re-equilibrations required 30 min at a rate of 2 ml/min of 0.025 M acetate buffer. Since acetate anion has a significant absorbance at 210 nm, it is replaced with Cl^-. This requires a 10 min wash with 1 M NaCl at 2 ml/min. The column then is washed with distilled water for 10 min at 2 ml/min. Finally, the column is washed with methanol for 10 min at 2 ml/min, prior to the injection of the sample. This equilibration sequence is repeated after each run.

Two-hundred-ten nm was chosen as the optimal wavelength for monitoring the column eluent, for several reasons. The spectra of standard sphingolipids all show absorption peaks between 210 and 212 nm but have only minimal absorption at higher wavelengths. All have a high absorbance, if not a maximum, at 210 nm. Many of the potential contaminants, such as phospholipids, also show significant absorption at 210 nm, thereby providing information about potential impurities in the samples as well. The baseline falls off in the later part of the run because the aqueous solvent has a lower absorbance at 210 nm than methanol.

Thin layer chromatography (TLC) was performed on Brinkmann precoated aluminum-backed sheets of silica

gel 60, F-254. Solvent was chloroform/methanol/2.5 M NH_3 (60:35:8, v/v/v). Detection was by phenol-sulfuric acid spray (14), in order to detect saccharide-containing contaminants as well as gangliosides. Sialic acid, glucose, galactose, galactosamine and individual fatty acids were detected by gas chromatography on an HP5840A gas chromatograph, equipped with a flame ionization detector and a 30 m SE-30 capillary column. Helium carrier gas at a flow rate of 2 ml/min was used in a program of 110 C to 250 C at 4 C/min. Each sample injection of 4 to 7 μl was side-vented for 10 sec. Each program began with the column at 50 C, followed by a 30 C/min increase for two min. Every experimental preparation was compared to a similarly sized sample of a known ganglioside standard, processed through the same analytical procedure at the same time. Methanolysis and trimethylsilyation of all samples was by the method of Yoshida and Mega (15).

Column fractions were initially desalted by the method of Ueno et al. (16). However, the microdialysis technique of Lau and Fujitaki (17) proved to be more convenient. Microdialysis was performed in a volume of 500–600 μl in 750 μl microfuge tubes, with the tops of the caps cut off and replaced by dialysis membrane. Dialysis against two changes of distilled water is complete in 24 hr, can be done simultaneously with multiple samples and gave no detectable loss of glycolipids (see Results).

Fresh frozen tissue (bovine pineals) was dehydrated by extraction with nine vol of acetone, which also removed much neutral lipid, and the resulting powder was extracted first with 19 vol of chloroform/methanol (2:1, v/v), then by 10 vol of chloroform/methanol (1:1, v/v) containing 5% water, and finally with 10 vol of chloroform/ethanol (1:2, v/v) containing 5% water (18). The combined chloroform/methanol extract, enriched in glycosphingolipids, was dried, suspended in a small volume of chloroform and applied to a silica column. One g of silica was used for each 25 mg of dried lipid extract. The column was eluted by batches, with each batch being seven column vol of solvent. Eluents were pure chloroform (C), followed by 25% acetone (A) in chloroform (all proportions are v/v), 50% A/C, 75% A/C and pure acetone. This series then was followed by 25% methanol (M), 50% M/C, 75% M/C and pure methanol. This procedure is similar to that of Ishizuka et al. (4). Most gangliosides of pineal are in the 50% methanol fraction. A large fraction of the phospholipids was removed in the preparation of the original acetone powder, but some polar phospholipids remained with the gangliosides. These were removed by subjecting the 50% methanol fraction to 0.1 N methanolic sodium hydroxide at 35–40 C for 2–3 hr (19), followed by dialysis and evaporation. The resulting solid is an "enriched crude ganglioside" preparation.

RESULTS

Standard procedures and the establishment of ganglioside elution patterns. After a number of trials 1 M NaCl was selected as the aqueous salt solution, which is added to the methanol in geometrically increasing proportion. The equation describing the curve is

$$x = 100\left(\frac{t - t_o}{t_i - t_o}\right)^2$$

where

- x = percent aqueous solvent of the total
- t = elapsed time from injection
- t_o = time between injection and beginning of gradient (isocratic methanol elution)
- t_i = time between injection and achievement of 100% aqueous solvent

In our most frequent format, t_o is 15 min and t_i is 60 min. The elution profile of standard GM_1 resulting from this procedure is shown in Figure 1A; GD_{1a} is shown in Figure 1B and standard GT_1 is shown in Figure 1C. The purchased standard of GT_1 stated that it contained 10–30% of GD_{1b}. Figure 2 shows the elution pattern of all three standard samples combined and run in the same way, with the baseline subtracted for clarity.

Glycolipids were dissolved in methanol for application to the column. The flow rate was maintained at 1 ml/min throughout the sample run. As a result of the above format, neutral uncharged sphingolipids eluted with

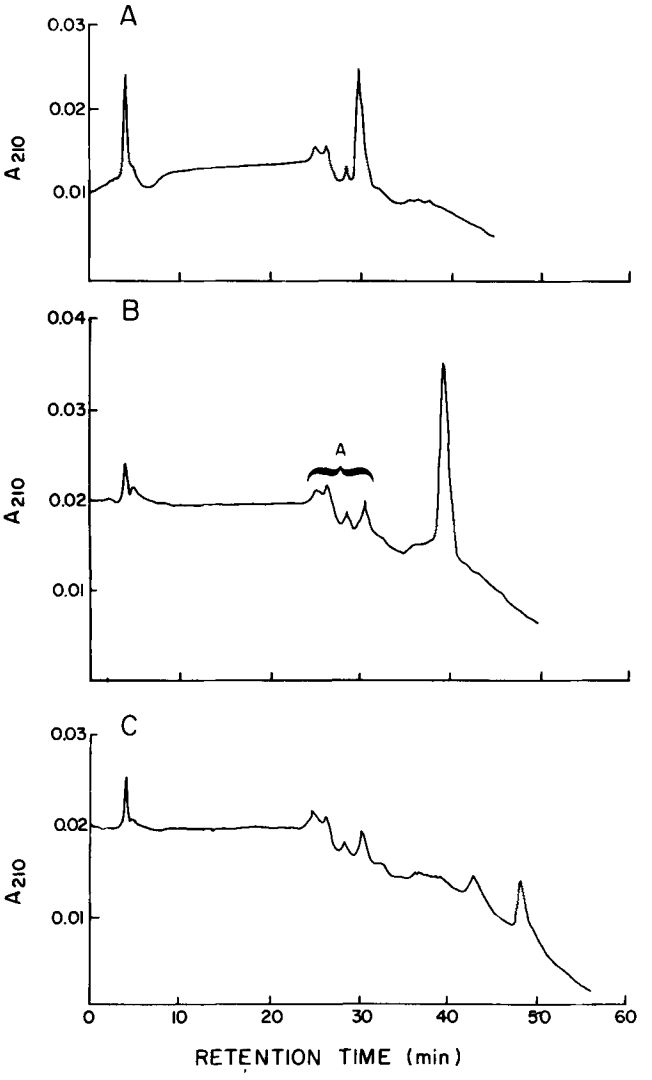

FIG. 1. Absorbance at 210 nm of purchased ganglioside standards run by the described HPLC procedure. GM_1, GD_{1a} and GT_1 samples are shown separately in A, B and C, respectively. See text for explanation.

isocratic methanol and sphingomyelin (a neutral zwitterion) was displaced from the column in the first five min of the gradient (Fig. 3 and pineal results) when the salt concentration was less than 15 mM. Monosialogangliosides eluted in the next 15 min, disialogangliosides during the 10-min period following and trisialogangliosides during the remaining 15 min of the gradient. This format was selected because it proved to be optimal for pineal sphingolipids, which include large amounts of neutral molecules and wherein mono- and disialogangliosides represent more than 99% of total ganglioside content.

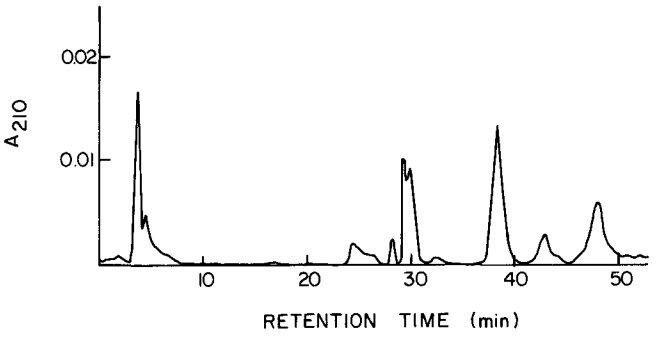

FIG. 2. Absorbance at 210 nm of combined standard gangliosides GM_1, GD_{1a} and GT_1, run by the described HPLC procedure. Baseline is corrected for clarity.

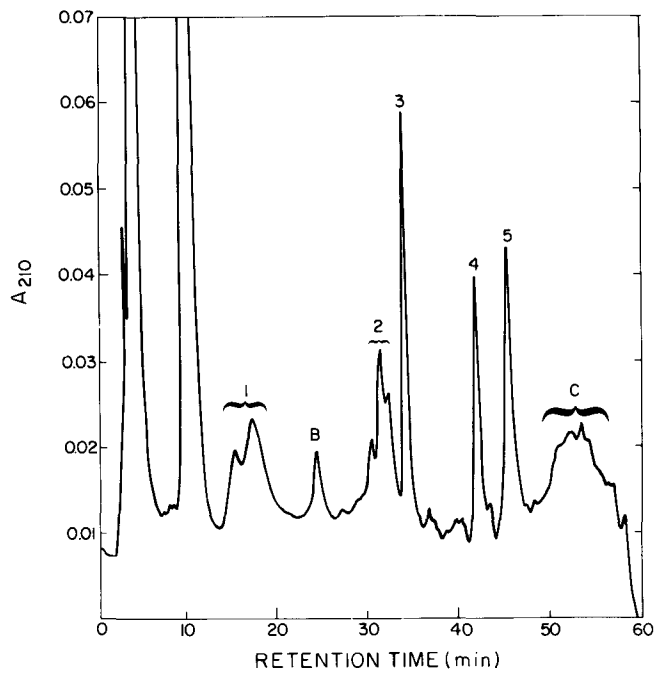

FIG. 3. Absorbance at 210 nm of "enriched crude ganglioside" preparation of bovine pineal gland, after mild alkaline hydrolysis. Letters refer to peaks produced by the procedure: B is present in blank runs, C appears only after the mild alkaline treatment. Peak 1 may be sphingomyelin, peak 3 is unidentified. The complex designated 2 contains GM_1 and GM_3. Peaks 4 and 5 both contain GD_3; peak 4 also contains GD_{1a}. Identifications were made by both thin layer and gas chromatography. For details, see text.

There was an injection artifact at four min. Four small, highly reproducible peaks between 22 and 32 min (labeled A in Fig. 1B) appeared even in blank runs. These small peaks yielded no detectable material by TLC or gas liquid chromatography (GLC) analysis, and may be refractive index changes.

Purified samples of hydroxycerebroside peak at five min after injection; globoside peaks at six min. Sphingomyelin and gangliosides GM_1 and GD_{1a} were identified after elution by TLC with purchased standards run simultaneously on the same plate. GM_1, which elutes at 29.8 min (Fig. 1A), and GD_{1a}, which elutes at 38.3 min (Fig. 1B), were compared to standard samples by GLC and gave ratios of sialic acid/galactose/glucose/N-acetyl galactosamine/fatty acid that were indistinguishable from standards run at the same time. In repeated HPLC elutions of the same molecules, the GM_1 peak varied ± 0.27 min and GD_{1a} ± 1.1 min (standard deviation). Recovery of GD_{1a} from HPLC and desalting procedures, as determined by gas chromatography, was 99.8% of stearic acid and 98.8% of glucose.

The GT_1 standard sample literature stated that the sample contained 10–30% GD_{1b}. Figure 1C shows two peaks, a minor one at 42 min and a major one at 48 min, from this standard. Both peaks gave the same R_f on TLC in chloroform/methanol/2.5 M ammonia (60:35:8, v/v/v).

Chloride salts of monovalent cations (e.g., ammonium and sodium) gave similar results. The divalent cation Ca^{++}, however, changed the elution behavior of standard gangliosides. When 0.5 M calcium chloride was used as the eluting salt, commercial standards of GT_1 and GD_{1a} were unseparated from each other and were displaced much earlier in the gradient than when the standard salt solution was used. Standards of GM_1 were unaffected in elution behavior.

Application of method to the purification of gangliosides of bovine pineal. Figure 3 shows the elution profile of the enriched crude ganglioside preparation from bovine pineals using the HPLC procedure described above. Peak B is artifactual; it contained no discernible material and was present in blank runs. Peak C appeared only after the mild alkaline hydrolysis. Peaks 1 and 3 are not gangliosides, as they contained no sialic acid as determined by GLC. The double peak at 1 gave only a single spot on TLC, which is indistinguishable from a standard of sphingomyelin run on the same plate. It was resistant to alkaline hydrolysis and eluted in 5–15 mM NaCl. Although sphingomyelin is neutral, it is zwitterionic and it may bind weakly to the column. Peak 3 has not yet been identified. Peaks 4 and 5 and the complex designated 2 contained the major gangliosides of bovine pineal. Peak 5 gave a single spot on TLC and yielded glucose, galactose and sialic acid as the only detectable carbohydrates by GLC. Using correction factors determined from standards of GD_{1a} in the same batch of samples and normalized to glucose, the ratio of sialic acid/galactose/total fatty acid/glucose was 2.01:1.02:0.92:1.0. No amino sugar was detectable and this peak appears to be pure GD_3. This represents the major ganglioside of bovine pineal. The eluent from the tubes containing peak 4 was examined by TLC and separated into GD_3 and a second, much smaller amount of material with an R_f identical to standard GD_{1a}. The combined peaks at 2 separated by TLC

into a small amount of material with the same R_f as standard GM_1 and a larger amount of material with higher mobility. After elution and GLC, the higher mobility material yielded a ratio of sialic acid/galactose/total fatty acid/glucose of 0.97:0.94:0.77:1.0 and is probably GM_3. This represents the second major ganglioside of bovine pineal.

DISCUSSION

It is customary to separate neutral glycolipids from gangliosides by liquid chromatography and to separate gangliosides from each other by TLC on silica gel (2). HPLC techniques have the advantages of speed and adaptability to small quantities of sample and often give a much better recovery than liquid chromatography on silica gel. Early HPLC procedures for gangliosides required derivatization, with the attendant problems of removing the derivatized groups later (10). Tjaden et al. were among the first to use HPLC successfully on underivatized gangliosides, using silica columns and a hot-wire detector (11). The use of strong acid in the eluent, however, was a possible source of degradation (11). Kundu and Scott (13) also used silica gel columns and achieved purification of gangliosides without acid or salt in the solvents. When using a complex mixture of gangliosides, Watanabe and Tomono, using a DEAE controlled pore glass (CPG) column in sequence with two other CPG columns, achieved good separation of mono-, di- and trisialogangliosides into classes, and also achieved separation of neutral glycolipids (12). Their system avoided acid and used lithium acetate as the salt.

The method described herein uses an aqueous salt solution, in common with other procedures that give good purification of gangliosides. We require only commonly available, standard NH_2 columns for HPLC, which then are used in an ion exchange mode. By monitoring at 210 nm, we achieve a sensitive, continuous and nondestructive monitoring of the column effluent which also allows sensitive detection of many impurities. We have found it to be highly reproducible in the 8–100 nmol range. The procedure should be adaptable to much larger quantities when needed. A single column can be reused without loss of resolving power at least 50 times. Individual NH_2 columns from the same supplier yield slightly different elution positions for a GD_{1a} standard, but a single column is highly reproducible. Recovery from the HPLC and microdialysis desalting procedure is greater than 98%.

We have used the procedure for the study of bovine pineal gangliosides and achieved a good purification of GD_3, the major ganglioside of the tissue, directly from the enriched crude ganglioside preparation.

Standard gangliosides, as well as those from a biological sample, are well separated by this procedure into mono-, di- and trisialoganglioside classes, which then can be purified in a single additional step by a TLC system or by the HPLC method of Kundu (13).

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Papers from the **Brian L. Walker Memorial Symposium** on

Lipids and Cancer

presented at the 76th AOCS Annual Meeting in Philadelphia, Pennsylvania, May 1985

In Remembrance of Brian L. Walker

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Brian Lawrence Walker died of cancer at his home in Guelph, Ontario, on April 12, 1984, at the age of 47. He was trained as an industrial chemist at the Liverpool College of Technology and obtained his Ph.D. in Food Chemistry at the University of Illinois in 1962 under the supervision of Dr. F. A. Kummerow. He remained at Illinois for two years as a postdoctoral fellow before joining the Department of Nutrition at the University of Guelph, where he spent the rest of his career. He was an innovative researcher in lipid biochemistry, and was engaged in research on the effects of *trans* fatty acids on membrane function prior to his death.

Brian was an active member of the American Oil Chemists' Society, and chaired the organizing committee for the society's meeting in Toronto in 1982. He served on the editorial boards of *Lipids* and the *Journal of Nutrition.* He was awarded the Borden Award of the Nutrition Society of Canada for research achievements prior to the age of 40.

As a senior faculty member of the Department of Nutrition, Brian coordinated the undergraduate program in nutrition and biochemistry for more than a decade. During his terminal illness, he was consulted by his colleagues regarding his preference of a commemoration of his service to the university. He was quite emphatic that he wished to be remembered for his association with undergraduate students. In accord with this wish, following his death the Brian L. Walker Memorial Scholarship was established. It was awarded for the first time in 1985 to the student with the best academic record in the undergraduate program in nutrition and biochemistry.

Brian left his wife Karen and their three children: Kimberley, 16, Valerie, 13, and Colin, 9. They are all active members of the Guelph Figure Skating Club, which Brian served as president. To honor his memory, the club established a trophy in his name for excellence in figure skating.

Brian Walker set an example of how to combine teaching, research and family activities. He also set an example of how to deal with death.

Calories, Fat and Cancer

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The experiments reported are part of our effort to dissociate the tumor-enhancing effects of dietary fat and high caloric intake. Rats either were fed ad libitum diets containing 4% corn oil or their calories were restricted by 40% and their diets contained 13.1% corn oil. Incidence of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors was 80% in rats fed ad libitum and 20% in those fed the calorie-restricted diets. Incidence of 1,2-dimethylhydrazine (DMH)-induced colon tumors was 100% in rats fed ad libitum and 53% in those whose caloric intake was restricted by 40%. The tumor yield (tumors per tumor-bearing rat) was significantly lower in rats on caloric restriction. In another series, rats were fed diets containing 5, 15 or 20% corn oil ad libitum or were fed calorie-restricted (by 25%) diets which provided 20 or 26.6% corn oil (therefore, the same absolute amount of fat was consumed in each of the pair-fed groups). Tumor incidence and tumor yield in the two calorie-restricted groups were similar to those seen in the rats fed 5% fat ad libitum; tumor burden (total g of tumor) was 45–65% lower in the calorie-restricted rats. The data suggest that caloric intake is a more stringent determinant of tumor growth than fat intake.
***Lipids 21,* 272–274 (1986).**

In 1909, Moreschi (1) reported that sarcomas implanted in mice maintained on restricted diets did not grow as well as those implanted in mice fed ad libitum. He also demonstrated that inhibition of tumor growth was related to degree of dietary restriction. Moreschi's findings were confirmed by Rous (2) and Bischoff et al. (3). Sivertsen and Hastings (4) found that albino mice maintained on severely restricted food intake exhibited 1/5 as many spontaneous mammary tumors as those permitted free access to food. Tannenbaum began the first systematic study of the influence of underfeeding on tumorigenesis, experimental and spontaneous. He found that dietary restriction led to significant inhibition of spontaneous mammary tumors or benzpyrene-induced skin tumors in mice (5). In the early experiments, food intake was manipulated to maintain the mice at a preselected body weight. In later experiments (6) Tannenbaum studied caloric restriction per se using a regimen of commercial dog food, skim milk powder and corn starch. The level of corn starch was manipulated to change caloric content of the diet. Incidence of spontaneous mammary tumors was significantly reduced in virgin and parous mice maintained on a diet low in calories (7).

The caloric content of the diet appeared to be of prime importance. Mice fed 8.5 cal daily of a diet containing 3% fat exhibited a 14% incidence of methylcholanthrene-induced skin tumors (8). When intake was raised to 10 cal/day (2% fat), tumor incidence was 22%; when the mice were fed 8.5 cal and 18% fat, tumor incidence was 23%. Lavik and Baumann (9) showed that mice fed a low fat, low calorie diet showed no methylcholanthrene-induced skin tumors. Mice fed a high calorie, low fat diet had twice the incidence of skin tumors as mice fed a diet low in calories but high in fat.

In our investigation (10) of the opposing effects of dietary fat and caloric restriction on initiation and promotion of DMBA-induced mammary tumors in virgin female Sprague-Dawley rats, we found that rats fed a diet with 40% fewer calories than controls exhibited significantly fewer tumors despite the consumption of more than twice as much fat by the rats fed the restricted diet. Those diets contained coconut oil with enough corn oil to provide adequate levels of essential fatty acids. The studies reported below used diets in which the only fat was corn oil.

MATERIALS AND METHODS

Virgin female Sprague-Dawley rats were used for studies of mammary tumors and male F344 rats were used in studies of colon cancer. All rats were housed individually in an air-conditioned room maintained at 21 C with a 12-hr light-dark cycle. There were 20 rats in each treatment group.

At the age of 50 days each female rat was given 5 mg of DMBA in 0.5 ml corn oil by gastric intubation. Food consumption was monitored daily and body weight and palpable tumors were checked weekly. Male rats were given six weekly doses of a solution of DMH, 30 mg/kg beginning at 6 wk of age. DMH hydrochloride was dissolved in physiological saline and the solution adjusted to pH 7.2 with sodium bicarbonate. Food consumption was monitored daily. The animals on DMBA were killed after 20 wk on the dietary regimen and those on DMH after 28 wk.

Rats were killed by intraperitoneal injection of sodium pentobarbital. Following midline ventral incision of the skin, blunt dissection was used to reflect the dermis. Position of all mammary tumors was noted, and the tumors were measured in three perpendicular directions, weighed and fixed in 10% buffered formalin. Sections were cut at 5 μm and stained with hematoxylin and eosin for histological examination. Intestinal tumors were classified according to the scheme of Pozharisski (11).

Statistical analysis of tumor incidence and yield was by chi-square analysis of contingency tables; other parameters were analyzed by the Kruskal-Wallis analysis of variance by ranks for nonparametrically distributed data. All analyses were performed on a microcomputer using Statpak programs (Northwest Analytical, Portland, Oregon).

In the first mammary and colon cancer studies, we restricted calories by 40%. The rats were pair-fed the restricted diet formulated to provide fewer calories, twice the level of fat and no reduction in dietary protein, fiber, minerals or vitamins (Table 1).

In the second mammary tumor experiment we compared the effects of ad libitum diets containing 5, 15 or 20% corn oil with diets in which caloric intake was restricted by 25% but absolute fat intake was maintained at levels equal to those in the groups fed 15 or 20% (Table 2).

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TABLE 1

Ad Libitum and Restricted Diets (40% Calorie Restriction)

Ingredient	Ad libitum diet (%)	Restricted diet	
		As formulated (%)	As fed (parts per 60)
Sucrose	59.0	25.0	15.0
Casein	21.6	36.2	21.7
DL-methionine	0.3	0.5	0.3
Corn oil	4.0	13.1	7.9
Choline	0.2	0.3	0.2
Cellulose	10.1	16.8	10.1
Mineral mix	3.8	6.4	3.8
Vitamin mix	1.0	1.7	1.0
Calories	360	365	220

TABLE 2

Ad Libitum and Restricted Diets (25% Calorie Restriction)

Ingredient	Diet[a]			
	15% Fat ad libitum	Pair-fed restricted	20% Fat ad libitum	Pair-fed restricted
Sucrose	48.0	30.7	43.0	23.9
Casein	21.6	28.8	21.6	28.8
Methionine	0.3	0.4	0.3	0.4
Corn oil	15.0	20.0	20.0	26.6
Choline	0.2	0.3	0.2	0.3
Cellulose	10.1	13.5	10.1	13.5
Mineral mix	3.8	5.1	3.8	5.1
Vitamin mix	1.0	1.3	1.0	1.3
Calories	415	420	440	452

[a]As fed, the restricted diets provided 314 and 337 calories and the same absolute amounts of fat as the 15 and 20% fat diets, respectively.

The mineral mix used throughout followed the Bernhart-Tomarelli formula (12); the AIN-76A vitamin mix was used (13).

RESULTS AND DISCUSSION

The results of the experiments in which mammary and colon tumors were induced experimentally in female Sprague-Dawley and male F344 rats, respectively, are detailed in Tables 3 and 4. In both experiments the rats fed ad libitum were compared with a group whose caloric intake was restricted by 40%.

In our first study (10) the fat used was mostly coconut oil and the rats on the restricted diet exhibited no tumors. We expected a greater incidence of tumors in the current experiment since it has been shown (14) that rats fed saturated fat exhibit fewer tumors than those fed unsaturated fat. As Table 3 shows, the incidence of tumors rose in both freely fed and restricted groups but the difference was still highly significant. Rats fed the ad libitum diet ingested about 18 g of diet per day, which translates to 65 kcal and 0.7 g fat daily. Rats restricted to 60% of

TABLE 3

DMBA-Induced Mammary Tumors in Sprague-Dawley Rats Fed a Diet Restricted in Calories by 40%

	Diet		Statistical significance
	Ad libitum	Restricted	
Incidence	16/20 (80%)	4/20 (20%)	$p < 0.001$
Tumors/tumor-bearing rat	4.0 ± 0.5	1.0 ± 0	$p < 0.001$
Total tumor yield	64	4	$p < 0.001$
Mean tumor weight (g)	2.8 ± 1.1	0.2 ± 0.1	$p < 0.01$
Mean tumor burden (g)	11.1 ± 4.4	0.2 ± 0.1	$p < 0.001$
Final body weight (g)	319 ± 10	198 ± 4	$p < 0.001$

TABLE 4

DMH-Induced Colon Tumors in F344 Rats Fed a Diet Restricted in Calories by 40%

	Diet		Statistical significance
	Ad libitum	Restricted	
Incidence	19/19 (100%)	10/19 (53%)	$p < 0.001$
Tumors/tumor-bearing rat	3.5 ± 0.4	2.1 ± 0.6	$p < 0.02$
Total tumor yield	63	20	$p < 0.001$
Extracolonic tumors	6/19 (32%)	2/19 (11%)	$p < 0.05$
Final body weight (g)	372 ± 6	216 ± 4	$p < 0.001$

calories ingested about 11 g, or 39 kcal containing 1.4 g fat, per day. The calorie-restricted rats ingested twice as much fat yet had significantly fewer tumors.

The effect of caloric restriction on DMH-induced colon tumors was marked. The rats on the calorie-restricted regimen exhibited a colon tumor incidence about half that of controls and 2/3 as many tumors per tumor-bearing rat, resulting in a total tumor yield 1/3 that in the rats fed ad libitum. Rats fed the restricted diet also exhibited 1/3 as many extracolonic tumors.

The studies described above were carried out in rats ingesting relatively low fat diets. In DMBA-induced carcinogenesis a sharp rise in tumor incidence is seen when the fat intake rises from 5 to 10% (15). Progressing beyond 10% dietary fat does not result in appreciable increase in tumor incidence. To test the influence of a diet higher in fat we repeated our earlier studies using diets containing 15 or 20% fat. The caloric intake was restricted by only 25%. The 5% fat diet is similar to that described in Table 1 except corn oil is increased to 5% at the expense of sucrose. The two restricted diets are shown in Table 2.

The results are summarized in Table 5. The sharp increase in tumor incidence and size seen in going from 5 to 15 or 20% fat confirms earlier reports (15). Significant reductions are seen in tumor incidence, frequency and size in rats fed the diets restricted in calories by 25%. In fact, the tumor burden of rats fed 20 or 26.7% corn oil in calorie-restricted diets is 65 and 45% less than that seen in ad libitum-fed rats ingesting a diet with only 5% fat. The actual fat intake in the rats freely fed diets containing 5, 15 or 20% corn oil was 1.39, 3.61 and 4.55 g/

TABLE 5

Effect of Fat Level and Caloric Restriction on DMBA-Induced Mammary Tumors in Sprague-Dawley Rats[a]

Diet	Incidence (%)	T/TBR[b]	Tumor wt (g)	Tumor burden (g)	Final body wt (g)
Ad libitum					
5% CO	65	1.9 ± 0.3	2.0 ± 0.7	4.2 ± 1.9	359 ± 11
15% CO	85	3.0 ± 0.6	2.3 ± 0.7	6.6 ± 2.7	368 ± 13
20% CO	80	4.1 ± 0.6	2.9 ± 0.5	11.8 ± 3.2	391 ± 12
Restricted[c]					
20% CO	60	1.9 ± 0.4	0.8 ± 0.2	1.5 ± 0.5	287 ± 11
26.7% CO	30	1.5 ± 0.3	1.4 ± 1.0	2.3 ± 1.6	310 ± 11
Statistical significance	$p < 0.005$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

[a]Calories restricted to 75% of rats fed ad libitum.

[b]Tumors/tumor-bearing rat.

[c]Rats received 75% of calories but same absolute amount of fat as ad libitum groups fed 15 or 20% corn oil.

100 cal, respectively, while in the two calorie-restricted groups it was 4.76 and 5.88 g/100 cal, respectively.

The rats fed the restricted diets gained less weight than the controls but gave no evidence of malnutrition. The final body weights in all restricted groups were close to the expected reductions of 40% or 25% vs the respective control groups (Tables 3-5). In the groups fed 5, 15 or 20% fat ad libitum, there was a trend toward increased weight with more fat in the diet, but the differences among the groups were not significant. It has been demonstrated that fewer tumors are seen in rats in whom growth is retarded by caloric deprivation (16-18). The experiments described above show that caloric reduction significantly inhibits tumor growth even when the diets are relatively high in fat.

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Mammary Tumorigenesis in Rats Fed Diets High in Lard

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Studies were performed to examine the effect of a lard diet on tumorigenesis by 7,12-dimethylbenzanthracene (DMBA), given parenterally rather than by gavage, to eliminate any effect of the high lard diet on carcinogen absorption. In addition, the effect of low dietary levels of the antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in the tumor model was evaluated. The lards fed were analyzed for fatty acid composition and content of certain potential contaminants. DMBA induced tumors when given by intravenous or subcutaneous injection. The high lard diet appeared to enhance tumorigenesis in rats given a dose of 0.25 mg (10% of the gavage dose) by injection into the mammary gland, although the effect was not statistically significant. In other experiments using lard from different sources and DMBA given by gavage, significant enhancement of tumorigenesis was limited to groups fed the high lard diets throughout the experiment or beginning after DMBA exposure. In contrast to earlier results, there was no demonstrable effect of feeding the high lard diets before DMBA administration. Addition of BHA and BHT to the lard at the concentration assayed in commercial lard samples or at the maximum concentration permitted did not influence the tumorigenesis. In groups in which tumorigenesis was enhanced by the high lard diet, the incidence of malignant, invasive tumors was higher than in other groups.
Lipids 21, 275–280 (1986).

Breast cancer incidence is increased in human populations and in laboratory rodents that ingest high fat diets (1–3). Design of preventive dietary measures requires identification of the types and amounts of fats responsible and knowledge of the ages or time periods in carcinogenesis at which fat is influential.

The enhancement of mammary carcinogenesis in DMBA- or N-nitrosomethylurea (NMU)-treated female rats is one of the most consistent dietary effects on carcinogenesis known (1–8). It has been demonstrated in many laboratories by investigators using protocols that differ in several respects, a fact that greatly strengthens its significance. There is, in general, increased mammary tumorigenesis enhancement with increased dietary content of linoleic and perhaps other unsaturated fatty acids in the high fat diets, but the effect varies with the structure of the fatty acid (2). Essential fatty acid (EFA) content, content of EFA plus oleate, ratios of content of poly- plus monounsaturated fatty acids to saturated fatty acids or to total fat all have been reported to be significant factors determining the dietary fat effect on mammary gland carcinogenesis (1,2,4 and reviewed in 8).

There is less agreement on the timing of the fat effect on tumorigenesis. Corn oil, by far the most intensively investigated fat, exerts its major detectable effect on tumorigenesis when fed at 20–25% of the diet (by wt) beginning after carcinogen exposure (1,5,8). There may be some effect at or before initiation; Dao and Chan (9) concluded that corn oil acted at initiation as well as during promotion and, further, that the duration of high fat intake, regardless of its timing, was an important factor. Kritchevsky et al. (10) reported enhanced tumorigenesis in rats fed a high mixed fat diet before DMBA exposure, again demonstrating an effect of fat at initiation of tumorigenesis.

Feeding a high lard diet to rats given DMBA, we found evidence of enhancement of tumorigenesis by lard fed only before initiation as well as when fed during promotion, but the timing and magnitude of the effect of lard varied from one experiment to another (5,6,11).

The studies reported here were undertaken to examine further the timing of the effect of the high lard diet on tumorigenesis using intravenous or subcutaneous administration of DMBA in an attempt to reduce the variability of results. It seemed possible that the high lard diet altered gastrointestinal absorption or hepatic metabolism of DMBA to change exposure of the gland and initiation of tumorigenesis.

A second possible explanation for the inconsistent activity of the high lard diet at initiation was the potential presence of carcinogens or cocarcinogens in some batches of lard. The lards fed were assayed for chlorinated hydrocarbon and estrogenic contaminants, as well as for fatty acid content. A third question examined was the contribution, if any, of the antioxidants added to retard development of rancidity. They would be expected to reduce rather than enhance tumorigenesis since BHT and BHA supplementation of diets at high levels, 1000 to 6000 ppm, inhibited DMBA-induced mammary carcinogenesis (12–14). An effect of the two antioxidants fed in the much lower amounts found in lard (about 10 ppm of each) or permitted in lard (100 ppm of each) had not been reported.

MATERIALS AND METHODS

Experiment 1. Weanling (21 days old) female Sprague-Dawley rats (40–50 g) (Charles River Laboratories, Wilmington, Massachusetts) were randomized to final treatment groups of 20 rats each and fed high lard or control diet (Table 1) to age 53 days. All rats were fed control diet from 53 to 57 days of age and given DMBA at 55 days, the protocol followed in our earlier studies in which DMBA was given by gavage. DMBA, emulsified with Emulphor® (EL-610, GAF Corp., New York City, New York) and ethanol, was given by intravenous (iv) injection (2.5 mg DMBA) or subcutaneous (sc) injection into the R3 mammary gland (1 or 0.5 mg DMBA) under ether anesthesia. At 57 days rats fed the high lard diet before DMBA treatment were divided into two groups of 20 each; one group was returned to that diet and the other was fed control diet for the remainder of the experiment.

Experiment 2. Rats were obtained and assigned to treatment groups as above; they were given DMBA,

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TABLE 1

Composition of High Lard and Control Diets

Component	% in diet[a]	
	Control	High lard
Casein (vitamin-free)	19.9	24.4
Sucrose, dextrose, dextrin[b]	71.2	46.4
Lard[c]	4.0	23.3
Corn oil (Mazola®)	1.0	1.2
Minerals (AIN-76®)	3.5	4.2
Vitamins[d]	0.4	0.5

[a]Diets formulated to give equivalent nutrient intake on a caloric basis and incorporated into 5% aqueous agar.

[b]Equal parts.

[c]Obtained from two different sources, analyzed for fatty acid, BHA and BHT content, estrogenic activity and organochlorine contaminants (see text).

[d]Content per kg control diet: vitamin A, 6600 IU; vitamin D_2, 3660 IU; vitamin E, 112 IU; menadione, 1.0 mg; thiamine HCl, 8.2 mg; riboflavin, 4.0 mg; niacin, 20.0 mg; calcium pantothenate, 20.0 mg; vitamin B_6, 8.0 mg; folic acid, 10.0 mg; inositol, 250.0 mg; choline Cl, 3100 mg; vitamin B_{12}, 50 μg.

TABLE 2

Fatty Acid Composition, BHA, BHT and Chlorinated Hydrocarbon Content of Lard Fed in Experiment 2 and in Earlier Experiments

Fatty acid	Wt% of fatty acid			
	Lard A[a], 1984	Lard B[b], 1984	Lard A, 1979	Lard A, 1982
14:0	1.4	1.4	1.4	1.4
16:0	23.8	23.8	23.6	24.2
16:1	2.9	3.3	2.7	2.6
18:0	14.0	13.0	13.6	14.5
18:1	43.3	43.5	41.1	40.4
18:2	10.8	11.4	10.3	9.9
18:3	1.6	1.4	0.6	0.7
BHA (ppm)	8.3	[c]	2.3	1.3
BHT (ppm)	10.2	[c]	3.6	6.3
Chlorinated hydrocarbons (ppb)	[d]	[d]	90	10

[a]Lard A was fed at 4% or 23.3% of the diet (Table 1).

[b]Lard B was fed at 4% or 23.3% of the diet (Table 1); rats were further subdivided into groups fed lard B with no detectable BHA or BHT, with 13 ppm each of BHA and BHT or with 105 ppm BHA and 101 ppm BHT.

[c]None detected by an assay that detected at least 5 ppm.

[d]None detected by an assay that detected at least 10 ppb.

0.25 mg sc on day 57. The feeding protocol was the same as in experiment 1 except that 1/2 of the rats fed control diet from weaning were changed to the high lard diet after DMBA administration, giving four final groups rather than three (see Table 3).

Experiment 3. Female Sprague-Dawley rats (40–50 g, 21 days old) (Charles River Laboratories, Wilmington, Massachusetts) were randomized to treatment groups of 25 rats each and fed high lard or control diet (Table 1); in this experiment the diets were further subdivided by the source of the lard and the amounts of BHA and BHT it contained (Table 2). All lards were food grade; lard A was purchased from the same source as in our earlier experiments and lard B was obtained from a second supplier without addition of BHA and BHT. It was fed as obtained or after addition of BHA and BHT in amounts to match approximately the content of lard A (13 ppm of each) or in the maximum amounts permitted (100 ppm of each). The amounts present were verified by assay; the higher amounts were actually 105 ppm BHA, 101 ppm BHT. Both lards were assayed for fatty acids and contaminants (Table 2). The 1979 and 1982 assays of lard A were performed by Hazelton-Raltech Inc. (Madison, Wisconsin). Lard B was obtained, prepared and analyzed by Mark Bieber and Robert Landers, Best Foods Research and Engineering Center (Union, New Jersey). They performed also the 1984 assay of lard A.

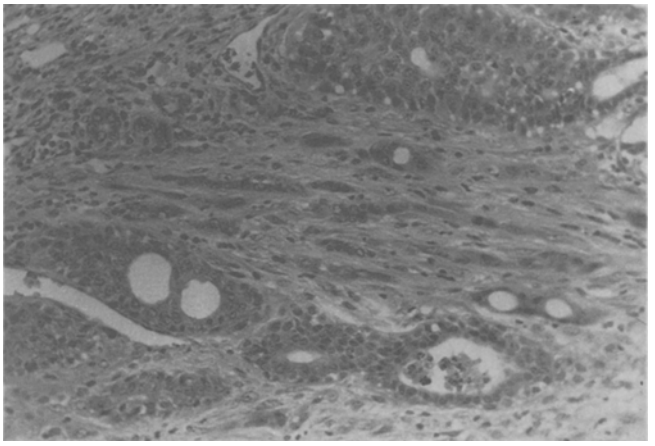

FIG. 1. Invasion of connective tissue surrounding a DMBA-induced mammary tumor by tumor cells growing in single cell cords. Experiment 3, rat fed high lard diet containing lard B. H&E, × 150.

Eight diets were fed: high lard and control using each of the two lards, A and B, and also using lard B with the lower or higher amounts of BHA and BHT added. Rats were fed and given DMBA according to the protocol in experiment 1, except that DMBA was given by gastric gavage in a dose of 2.5 mg per rat. In the groups fed lard A, or lard B with no antioxidant added, the rats were changed from high lard to control or control to high lard after administration of DMBA as in experiment 2.

In all experiments rats were housed individually in environmentally controlled quarters and given distilled water and diet ad libitum. They were weighed and palpated for tumors weekly. Tumors were measured in two diameters using vernier calipers. Rats were killed and necropsied at the times indicated; all mammary gland masses were measured, weighed and examined histologically.

Tumors were evaluated without knowledge of treatment group. They were diagnosed as fibroadenoma with or without epithelial hyperplasia and with or without atypia or as adenocarcinoma. The diagnosis of adenocarcinoma was reserved for tumors that showed stromal invasion and pleomorphism (Fig. 1) applying the criteria of

TABLE 3

Experiments 1 and 2: Mammary Tumors Induced by Parenteral DMBA

DMBA				Mammary tumor incidence (%)		Mammary tumors	
Route	Dose (mg)	Diet[a]	Latency[b] (wk)	All tumors	Adenocarcinoma	Wt[c] (g)	No.[b]
Subcutaneous	1[d]	C	6	84	32	2.4	2.1
		HL	8	95	35	1.8	2.0
		HL-C	5	85	55	1.9	2.8
Subcutaneous	0.5[e]	C	15	79	21	1.2	1.7
		HL	11	70	15	1.7	1.5
		HL-C	14	74	37	1.6	2.0
Subcutaneous	0.25[f]	C	11	80	21	7.7	1.6
		HL	9	95	30	6.6	1.5
		C-HL	10	90	55	5.6	1.2
		HL-C	9	80	30	4.5	1.3
Intravenous	2.5[g]	C	13	83	28	2.0	1.8
		HL	9	79	37	2.0	2.2
		HL-C	10	68	42	2.5	1.6

[a]C, control; HL, high lard; C-HL, control before and HL after DMBA; HL-C, high lard before and control after DMBA. Twenty rats per group.
[b]Average time to first tumor in rats that developed tumors.
[c]Per tumor bearing rat at necropsy, including palpable and nonpalpable, histologically benign and malignant tumors.
[d]Experiment 1; necropsied 9 to 19 wk after DMBA.
[e]Experiment 1; necropsied 9 to 25 wk after DMBA.
[f]Experiment 2; necropsied 13 to 17 wk after DMBA.
[g]Experiment 1; necropsied 7 to 19 wk after DMBA.

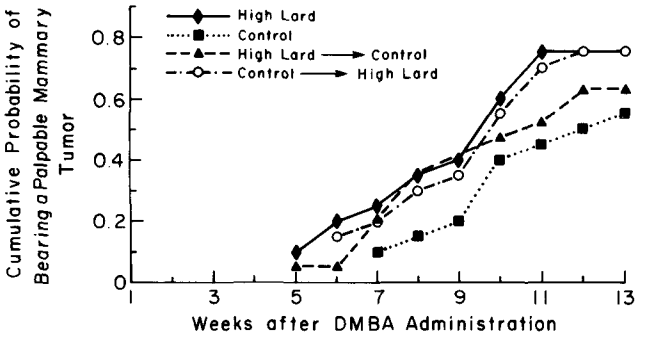

FIG. 2. Cumulative probability of bearing a mammary tumor in rats given DMBA, 0.25 mg by injection into the mammary gland and fed the experimental diets. For all three groups fed the high lard diet, the difference from the control group yields a p value of 0.08.

Greaves (15). Using the broader criteria of Young and Hallowes (16), the adenomas with hyperplasia and atypia would be included as carcinomas.

Statistical analysis of cumulative probability of bearing tumor was made using the survival statistics methods in the BMDP[79] program (17). Tumor weight and number were analyzed by ANOVA in the same program; tumor incidences were compared by chi-square analysis.

RESULTS

Subcutaneous administration of DMBA—experiments 1 and 2. Tumor development was rapid in rats given 1 mg DMBA sc; there was an average latency to the first palpable tumor of only 5–8 wk in the three diet groups. In rats given 0.5 mg sc average latency was 11–15 wk (Table 3). There was no consistent influence of the high lard diet on tumor latency, incidence or number. One or more tumors occurred in the treated gland and, occasionally, in an adjacent gland, particularly in rats given 1 mg; there were no tumors in distant glands. Rats were killed and necropsied when they bore a 2-cm tumor; there was no detectable effect of dietary lard on time to 2-cm tumor.

In experiment 2 in which rats were given the smallest dose of DMBA (0.25 mg), tumors appeared earlier in rats fed the high lard diet throughout the experiment than in rats fed the control diet. In the two groups fed the high lard diet only before or only after DMBA administration, time to detection of first tumor was intermediate between the two groups fed one of the diets throughout (Fig. 2). The results followed the pattern of previous studies using intragastric administration of DMBA, although the differences between groups in time to palpable tumor did not reach statistical significance; $p = 0.08$ for high lard vs control groups. Final tumor incidences, weights and histology are given in Table 3.

Intravenous administration of DMBA—experiments 1 and 2. After iv DMBA, tumors developed more rapidly initially in rats fed the high lard diet than in controls. At 9 wk the tumor incidences were 8% in controls and rats changed from high lard to control diet before DMBA and 46% in rats fed high lard diet before and after DMBA. Thereafter multiple tumors developed rapidly, and overall cumulative probability for bearing tumors and final incidences, numbers and weights of tumors (Table 3) were not significantly different in the three diet groups.

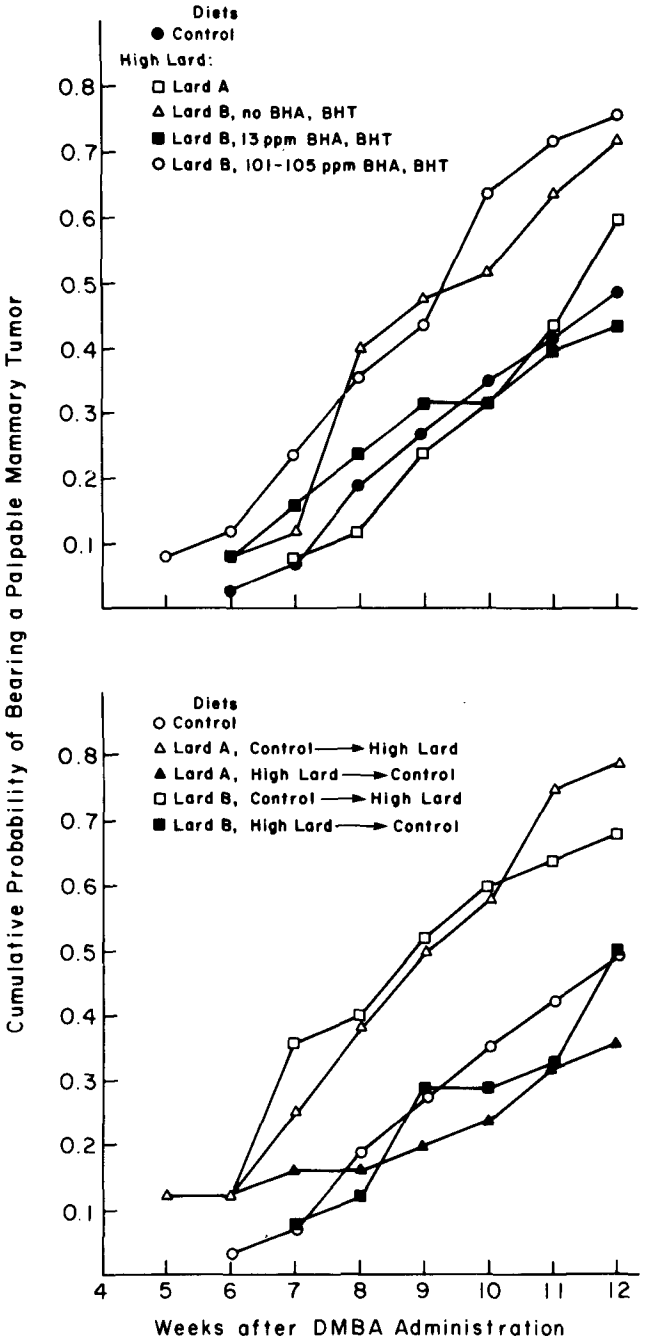

FIG. 3. Cumulative probability of bearing a mammary tumor in rats given DMBA, 2.5 mg by gavage, and fed the experimental diets throughout the experiment (top) or changed from one diet to another at the time of DMBA exposure (bottom). For statistical analysis of differences see Table 4.

Experiment 3. The cumulative probabilities of bearing a mammary gland tumor in rats fed the control diets (lard A, lard B, lard B + BHA and BHT at either level) were virtually identical and were therefore combined to give one control group. Groups of rats fed the high lard diets gave variable results. High lard diets fed throughout the experiment were associated with significantly earlier tumor appearance than control diet when the lard was lard B with no added BHA or BHT or with the higher level of both antioxidants; lard A did not enhance tumorigenesis when fed throughout (Fig. 3A). However, rats fed the high lard diet containing either lard A or lard B beginning after DMBA administration showed increased cumulative probability of bearing tumors compared to control rats; rats fed the high lard diets before DMBA and then changed to control diet were indistinguishable from the controls (Fig. 3B).

Final tumor incidences, numbers and weights showed a pattern of difference from control values similar to the pattern in development of the first palpable tumor (Table 4). Tumor histology was clearly malignant in a higher percentage of animals in the groups in which the high lard diets had an effect on the other parameters evaluated. Comparison of Table 4 and Figure 3 shows that tumor incidence and malignant histology correlated more strongly than tumor weight and number with the cumulative probability of tumor-bearing. Fibroadenomas occurred in only a small number of animals; tumors classified as fibroadenoma with hyperplasia with or without atypia occurred in incidences of 32-68% in all groups and showed no consistent relationship to dietary treatment.

Rats in all experiments grew well; the high lard diets supported slightly greater rates of weight gain in some but not all experiments. The differences in body weight, which ranged from 0-9% at DMBA administration and from 4-14% at termination, did not explain the differences in outcome of the experiments. We have shown previously in a pair-feeding experiment that enhancement of DMBA tumorigenesis by lard does not depend upon increased caloric intake and growth (11).

DISCUSSION

Interpretation of the results of the experiments reported, showing variable effects of a high lard diet on DMBA tumorigenesis, is not clear. The results are not consistent with results of our earlier experiments (6,11) and a recent report by Sylvester et al. (18) showing an effect of high lard diets on DMBA tumorigenesis at initiation as well as when fed throughout the period of tumor development.

The timing and magnitude of tumorigenesis enhancement by high lard diets may depend on major or minor constituents of the lard, but we could not demonstrate differences that appeared important. The fatty acid compositions of the four lards we have used (Table 2) are remarkably similar to each other and to a lard used in experiments reported by Carroll and Khor in 1971 (19). Since feed for pigs contains only low levels of fat and supplies calories largely as carbohydrate from plant sources, the fatty acid content of their adipose tissue is relatively constant and characteristic. There was a small difference in the content of linolenic acid (18:3) which accounted for 1.4-1.6% of the fatty acids in lards A and B compared to 0.6-0.7% in the batches of lard A used in our earlier experiments (6,11), in which lard consistently exerted its effect when fed throughout the experiment or only prior to initiation. Carroll's report of inhibition of tumorigenesis by fish oil in his recent experiments rather than the previously observed enhancement (2) suggests that mammary tumorigenesis may be highly susceptible to the balance of the different types of polyunsaturated fatty acids. The ratios of polyunsaturated or poly- plus

TABLE 4

Experiment 3: DMBA-Induced Mammary Tumors in Rats Fed Lard from Different Sources and with Different Content of BHA and BHT[a]

Diet[b]	Lard	BHA (ppm)	BHT (ppm)	Mammary tumor incidence (%) All tumors	Mammary tumor incidence (%) Adenocarcinoma[d]	Mammary tumors Wt (g)[c]	Mammary tumors No.[c]
C	A,B	0-100	0-100	78	28	5.9	3.7
HL	A			76	24	6.6	7.1
C-HL[e]	A			83	46	9.2	5.7
HL-C	A			68	28	4.7	3.4
HL[e]	B	<5	<5	92	52	8.7	5.3
C-HL[e]	B	<5	<5	92	40	7.9	6.3
HL-C	B	<5	<5	71	25	7.2	4.1
HL	B	13	13	76	28	5.0	4.2
HL[e]	B	105	101	96	60	9.5	4.7

[a]Ninety-nine rats fed control diets; 24-25 rats per high lard group. All rats given 2.5 mg DMBA by gastric gavage at age 55 days and necropsied 10-12 wk later.

[b]C, control; HL, high lard; C-HL, control before and HL after DMBA; HL-C, high lard before and control after DMBA. Twenty rats per group.

[c]Average time to first tumor in rats that developed tumors.

[d]Tumors that showed histologic evidence of invasion.

[e]Significantly different from control in cumulative probability of bearing a tumor ($p < 0.05$ using survival distribution as programmed in BMDP, ref. 17) (Fig. 3).

TABLE 5

Experiment 3: Summary of DMBA-Induced Tumors with Different Lard Groups Combined[a]

Diet	Mammary tumors Incidence (%)	No.	Wt (g)	Adenocarcinoma (%)
C	78	3.7	5.9	28
HL	85	5.3	7.6	41
C-HL	88	6.0	8.5	43
HL-C	69	3.7	6.0	26

[a]See Table 4 for detailed data and footnotes. The data have been combined for all four groups fed high lard diet throughout or two groups changed from high to control or control to high.

monounsaturated to total fatty acids were slightly lower in the A lard assayed in 1979 and 1982 than in the two lards assayed in 1984, but the differences appear unimportant.

A second possibility we examined in some detail in experiment 3 is that lard's content of BHA and BHT might influence DMBA tumorigenesis. Antioxidants are added in relatively small amounts to lard since peroxidation is not a significant problem, and the amounts we measured were uniformly low. In rats fed throughout the experiment lard B containing no BHA or BHT or approximately 100 ppm of each, there was equivalent enhancement of tumorigenesis, but lard B containing 13 ppm of each of the two antioxidants gave no enhancement of tumorigenesis. In rats fed the diets high in lard A, which contained 8 and 10 ppm, respectively, of the two antioxidants, or lard B with no added antioxidant beginning after DMBA exposure, there was significant enhancement of tumorigenesis; however, lard A showed no effect when fed throughout the experiment.

A third possible contributor to the variation in the effect of lard is the declining content of chlorinated hydrocarbon insecticides in later samples; however, assay of the lard fed by Sylvester et al. (18) has shown no detectable insecticide contamination. None of the lard samples showed evidence of estrogenic activity in a bioassay (Bieber, Mark, unpublished observations).

The control groups for each lard sample gave data similar in all respects and were combined. If the data for the four high lard groups were combined as well as the data for the two groups each switched from control to high lard or from high lard to control, then the results were as shown in Table 5. The cumulative probability for the first palpable tumor was significantly greater than control in the combined groups fed the high lard diet both before and after DMBA exposure ($p = 0.05$) and the groups fed the high lard diet only after exposure ($p = 0.0005$). The feeding of the high lard diet before exposure only had no effect.

The only conclusion which can be made from these experiments is that, overall, the high lard diet enhanced post exposure events, regardless of the content of BHA and BHT within the range tested of <5-100 ppm of each, but there was variability between groups in expression of the enhancement. Factors that influence expression of the enhancing effect are not known. Assay of fatty acid composition, chlorinated hydrocarbon and other con-

taminants and estrogenic activity revealed no explanation for the variation in results.

We had hoped that parenteral rather than intragastric DMBA administration would yield more consistent data by eliminating variations resulting from gastrointestinal absorption and hepatic metabolism. Our results using injection of 0.25 mg DMBA into the mammary gland and fat pad were similar to our earlier results, although the dietary effects were not statistically significant at the group size of 20 rats. At higher parenteral doses, tumor development was rapid and no significant effect of the high lard diet was observed. We had hoped also that the parenteral experiments would clarify the timing of the lard effect. The results of experiment 2 suggest an influence both before and after DMBA exposure, but do not give a definitive answer. Experiment 1, designed to look only for an influence at initiation, gave no evidence of an effect at that time. Resolution of the questions raised may be possible by using sc administration directly into the gland and fat pad of DMBA at a low dose in the range of 0.25 mg and by studying earlier as well as later time periods in larger groups of rats.

Second, a more detailed histological analysis of tumors than customarily made may be useful. Dietary effects on tumor histology have not been emphasized previously. We and others have generally followed the criteria illustrated by Young and Hallowes (16) derived from studies in several models. The lesions they illustrate, and many of the tumors found in the DMBA-treated rat at the doses we have used, appear to be intraductal papillomas or fibroadenomas with varying degrees of epithelial hyperplasia and atypia but without clear histological evidence of malignancy. Histologically malignant tumors, showing clear stromal or muscle invasion and malignant nuclear features, occur in lower incidence. Investigations of the appearance, development and growth rate of the more clearly malignant tumors in relationship to dietary treatment are in progress in our laboratory.

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Effects of High Levels of Dietary Fats on the Growth of Azaserine-Induced Foci in the Rat Pancreas[1]

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Azaserine induced two phenotypically different populations of foci, namely, acidophilic and basophilic foci. The effects of dietary modification during the post-initiation phase of carcinogenesis were examined. A diet of 20% (w/w) unsaturated fat (unsat) compared to a 20% saturated fat (sat) diet or a control diet (5% unsaturated fat) increased the number of acidophilic foci, as well as the thymidine labeling index (LI) of their nuclei. While the basophilic foci are carcinogen-induced and at 2.5 mo post-initiation have a similarly high growth rate to the acidophilic foci, this rate is not sustained as indicated by examination of both the LI and mean size of foci at 4 mo post-initiation.
Lipids 21, 281–284 (1986).

In both Canada (3) and the United States (4), the incidence of pancreatic cancer ranks approximately eighth or ninth compared to the incidence of other cancers. However, pancreatic cancer is the fifth most common cause of death due to cancer; it is usually diagnosed late, thus precluding effective treatment, and with the exception of an association with the smoking of cigarettes, the etiology of this cancer is largely unknown (5). Using international data of dietary fat intake and cancer mortality, a positive correlation of pancreatic cancer mortality with per capita fat consumption has been shown (6,7). Studies of pancreatic cancer with experimental animal models have generally corroborated these findings. Treatment with the known pancreatic carcinogen azaserine and the concurrent feeding of a 20% unsaturated fat (unsat) but not a 20% saturated fat (sat) diet enhanced the incidence and multiplicity of pancreatic cancer in the rat as compared to the group fed a control AIN-76A purified diet for rat and mouse (1) (AIN) containing 5% unsaturated fat (8). Subsequent studies have shown that dietary unsaturated fat acts during the post-initiation phase of carcinogenesis (9,10). In this regard, there appears to be a parallelism between the rodent models for mammary and pancreatic carcinogenesis (11,12).

For the study of pancreatic carcinogenesis, rodent models using the rat (13) and the hamster (14) have been developed and characterized. Our laboratory has recently developed a short-term, quantitative version of the rat/azaserine model which depends on the identification and measurement of azaserine-induced foci or nodules of atypical acinar cells (15–18). Azaserine induces two phenotypically different populations of foci, namely, acidophilic and basophilic foci (16). The acidophilic population appears to have considerable growth potential, whereas the basophilic foci apparently have limited growth potential (16,19). Dietary unsaturated fat but not saturated fat diets increased the growth of the acidophilic foci but not the basophilic foci (16). In this short-term model, the feeding during the post-initiation phase of the unsat diet compared to the sat diet resulted in an enhanced number of acidophilic foci (16). There was not, however, a statistically significant effect on the mean focal diameter of the acidophilic foci, though they were larger in the pancreases of the rats fed the unsat diet. Neither the number nor the size of the basophilic foci were different for the two diets. To date, we do not know if the effects of the high levels of unsaturated fats selectively stimulate the carcinogen-induced foci or if there is a more general stimulus to the entire acinar cell component of the pancreas. In the present study, the growth of the pancreas and carcinogen-induced foci were assessed by quantitative microscopy and ^{3}H-thymidine LIs of the focal and nonfocal portions of the acinar pancreas.

METHODS

Animals. Suckling male Lewis rats (Charles River Breeding Laboratories Inc., Wilmington, Massachusetts) were each injected intraperitoneally (i.p.) with a single dose of 30 mg azaserine per kg body weight at 14 days of age to initiate pancreatic carcinogenesis. Azaserine (Calbiochem-Behring Corp., La Jolla, California) was dissolved in 0.9% NaCl solution. The dams were fed ad libitum a purified control diet, AIN (1,20), but without the antioxidant ethoxyquin. The pups were weaned at 21 days of age to the experimental groups. During this post-initiation phase of carcinogenesis, the rats were housed individually in wire mesh-bottom cages. They were weighed at least once per week, and diet and deionized water were available ad libitum. Groups of rats were autopsied at 2.5 and 4 mo after weaning.

Diets. One of three purified diets was fed during the post-initiation phase. Details concerning the composition of these diets have been published elsewhere (8,9) and are included in Table 1. One group of rats was maintained on the AIN diet which contained a total of 5% unsaturated fat (corn oil). In the experimental groups the fat content was increased to 20% total lipid by substituting on a weight basis fat for the carbohydrate in the AIN diet. The unsat diet used 20% corn oil and the sat used 18% hydrogenated coconut oil with 2% corn oil to supply essential fatty acids. A previous investigation has shown clearly that the high fat diets—whether formulated as were these diets by substitution or by isonutrient modification (increasing the lipid content at the expense of the carbohydrate content but also increasing the protein, fiber, vitamin and mineral contents so as to maintain the same ratio of lipid to nutrient) of the basic AIN-76A diet—are comparable in their effects on rat growth and on the development of azaserine-induced, pancreatic lesions (21).

DNA synthesis. For the DNA synthesis experiments, rats were fed either AIN or unsat diet beginning at seven wk of age. At day 7, 14 and 30 following introduction to the diets, five or six rats per dietary group were evaluated for incorporation of ^{3}H-thymidine into pancreatic DNA. At six, four and two hr prior to kill, all rats were injected i.p. with ^{3}H-methyl-thymidine (sp act 1.0 m Ci/0.0029 mg;

[1]A preliminary report has been presented as an abstract (2).

TABLE 1

Composition of Diets Expressed as Percentage by Weight[a]

Ingredients	AIN-76A (AIN)	20% Saturated fat (sat)	20% Unsaturated fat (unsat)
Casein	20.0	20.0	20.0
Methionine	0.3	0.3	0.3
Cornstarch	15.0	11.7	11.7
Sucrose	50.0	38.3	38.3
Cellulose	5.0	5.0	5.0
Unsaturated fat[b]	5.0	2.0	20.0
Saturated fat[c]		18.0	
Micronutrients[d]	4.7	4.7	4.7

[a]All diets were stored at 4 C prior to feeding. The diets were fed ad libitum as powders.

[b]Corn oil was the unsaturated fat used in these experiments.

[c]The saturated fat was 18% hydrogenated coconut oil with 2.0% corn oil added to provide for essential fatty acid requirements of the rats.

[d]The compositions of the vitamin and mineral mixtures are those recommended for the AIN diet (1).

TABLE 2

Body and Pancreatic Weights for Azaserine-Initiated Rats[a]

Diet	Final body weight (g)		Pancreas weight (g)	
	2.5 Mo	4 Mo	2.5 Mo	4 Mo
AIN	367 ± 19	475 ± 23	0.99 ± 0.05	1.07 ± 0.02
Sat	418 ± 27	540 ± 16	1.06 ± 0.07	1.23 ± 0.05
Unsat	386 ± 7	534 ± 19	1.00 ± 0.01	1.10 ± 0.09

[a]Values expressed as mean ± SE; three rats per group at 2.5 mo, four rats per group at 4 mo.

New England Nuclear Corp., Boston, Massachusetts) for a total dose of 60 μCi per 100 g body weight. Pancreatic nuclei were isolated and DNA was extracted according to well-described procedures (22). Radioactivity was quantitated using Biofluor (New England Nuclear) and scintillation spectroscopy (Beckman Instruments, Wakefield, Massachusetts). The quantity of DNA was determined by a fluorometric procedure (23).

Quantitative light microscopy. Azaserine-induced lesions of atypical acinar cells (henceforth called foci) were identified and classified as acidophilic or basophilic in general accord with the criteria of Rao et al. (19) and Roebuck et al. (16). Only transections of foci with 10 or more nuclei were evaluated. This corresponds to a minimum diameter of ca. 0.1 mm. The areas of the focal transections were measured with a camera lucida and an X,Y-digitizer (GTCO Corp., Rockville, Maryland). The areas of the tissue sections were measured with the Video Van Gogh hardware and programs (Tekmar Inc., Cleveland, Ohio) and a video camera (Dage-MTI Inc., Michigan City, Indiana). The entire system was operated by, and data processed with, an IBM/XT (IBM Corp., Boca Raton, Florida). From the observed number and area of the focal transections for each rat pancreas, the mean number and mean size of the foci were determined by the quantitative stereologic methods of Pugh et al. (24). Details of the application to pancreatic foci are published (16).

Nuclear labeling index. Into the same rats as used for quantitative, microscopic measurements of pancreatic foci, ^{3}H-thymidine was injected i.p., three injections per day at two hr intervals starting at 1000 hr for two successive days. This represented a total of six injections and a total of 2 μCi for the rats autopsied at 2.5 mo and 1 μCi for the rats autopsied at 4 mo post-initiation. This allowed for extensive labeling of acinar cells. Autoradiographs were prepared by dipping the deparaffinized slides into Kodak NTB-3 emulsion (Kodak Co., Rochester, New York). The slides were exposed for four wk, processed by standard techniques and stained with hematoxylin and eosin. The LI is defined as the percentage of labeled nuclei (5 grains or more). Approximately 5000 nuclei of either nonfocal or acidophilic foci were counted per rat. Because of their small size and low LI, all nuclei of basophilic foci were counted; usually there were 1000–2000 nuclei per tissue section.

RESULTS AND DISCUSSION

As expected from considerable experience with this model (8,9,25), the rats fed the sat and unsat fat diets gained more weight than did the rats fed the AIN control diet (Table 2). Over the 2.5–4 mo period, growth of the pancreas was as expected. There was no statistically significant difference ($p > 0.05$, ANOV) in the pancreatic weights between the three diet groups at either 2.5 or 4 mo post-initiation. The lack of difference in weights of pancreases from rats fed these three diets implies that fat per se or more specifically unsaturated fat does not stimulate a permanent hypertrophy or hyperplasia in the organ. This observation is also supported by data of a study with larger numbers of rats (25). In that study, rats not treated with a carcinogen but fed for one yr either the AIN or the unsat diet had pancreases that did not differ significantly in weight.

A direct measure of the effects of the unsat diet compared to the AIN control diet was made by measuring in vivo, pancreatic DNA synthesis. ^{3}H-Thymidine incorporation into pancreatic DNA expressed as dpm/μg DNA (mean ± SE) in rats fed either AIN or unsat diets for seven days was 20.9 ± 1.4 and 18.2 ± 0.9; for 14 days it was 13.8 ± 1.6 and 19.1 ± 2.2; and for 30 days it was 9.8 ± 0.9 and 14.6 ± 2.1, respectively. Statistical significance at a specific time point (t-test) or for a trend (linear regression) could not be shown. Dietary exposure for longer times might be necessary. However, these results support the gross observations of pancreas weight, that is, a diet high in unsaturated fat does not stimulate pancreatic growth as compared to either the control diet (AIN) or the sat diet. Additionally, we have seen no histologic evidence for any alterations by these diets in the proportions of the specific cell types within the non-carcinogen-treated pancreas.

As expected from previous studies (16–18), two phenotypically different populations of foci were observed in the pancreases of these azaserine-initiated rats at both post-initiation times (Table 3). These are the so-called acidophilic and basophilic foci. Based on results of

TABLE 3

Effect of Diets Fed During the Post-initiation Phase on Pancreatic Foci Induced by Azaserine[a]

Time post-initiation (mo)	Diet	Observed transectional data of foci		Calculated volumetric data of foci		
		No. per cm^2	Mean area ($mm^2 \times 100$)	No. per cm^3	Mean diameter (μm)	Volume as % of pancreas
Acidophilic foci						
2.5	AIN	8.9 ± 2.8	7.1 ± 0.5	272 ± 85	327 ± 7	0.661 ± 0.231
	Sat	5.9 ± 2.1	6.2 ± 0.5	177 ± 74	355 ± 32	0.340 ± 0.091
	Unsat	6.1 ± 1.4	11.7 ± 0.1	150 ± 31	407 ± 22	0.715 ± 0.162
4	AIN	13.5 ± 2.0	15.8 ± 1.5	288 ± 50	477 ± 15	2.122 ± 0.362
	Sat	8.9 ± 1.9	12.2 ± 1.9	234 ± 59	403 ± 56	1.165 ± 0.381
	Unsat	20.4 ± 1.0	22.4 ± 4.3	369 ± 37	561 ± 34	4.544 ± 0.892
Basophilic foci						
2.5	AIN	3.2 ± 1.0	2.3 ± 0.4	181 ± 65	187 ± 16	0.073 ± 0.024
	Sat	2.7 ± 0.2	2.7 ± 0.9	160 ± 52	201 ± 48	0.072 ± 0.021
	Unsat	1.6 ± 0.4	1.8 ± 0.1	102 ± 13	155 ± 15	0.029 ± 0.005
4	AIN	5.1 ± 0.5	3.2 ± 0.5	279 ± 22	184 ± 11	0.170 ± 0.036
	Sat	4.1 ± 0.6	2.2 ± 0.2	253 ± 29	162 ± 8	0.094 ± 0.024
	Unsat	4.1 ± 0.6	3.2 ± 0.4	196 ± 36	214 ± 11	0.135 ± 0.028

[a]Values are mean ± SE; three rats per group at 2.5 mo, four rats per group at 4 mo.

quantitative stereology, the calculated volumetric data of the foci from these smaller groups of rats closely agree with previous experiments (16,17). Therefore only the salient features will be briefly summarized below as the thrust of this investigation was on the LIs of focal and nonfocal acinar tissue.

At both post-initiation times examined, in the AIN and sat groups the total number of pancreatic foci based upon the calculated volumetric data was approximately equally divided between the acidophilic and basophilic phenotypes (Table 3). In the unsat group, there were equal numbers of the two types of foci at 2.5 mo, but by 4 mo the acidophilic foci were present in twice the number as the basophilic foci. Over the 2.5 to 4 mo period, the acidophilic foci increased in number significantly ($p < 0.01$, t-test) in the unsat group, but not in the other two. With respect to the size of the foci, the acidophilic foci were generally 2-3 times larger in diameter than the basophilic foci.

For all three diet groups, the basophilic foci showed no consistent increase in size from 2.5 to 4 mo. In contrast, the acidophilic foci of all three groups were ca. 30% larger at 4 mo than at 2.5 mo post-initiation. The volume of pancreas occupied by foci is an expression of "tumor" burden. From Table 3, it is clear that the acidophilic foci contribute most to the tumor burden. This is due to both their greater number as well as size. The enhancement of focal growth by the intake of a diet high in unsaturated fat is dramatically illustrated by comparing the pancreatic tumor burden for the acidophilic foci in the unsat group to that in the AIN and sat groups. From 2.5 to 4 mo, the tumor burden (focal volume as percentage of pancreas) for acidophilic foci of the AIN and sat groups increased threefold; however, in the unsat group the increase was sixfold. Over the same time span, the basophilic population of foci grew as indicated by an increase in the tumor burden. However, the observed number of foci was so small for this population that the accurate assessment of the effect of diet on their growth was not possible.

An evaluation of the LI provides a direct measure of hyperplastic growth of the acinar cell tissue and, thus, the potential influence of diet on the growth of the foci. The LIs for nonfocal pancreatic acinar tissue and the azaserine-induced foci (described above and in Table 3) at both 2.5 and 4 mo post-initiation are presented in Table 4. Direct comparisons between the LIs measured at 2.5 and 4 mo post-initiation cannot be made, as different quantities of ^{3}H-thymidine were used at the two times. In no case did the LI of nonfocal acinar cells of the rats treated with azaserine differ significantly (paired t-test) from the LI of acinar cells from rats fed similar diets but not receiving azaserine. Thus, azaserine per se does not affect DNA synthesis of the nontransformed acinar cells. This is not surprising since considerable time had elapsed since initiation (15).

The effects of high intakes of fats and unsaturated fat in particular on pancreatic DNA synthesis (LI) were evaluated. For the basophilic population of foci, there was not a statistically significant difference ($p > 0.05$, ANOV) in LIs between the three diets at either 2.5 or 4 mo post-initiation. The LIs of the acidophilic population of pancreatic foci were similar among all three diet groups at 2.5 mo, but at 4 mo post-initiation the LI of the acidophilic foci in the unsat group was significantly larger ($p < 0.05$, ANOV) than the indices of either the AIN or the sat groups. This more rapid growth apparently accounts at least in part for the larger acidophilic foci seen in the pancreases of rats fed the unsat diet. Interestingly, at 2.5 mo the basophilic foci have LIs similar to the acidophilic foci, but by 4 mo post-initiation, the acidophilic foci have at least three times the labeling as the basophilic foci. In rats initiated with 4-hydroxyquinoline-1-oxide and evaluated one yr later, Rao et al. (19) showed that acidophilic foci had a 20-fold higher LI than the basophilic foci. Thus, it appears that the basophilic foci

TABLE 4

[3]H-Thymidine Labeling Index (LI) for Nonfocal Pancreatic Acinar Tissue and Azaserine-Induced Foci at 2.5 and 4 Mo Post-initiation[a,b,c]

Diet	Saline-treated control	Azaserine-initiated		
	Acinar tissue	Nonfocal acinar tissue	Basophilic foci	Acidophilic foci
2.5 Mo post-initiation				
AIN	0.80 ± 0.31	1.08 ± 0.01	4.35 ± 4.16	10.39 ± 3.18
Sat	0.92 ± 0.05	0.95 ± 0.19	5.84 ± 4.73	9.01 ± 1.61
Unsat	0.93 ± 0.35	1.19 ± 0.23	10.45 ± 3.83	10.75 ± 1.07
4 Mo post-initiation				
AIN	0.13 ± 0.02	0.19 ± 0.06	1.41 ± 0.71	3.86 ± 0.66
Sat	0.09 ± 0.02	0.12 ± 0.03	0.99 ± 0.64	3.41 ± 0.93
Unsat	0.09 ± 0.03	0.14 ± 0.05	0.94 ± 0.30	5.68 ± 0.27

[a]LI is expressed as percentage of total nuclei (mean ± SD).

[b]At 2.5 mo there were two rats and at 4 mo three rats per group (except for saline groups with two rats each).

[c]At 2.5 mo, rats received [3]H-thymidine at 2 μCi/g and at 4 mo 1 μCi/g.

may have a high rate of cellular growth initially and then their growth tapers off, while the acidophilic foci continue to grow.

In conclusion, the unsat diet, but not the sat or AIN diets, has been associated with the enhancement of experimental pancreatic carcinogenesis. Evidence from these studies indicates that the unsat diet does not stimulate a general, nonspecific growth of the rat pancreas. Quantitative stereologic measurements of azaserine-induced pancreatic foci confirm the previous observations of the general lack of growth potential by the basophilic foci as compared to the acidophilic foci. The basophilic foci show a high LI at 2.5 mo post-initiation, but by 4 mo their growth is considerably less than the acidophilic foci. There appeared to be no specific effect of diet on the basophilic population of foci. The rate of growth as measured by vol % of the basophilic foci from 2.5 to 4 mo was quite similar to the rate of growth of the acidophilic foci irrespective of the diet fed. The acidophilic foci had a higher LI than the basophilic foci four mo after initiation. The unsat diet compared to either sat or AIN diet increased the number of acidophilic foci observed at 4 mo post-initiation. Acidophilic pancreatic foci of rats fed the unsat diet had a higher LI than did similar foci of the other two diet groups. The mechanism(s) of enhancement of the acidophilic foci by unsat diet is unknown, though it appears to be a relatively specific growth stimulus to only the acidophilic foci.

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Dietary Polyunsaturated Fat in Relation to Mammary Carcinogenesis in Rats

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High fat diets promote the development of mammary tumors induced in rats by 7,12-dimethylbenz(a)anthracene (DMBA), and polyunsaturated fats are more effective than saturated fats. This difference is related to the linoleic acid content of polyunsaturated vegetable oils, but the amount of linoleate required for maximum tumor promotion appears to be higher than indicated by earlier experiments. Comparison of the effects of a polyunsaturated vegetable oil (corn oil) containing linoleate with a fish oil (menhaden oil) containing polyunsaturated fatty acids derived from linolenic acid showed that higher dietary levels of corn oil increased the yield of DMBA-induced mammary tumors, while corresponding levels of menhaden oil had an inhibitory effect. This is further evidence that promotion of mammary tumorigenesis by polyunsaturated vegetable oils may be mediated by prostaglandins or other biologically active eicosanoids derived from n-6 fatty acids.
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Earlier experiments in our laboratory showed that rats treated with DMBA and fed diets containing high levels of polyunsaturated fats develop more mammary tumors than rats exposed to the same dose of carcinogen and fed low fat diets or diets containing high levels of saturated fats (1,2). In further studies, it was found that diets containing a high level of saturated fat plus a small amount of polyunsaturated fat increased the tumor yield to about the same extent as diets high in polyunsaturated fat (3). It appeared that the polyunsaturated fat was required as a source of essential fatty acids because the tumor yield was also enhanced by adding ethyl linoleate, but not ethyl oleate, to a high saturated fat diet (4).

In the above experiments, diets containing a mixture of 3% sunflowerseed oil with either 17% coconut oil or 17% beef tallow gave much higher tumor yields than a diet containing 3% sunflowerseed oil alone (3). That the saturated fats themselves contain very little linoleic acid suggested that they were modifying the distribution or metabolism of the linoleic acid in the sunflowerseed oil in some way which made it more effective in promoting tumorigenesis. Before investigating this possibility further, however, it was decided to do another feeding experiment with mixtures of sunflowerseed oil and coconut oil to compare the effects on tumor yield with those observed previously.

In earlier studies, it was also observed that a group of rats treated with DMBA and fed a diet containing 3% fish oil (menhaden oil) and 17% coconut oil developed nearly as many tumors as the group fed 3% ethyl linoleate and 17% coconut oil (4). Menhaden oil contains substantial amounts of polyunsaturated fatty acids derived from linolenic acid (n-3) but very little linoleic acid (n-6). The fact that it gave a marked increase in tumor yield therefore suggested that the requirement for polyunsaturated fat could be satisfied by fatty acids of either the n-3 or n-6 families.

Experiments of Abraham and associates (5,6) have shown that the growth-promoting effect of polyunsaturated fat on transplantable mammary tumors in mice can be inhibited by 5,8,11,14-eicosatetraynoic acid, an acetylenic analogue of arachidonic acid, and by indomethacin, an inhibitor of prostaglandin synthesis. More recently, indomethacin has also been found to block the stimulatory effect of dietary fat on mammary tumors induced by DMBA (7) and on a transplantable mammary tumor in rats (8). These results suggest that the promoting effects of dietary polyunsaturated fat on mammary tumors are mediated by prostaglandins or other biologically active compounds derived from polyunsaturated fatty acids.

The above observations were inconsistent with our finding that fatty acids of both the n-3 and n-6 families were effective in promoting mammary tumorigenesis. Prostaglandins derived from n-3 fatty acids differ in structure and properties from those derived from n-6 fatty acids. Furthermore, eicosapentaenoic acid [20:5(n-3)] is a poor substrate for the enzyme, cyclooxygenase, which catalyzes the first step in formation of prostaglandins and related compounds but can compete with arachidonic acid [20:4(n-6)] for this enzyme and thus affect the production of such compounds from arachidonic acid (9).

In view of the apparent dichotomy between our results and those reported from other laboratories, another experiment was designed to compare the effects on DMBA-induced mammary carcinogenesis in rats of feeding diets containing 3, 10 or 20% of either corn oil or menhaden oil. Corn oil was chosen for this experiment because it is less polyunsaturated than sunflowerseed oil and thus more comparable to menhaden oil in degree of unsaturation.

MATERIALS AND METHODS

Female Sprague-Dawley rats, 40 days old, were purchased from Charles River Canada (St. Constant, Quebec, Canada). They were housed in galvanized iron, suspended cages with wire mesh bottoms in a temperature-controlled room with a 12 hr light-dark cycle and were fed standard rat chow (Ralston Purina Co., St. Louis, Missouri). At 50 days of age they each were given a single intragastric 5 mg dose of DMBA (Eastman Kodak, Rochester, New York) in 0.25 ml of olive oil. One week later they were divided randomly into groups of 20 rats each and fed semipurified diets as described for the individual experiments. The rats were weighed weekly and palpated for the presence of tumors, and the size and location of each tumor was noted. At the end of the experiment, the rats were killed and the total number of tumors recorded.

The composition of the semipurified diets is given in Table 1, and the fatty acid composition of the dietary fats and oils is given in Table 2. The coconut oil was supplied by Canada Packers Ltd. (Toronto, Ontario, Canada) and

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TABLE 1

Composition of Semipurified Diets[a]

	3% Fat	10% Fat	20% Fat
Casein	190	210	230
Dextrose	705	612.5	490
Fat	30	100	200
Salt mixture	25	27.5	30
Celluflour	50	50	50

[a]Expressed as g/kg of diet.

TABLE 2

Fatty Acid Composition of Dietary Fats

	Wt% of total fatty acids			
Fatty acid	Coconut oil[a]	Sunflowerseed oil[a]	Corn oil[a]	Menhaden oil[b]
8:0	5.9	—	—	—
10:0	5.5	—	—	—
12:0	49.6	—	—	—
14:0	19.1	0.9	—	7.4
16:0	8.6	7.0	10.1	13.9
16:1	—	0.1	—	13.1
16:2	—	—	—	2.6
16:3	—	—	—	1.5
16:4	—	—	—	2.3
18:0	2.9	4.3	1.6	2.7
18:1	5.8	12.9	31.4	11.6
18:2	2.4	73.2	56.3	2.0
18:3	—	0.2	0.4	2.1
18:4	—	—	—	4.8
20:0	—	0.2	—	—
20:1	—	—	—	1.5
20:4	—	—	—	1.3
20:5	—	—	—	17.2
22:0	—	0.6	—	—
22:5	—	—	—	2.2
22:6	—	—	—	9.0

[a]Methyl esters of the oils were analyzed as described previously (10).
[b]Fatty acid analysis provided by Zapata Haynie Corp. (Reedville, Virginia).

the menhaden oil by the Zapata Haynie Corp. (Reedville, Virginia). The corn oil and sunflowerseed oil were purchased locally. The sources of other dietary ingredients and the composition of the vitamin mixture used for the first dietary experiment were as reported previously (3). For the second experiment, 22 g of Vitamin Diet Fortification Mixture (ICN, Life Sciences Group, Nutritional Biochemical Division, Cleveland, Ohio) was added at the expense of dextrose.

RESULTS

Effect of mixtures of polyunsaturated vegetable oil and saturated fats on mammary tumorigenesis. For this experiment, 100 rats were treated with DMBA and subsequently divided into five groups, each consisting of 20 rats housed in pairs. The groups were fed semipurified diets containing 3, 10 or 20% sunflowerseed oil, or mixtures of 3% sunflowerseed oil with either 7% or 17% coconut oil.

The final body weights were similar in all of the dietary groups, although the average of the low fat group was slightly lower than those of the high fat groups (Table 3). At autopsy, the groups fed 10% or 20% sunflowerseed oil had substantially more tumors than rats from the other dietary groups, and the groups fed 3% sunflowerseed oil mixed with coconut oil had only about the same number of tumors as those fed 3% sunflowerseed oil alone (Table 3).

Comparison of effects of polyunsaturated vegetable oil and fish oil on mammary tumorigenesis. In this experiment, 120 rats were treated with DMBA and one week later were divided into six dietary groups of 20 rats each, housed in groups of four. These groups were fed semipurified diets containing 3, 10 or 20% of either corn oil or menhaden oil.

The fish oil used for this experiment is very susceptible to lipoperoxidation because of the highly unsaturated fatty acids present and the lack of natural antioxidants such as tocopherols. A preliminary experiment showed, however, that diets containing this oil were readily accepted by rats provided the food cups were cleaned and fresh diet was provided daily. When this was done, the rats fed the diets containing menhaden oil weighed about the same as those fed the diet containing 3% corn oil, but rats fed diets containing higher levels of corn oil were somewhat heavier (Table 4).

The diets containing 3% corn oil or menhaden oil had somewhat similar effects on mammary carcinogenesis, but the results of feeding higher levels of the oils were quite different (Table 4). As expected, the tumor yield increased when higher levels of corn oil were fed, but higher levels of menhaden oil seemed to inhibit carcinogenesis. The latent period was also longer in rats fed menhaden oil compared to those fed corn oil (Table 4).

For this experiment, the tumors obtained at autopsy were examined histologically. Nearly all of the tumors found in the groups fed corn oil were adenocarcinomas but some of the suspected tumors detected by palpation in the animals fed fish oil were identified pathologically as cystic hyperplasia or fibrosis of mammary tissue.

DISCUSSION

In the present study, a diet containing 3% sunflowerseed oil and 17% coconut oil failed to increase the number of DMBA-induced mammary tumors over that obtained by feeding 3% sunflowerseed oil alone. This result differs from those of earlier experiments in our laboratory (3,4) but does not invalidate the idea that promotion of mammary tumorigenesis by dietary fat depends on the content of polyunsaturated fatty acids as well as the level of dietary fat.

Experiments by Tinsley et al. (11) and by Chan et al. (12) have shown that mammary tumorigenesis in mice and rats is positively correlated with the linoleic content of dietary fat. McCay et al. (13) reported that a diet containing 2% linoleic acid and 18% tallow gave a tumor yield intermediate between those obtained with a low fat diet and diet high in polyunsaturated fat. In a recent study, Ip et al. (14) found that tumor incidence and yield were correlated with the essential fatty acid content of the diet

TABLE 3

Mammary Tumors in DMBA-Treated Rats Fed Diets Differing in Amount and Type of Fat

Dietary group	Linoleic acid content of diet (% wt)	Final body weight[a] (g)	No. of rats with tumors	No. of tumors/ tumor-bearing rat[a]	Cumulative no. of mammary tumors[b]	Latent period[a,c]
20% Sunflowerseed oil	14.6	313 ± 8.8	18	6.1 ± 1.04	110	63 ± 4
10% Sunflowerseed oil	7.3	309 ± 11.2	16	7.4 ± 1.25	124	67 ± 6
3% Sunflowerseed oil	2.2	282 ± 5.1	14	4.4 ± 1.4	58	76 ± 7
3% Sunflowerseed oil + 7% coconut oil	2.2	298 ± 5.7	17	3.8 ± 0.68	64	72 ± 5
3% Sunflowerseed oil + 17% coconut oil	2.3	312 ± 7.9	15	3.2 ± 0.95	47	75 ± 7

[a]Values are mean ±SE.

[b]Values include nonpalpable tumors found at autopsy.

[c]Days from time of treatment with DMBA to appearance of first palpable tumor.

TABLE 4

Mammary Tumors in DMBA-Treated Rats Fed Diets Containing Polyunsaturated Vegetable Oil or Fish Oil

Dietary group	Linoleic acid content of diet (% wt)	Final body weight[a] (g)	No. of rats with mammary adenocarcinomas	No. of adenocarcinomas/ tumor-bearing rat[a]	Cumulative no. of adenocarcinomas	Latent period[a,b]
20% Corn oil	11.2	352 ± 14.1	11	5.0 ± 1.2	55	78 ± 8
10% Corn oil	5.6	309 ± 8.7	9	4.0 ± 1.1	36	75 ± 12
3% Corn oil	1.7	292 ± 8.2	9	2.2 ± 0.4	20	81 ± 9
20% Menhaden oil	0.4	292 ± 5.9	6	1.8 ± 0.3	11	106 ± 11
10% Menhaden oil	0.2	276 ± 5.1	4	1.5 ± 0.3	6	96 ± 26
3% Menhaden oil	0.06	292 ± 6.5	7	3.6 ± 0.8	25	93 ± 8

[a]Values are means ±SE.

[b]Days from time of treatment with DMBA to appearance of first palpable tumor.

up to a level of 4.5% of the diet. It thus seems possible that the amount of linoleate provided by our diets low in polyunsaturated fat is close to the minimum required for tumor promotion, and the lack of consistency in the results may be due to animal variability or other factors such as antioxidant content of the dietary fat.

The experiment in which effects of dietary corn oil and menhaden oil were compared indicates that the requirement for polyunsaturated fat is specific for n-6 fatty acids such as linoleate. This also differs from our earlier conclusion, which was based on a single experiment with a diet containing 3% menhaden oil and 17% coconut oil (4). The reason for this discrepancy is not clear.

In the present study, the diets containing 3% menhaden oil and 3% corn oil gave somewhat comparable results, but at higher levels the corn oil promoted tumorigenesis while the menhaden oil had an inhibitory effect (Table 4). Jurkowski and Cave (15) reported similar effects of menhaden oil on the yield of mammary tumors induced by N-methyl-N-nitrosourea in rats. They also found that the inhibitory effect of the oil was greater at higher levels of intake. The results of these studies are also supported by studies of Karmali et al. (16) in which a dietary supplement enriched in n-3 fatty acids (MaxEpa) was found to reduce the growth rate of a transplantable mammary tumor in mice. More recently, Gabor and Abraham (17) have reported that the growth of transplanted mammary adenocarcinomas in mice fed a 10% corn oil diet was significantly greater than in mice fed a diet containing 10% menhaden oil.

The effect of fish oil in suppressing tumor growth is not limited to mammary tumors. O'Connor et al. (18) recently reported that a diet containing 20% menhaden oil significantly decreased the size and number of preneoplastic lesions of the pancreas induced in rats by L-azaserine compared to a diet containing 20% corn oil.

The inhibitory effect of dietary fish oil on mammary tumorigenesis may be mediated by eicosanoids. Competition between n-3 and n-6 fatty acids or decreased availability of dietary linoleic acid could result in lower production of eicosanoids of the 2-series. Prostaglandins of this series have been implicated in mammary tumorigenesis (7,15–17). The polyunsaturated fatty acids in fish oil are very susceptible to peroxidation and this may also influence tumorigenesis. Products such as malonaldehyde and hydrogen peroxide are known to have a direct toxic action on cells (19,20). Further studies are required to distinguish between these possible mechanisms of action.

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Effect of Dietary Components on the Pathobiology of Colonic Epithelium: Possible Relationship with Colon Tumorigenesis

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The concept that diet plays an important role in the initiation and/or development of various types of tumors in man and experimental animals is well documented. The etiology of colon cancer is complex and multifactorial in nature, and there is little information on the dietary components that may act as initiators during colon tumorigenesis. We have evaluated various dietary heterocyclic mutagenic amines present in a typical "Western" diet for their nuclear damaging effect (presumably a genotoxic response) on the colonic epithelium of C57BL/6J mice in vivo. Among the mutagenic amines studied, 2-amino-3,4-dimethylimidazo(4,5-f)quinoline and 2-amino-3-methylimidazo(4,5-f)quinoline were very potent inducers of nuclear aberrations. These observations provide us with clues that our daily diet may contain colon-specific genotoxic components. Promotional effects of dietary fat and/or bile acids on colon tumorigenesis have been well studied. Dietary levels of calcium (0.1, 0.5 or 1.0% by weight) appear to modify the toxicity of orally administered fat or cholic acid (assessed by quantifying cell proliferation). The colons of animals consuming 0.1% or 0.5% calcium diet were more susceptible to the toxicity, whereas the colons of those consuming a 1.0% calcium diet appeared more like control colons. These studies demonstrate a profound effect of dietary constituents on the pathobiology of the colonic epithelium which may have a marked influence on the colon tumorigenesis.
Lipids 21, 289–291 (1986).

Epidemiological studies have suggested that diet plays an important role in the etiology of colon cancer. It is known that dietary components may initiate, promote or inhibit tumorigenesis in animal models. However, limited studies have evaluated the biological effects of dietary components on the colonic epithelium in vivo. It is important to recognize early changes in the colonic epithelium which may lead to evolution and expression of neoplastic cells and their progression into well-developed tumors. Some early histological and proliferative abnormalities have been noted in rodent colons treated with colon carcinogens or in human colons predisposed to cancer development (1–4). The influence of diet or dietary components on the genesis of some of these histological or proliferative abnormalities has not been explored. Experimental findings reported here demonstrate that both of these abnormalities can be induced and modulated by dietary components.

MATERIALS AND METHODS

Animals. In all studies eight- to 10-week-old C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) were used. The animals were housed in plastic cages with wire tops and sawdust bedding with a 12 hr light and 12 hr dark cycle. The animals were fed laboratory chow ad libitum unless specified otherwise and had free access to water.

Toxicity of food mutagens to colonic epithelium. The mutagenic amines tested with Trp-P-1 acetate [CAS: 62450-06-0; 1,4-dimethyl-5H-pyrido(4,3-b)indole-3-amine]-acetate; Trp-P-2 acetate [CAS: 62450-07-1; 1 methyl-5H-pyrido-(4,3-b)indole-3-amine]acetate (Nard Inst. Ltd., Osaka, Japan); Glu-P-1 [CAS: 67730-11-4; 6 methyldipyrido(1,2-a:3′2′-d)imidazol-2-amine (a gift from T. Sugimura, National Cancer Center Research Institute, Tokyo, Japan); IQ [CAS: 76180-96-6; 3-methyl-3H-imidazo(4,5-f)quinoline-2-amine]; and MeIQ [2-amino-3,4-dimethylimidazo(4,5-f)quinoline] (Toronto Research Chemicals, Toronto, Ontario, Canada). All test compounds were dissolved or suspended in saline and administered to the animals by oral intubation in the morning. The highest dose represented ca. 75% of the maximum tolerated dose of a test compound (5).

Animals were killed 24 hr after treatment; their colons were removed, fixed in 10% buffered formalin and processed for histology. Colonic sections were stained with Feulgen/fast green and evaluated for aberrated nuclei in the colonic crypts. Details have been described previously (5).

Diets. All diets were formulated based on the composition of AIN-76 diet (6) with the exception of calcium phosphate ($CaHPO_4$, diabasic) levels. A low calcium diet (0.1% by weight) was prepared by modifying the AIN-76 mineral mix which contained 100 g of $CaHPO_4$ rather than 500 g/kg of mineral mix. The AIN-76 diet represented ca. 0.5% calcium level; a high calcium diet (≅1% by weight) was prepared by adding 1.7 g of calcium phosphate to 100 g of AIN-76 diet. In all diets the ratio of Ca to P was approximately 1:1.

Influence of dietary calcium levels on the toxicity of orally administered beef tallow (BT) and bile acid. Animals were given AIN-76 or modified AIN-76 diets containing various levels of calcium (0.1, 0.5 or 100% by weight; Ca/P, 1:1). Animals (30 per group) were allowed to eat specific diets for two wk, then were given a bolus of BT (0.1 or 0.4 ml) or saline by oral intubation. Sixteen hr later five animals from each group were injected with colchicine (1 mg/kg body weight). Their colons were removed and processed for histology as described previously. Colonic sections stained with hematoxilyn and eosin were evaluated for the number of metaphase cells per crypt column. Five animals per group were not treated with colchicine, but their colons were removed 16 hr after the fat or saline treatment and processed in the same manner as the colchicine-treated colons to determine the number of mitotic cells per crypt (7).

Animals were given either AIN-76 or modified AIN-76 diets containing various levels of calcium as described above. Test diets contained 0.25% cholic acid (CA) by weight. Animals were allowed to eat designated diets for two wk. Ten animals from each group were injected with colchicine (1 mg/kg body weight) and two hr later their colons were removed and processed for histology. Ten to 20 crypts per animal were scored for arrested metaphase cells. Ten additional animals from each group were injected with [^{3}H]thymidine (1 μCi/g body weight, sp act 0.42 Ci/mmol; Amersham Corporation, Arlington

Heights, Illinois) in saline intraperitoneally. One hour later their colons were removed, cut open longitudinally, fixed flat in the buffered formalin and processed for histology. Unstained paraffin sections were stained with Feulgen, then dipped in NTB-2 emulsion (50% in distilled water at 40 C). The slides were kept in the dark for two wk, then developed by standard procedure. Ten longitudinal sections of full crypts were evaluated for the number of labeled cells along the crypt columns per animal.

RESULTS AND DISCUSSION

Toxicity of food mutagens to colonic epithelium. Table 1 shows comparative data on the nuclear damaging effect of dietary mutagenic amines and their mutagenicity reported by Sugimura (8). At the highest dose level MeIQ and IQ were the most potent inducers of nuclear aberration (NA) in the colonic epithelium. Their nuclear damaging effects appear to follow a trend similar to their mutagenicity. There was no apparent relationship between their nuclear damaging effects and toxicity.

These mutagenic amines are present in broiled and/or fried protein-rich food and constitute a major portion of the mutagenic activity present in a typical North American diet. However, their significance with respect to colon tumorigenesis in animal models or in humans remains unclear. Nevertheless, it is suggested that some of these amines may turn out to be colon carcinogens because of their structural similarity to the colon carcinogen, 2,3-dimethyl aminobiphenyl (9).

It has been suggested that the NA assay can be used to screen dietary components for colon specific genotoxic compounds (10–12). However, it should be noted that NA are cell-lethal events. Therefore, induction of NA in the colonic epithelium by a chemical compound suggests that the test compound, or its active metabolite, is capable of reaching the colonic epithelium and exerting nucleotoxic effects.

Influence of dietary calcium level on the toxicity of beef tallow and bile acid to colonic epithelium. There was no apparent effect of varying dietary calcium levels on the body weights of the animals after two weeks of feeding. However, animals fed cholic acid-supplemented diets exhibited slight depression in their body weights in all dietary groups (Table 2). Animals given 0.1 ml of BT on the 0.1% calcium diet exhibited significantly higher mitotic activity in the colon compared to those on 0.5 or 1.0% calcium diets (Table 3). We have shown previously that orally administered fat (BT or corn oil) damages the surface epithelium of the colons, which in turn increases cell proliferation within 16 hr, presumably a repair response (7). Therefore, cell proliferation can be used as an index of fat toxicity. Colons of animals on 0.1% calcium diet were more sensitive to fat-induced cell proliferation. It is noteworthy that the proliferative response induced by 0.4 ml of BT was unaffected by dietary treatments. This may suggest that the damaging effect of 0.4 ml of BT was too severe and obscures dietary effects. Intrarectal administration of free fatty acids or bile acids has been shown to damage the colonic epithelium. This is followed by a marked increase in the number of dividing cells per crypt. It is speculated that an intake of a high level of fat in a meal situation may expose the colonic epithelium to a higher level of lipids, including bile

TABLE 1

Comparison of Mutagenic Activity and NA[a] Inducibility of Mutagenic Heterocyclic Amines

	Mutagenic activity, S typhimurium TA 98 revertants/μg^b	NA/crypt	Most effective dose[c] (mg/kg body weight)
MeIQ	661,000	1.0	100
IQ	433,000	1.0	400
Trp-P-2	104,200	0.4	10[d]
Trp-P-1	39,000	0.4	200[d]
Glu-P-1	49,000	0.4	200[d]

[a]NA: nuclear aberrations.

[b]Sugimura (8).

[c]The dose at which maximum NA was observed for a particular amine.

[d]Very toxic to animals.

TABLE 2

Body Weights of Animals Fed Diets Varying in Calcium Levels With and Without Cholic Acid[a]

Treatment group	Initial body weight (g)	Final body weight (g)
1.0% Ca	16.10 ± 0.9	19.1 ± 1.4
1.0% Ca + CA	16.90 ± 0.9	18.7 ± 0.9
0.5% Ca	17.50 ± 1.1	20.0 ± 1.8
0.5% Ca + CA	17.20 ± 1.1	18.9 ± 1.2
0.1% Ca	16.70 ± 1.2	20.1 ± 1.4
0.1% Ca + CA	17.10 ± 1.1	19.4 ± 1.1

[a]CA represents 0.25% cholic acid in a semisynthetic diet. Body weights represent before (initial) and after (final) 2 wk of dietary treatments.

TABLE 3

Effect of Dietary Calcium (Ca) on Fat-Induced Cell Proliferation in the Colonic Epithelium[a]

	Mitotic figure/crypt	
Treatment groups	− Colchicine	+ Colchicine
Group I		
0.1% Ca	0.75 ± 0.30[b]	1.78 ± 0.32[b,c]
0.5% Ca	0.49 ± 0.04[c]	1.08 ± 0.18[b]
1.0% Ca	0.29 ± 0.03[d]	1.21 ± 0.15[c]
Group II		
0.1% Ca + 0.1 ml BT	1.18 ± 0.08[b,c]	5.1 ± 0.84[b]
0.5% Ca + 0.1 ml BT	0.67 ± 0.08[b]	2.72 ± 0.18[b]
0.1% Ca + 0.1 ml BT	0.6 ± 0.08[c]	2.2 ± 0.18[b]
Group III		
0.1% Ca + 0.4 ml BT	1.35 ± 0.23	5.11 ± 1.0
0.5% Ca + 0.4 ml BT	1.26 ± 0.16	4.66 ± 0.40
1.0% Ca + 0.4 ml BT	1.5 ± 0.2	4.75 ± 0.41

Mean ± SEM. Values sharing the same superscript in each group and each column are significantly different from each other ($P \leq 0.05$).

[a]Animals were fed different diets for 2 wk then were gavaged with 0.1 ml or 0.4 ml BT at 5 p.m. − Colchicine animals were killed between 9–10 a.m. the next day, while + colchicine animals were injected with colchicine (1 mg/kg body weight intraperitoneally) at 9 a.m. and killed 2 hr later. Colons from − colchicine or + colchicine animals were removed and processed for microscopic determination of MF/crypt as described in Materials and Methods.

TABLE 4

Effect of Dietary Calcium (Ca) on Bile Acid-Induced Cell Proliferation in the Colonic Epithelium

Diet group	MF/crypt[a]	Number of labeled cells/crypt
0.1% Ca	1.98 ± .32[b]	3.39 ± 0.56[b]
0.5% Ca	1.2 ± 0.10[b,c]	—
1.0% Ca	0.88 ± 0.13[b,c]	2.30 ± 0.40[c]
0.1% Ca + CA	4.92 ± 1.06[b,c]	7.6 ± 0.82[b,c]
0.5% Ca + CA	4.20 ± 1.3[b,c]	—
1.0% Ca + CA	2.20 ± 0.53[c]	5.0 ± 0.74[b,c]

Mean ± SEM. MF (mitotic figure)/crypt (5 animals/group, 10 crypts/animal were evaluated). Values sharing the same superscript in each column are significantly different from each other ($P \leq 0.05$).
[a]CA represents 0.25% cholic acid in semisynthetic diet.

acids, which in turn might be damaging (7).

The most proliferative response was found in colons of animals fed 0.1% calcium diet with 0.25% cholic acid (Table 4). Again this suggests the possibility that a high calcium diet reduces the toxicity of bile acids to colonic epithelial cells. Cholic acid feeding increases the deoxycholic acid level in animal feces which in turn is toxic and increases cell proliferation (13,14). The presence of cholic acid in the diet also exerts a promotional effect during colon tumorigenesis (15,16).

Autoradiographic analysis of the colonic sections revealed similar effects. The number of labeled cells per crypt was significantly higher in the 0.1% calcium group compared to that in the 1.0% calcium group. It is also apparent that even 1.0% calcium diet was unable to completely inhibit the effect of dietary cholic acid with respect to increase in cell proliferation (Table 4).

Other studies have shown a similar protective effect of calcium on bile acid and free fatty acid-induced toxicity to colonic epithelium (17,18). However, the experimental conditions were quite different from those described in the present study. Recently Lipkin and Newmark (19) showed that aberrant proliferative characteristics of human colons with high risk for developing cancer can be modified to a more normal situation after ingestion of calcium tablets for several months.

The exact mechanism by which a high calcium diet protects against fat or bile acid toxicity remains unclear. A possible mechanism has been suggested by Newmark et al. (20). They have hypothesized that ionized calcium present in the colonic lumen will bind with toxic free fatty acids and bile acids and form inert calcium soaps. To test this hypothesis, it is important to quantify calcium-bound free fatty acids or bile acids. So far this has not been possible due to lack of proper methodology.

In experimental diets the ratio of Ca to P was kept constant. Therefore, the phosphate level was changing with the level of calcium. In this situation it is difficult to suggest that the level of calcium alone modulated the effect of fat or bile acid toxicity. However, it was thought necessary to keep a constant ratio in all experimental diets because of the possible unfavorable effects (21-23). Further studies are required to investigate the effect of dietary calcium on the pathobiology of the colon.

In conclusion, it has been demonstrated that some dietary mutagens can reach the colonic epithelium and damage cells present in the proliferative compartment. Increased bile acid or fat levels in the colonic lumen are toxic to surface epithelium and affect cell proliferation within a very short period of time (two wk). This can be modulated by dietary calcium. A high calcium diet appears to counteract the effect of fecal bile acids or fatty acids. Observations reported here support the contention that diet may play an important role in the etiology of colon cancer and can influence the genesis of some early lesions possibly associated with tumorigenesis in the colonic epithelium.

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A Comparison of Lipids from Liver and Hepatoma Subcellular Membranes

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Subcellular fractions of nuclei, mitochondria, endoplasmic reticulum, plasma membrane and cytosol were prepared from liver and hepatoma 7288CTC. Marker enzyme activities, biochemical compositions and electron microscopy were used to establish purity. Hepatoma NADH: cytochrome C reductase and 5′-nucleotidase exhibited abnormal subcellular distributions.

The lipids from the subcellular fractions were examined in detail. Mitochondria and plasma membranes were characterized by elevated percentages of diphosphatidylglycerol and sphingomyelin, respectively, in both tissues. All hepatoma subcellular fractions contained dramatically elevated levels of sphingomyelin and cholesterol, two components that form preferential strong complexes in vitro. The fatty acid composition of hepatoma sphingomyelin differed markedly from liver and, unlike liver, did not exhibit organelle specific compositions. Some hepatoma lipid classes contained reduced percentages of palmitate while others contained higher levels. Hepatoma phosphatidylcholine and phosphatidylethanolamine from organelles contained lower percentages of long chain polyunsaturated fatty acids than liver. Generally, unique fatty acid profiles exhibited by individual phospholipid classes of liver subcellular fractions were absent or much reduced in the hepatoma. The ratios of oleate to vaccenate were near one for most of the phospholipid classes of most liver fractions, but all hepatoma classes, with few exceptions, contained a much higher percentage of oleate in all subcellular fractions. The hypothesis is proposed that the origin of some acyl moieties for the biosynthesis of various hepatoma lipid classes differs from liver sources. The possible changes in acyl pools, sources and compartments for complex lipid biosynthesis could result in change in the quantities of molecular species that could contribute to the abnormal properties of the hepatoma membranes.

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Neoplasia, or in vitro transformation, is accompanied by biochemical, morphological and growth-related changes (1). Lipids, major components of membranes, have been examined for their direct or indirect involvement in neoplasia (2). The lipid compositions of liver and hepatoma have been studied in detail (3–8) and in some instances the plasma membrane of these tissues has also been analyzed (9,10). Bergelson et al. (11) analyzed the lipids of the subcellar fractions of liver and hepatoma. Based on lipid composition studies, they proposed that the subcellular fractions from normal cells retain specificity in the lipid composition and de-differentiation (tumorigenesis) involves a loss of specificity. With this hypothesis in mind, we compared the membranes of several subcellular fractions from hepatoma 7288CTC and liver (12,13). The characterization of the subcellular fractions and the lipids found in these membranes are reviewed in this paper.

MATERIALS AND METHODS

Membrane preparation. Hepatoma (7288CTC) was grown on the hind legs of male rats (Buffalo strain, 250 g) for 30 days. Both normal and hepatoma-bearing animals were maintained on a chow diet and fed ad libitum. The hepatoma tissue was carefully dissected from muscle and connective tissue and pressed through a metal sieve and the cells were disrupted by nitrogen cavitation in a bomb at 1000 psi as described earlier (12). The resulting homogenate was separated into the subcellular fractions by differential and Ficoll density gradient centrifugation according to the procedures of Wallach and Kamat (14) and Allfrey et al. (15).

Liver from normal rats was homogenized with a Teflon-glass homogenizer and the subcellular fractions separated by differential, Ficoll density gradient and sucrose density gradient centrifugation according to the procedures of Fleischer and colleagues (16,17).

Chemical and analytical analyses. Protein was determined by the method of Lowry et al. (18) and phospholipid phosphorous by the method of Rouser et al. (19). DNA and RNA were precipitated by ice-cold 10% trichloracetic acid (TCA) (20). DNA was estimated colorimetrically using diaminobenzoic acid (21) and RNA by a modified orcinol reagent (22). The sialic acids in glycolipids and glycoproteins were determined colorimetrically using the thiobarbituric acid assay method of Warren (23). Total lipids from the freeze-dried subcellular fractions were extracted by the Bligh-Dyer procedure (24) and fractionated into neutral (NL) and phospholipids (PL) by silicic acid chromatography (25). Individual lipid classes were separated by thin layer chromatography (TLC) (3,13); methyl esters were prepared (26) and analyzed quantitatively by gas liquid chromatography (GLC) (27). Additionally, the methyl esters were separated according to degree of unsaturation and the position of the double bonds in the monoene fractions determined (5).

Enzymatic analyses. The enrichment of plasma membranes was assessed by determining the activities of 5′-nucleotidase (28) and ouabain-sensitive (Na^+-K^+) and insensitive (Mg^{++}) ATPases (29). NADH:cytochrome C reductase, a marker for ER and PM, was measured in the presence of 1 mM KCN, and activity of cytochrome C oxidase, a marker for mitochondria, was estimated by the difference in NADH:cytochrome C reductase acativity in the presence and absence of 1 mM KCN (30).

Materials. Sources of the high purity reagents, standards and glass-distilled solvents are given elsewhere (12).

RESULTS AND DISCUSSION

Unlike liver, hepatoma 7288CTC resisted disruption by Teflon-glass homogenization but was broken easily by the nitrogen cavitation technique (14). A similar situation has

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TABLE 1

Composition of Subcellular Fractions from Liver and Hepatoma 7288CTC[a,b]

Subcellular fraction	μg/mg Protein				nmol Sialic acid/mg protein				Total phospholipids μg P/mg protein	
	DNA		RNA		Protein-bound		Lipid-bound			
	L[c]	H[c]	L	H	L	H	L	H	L	H
Homogenate	24	54	42	43	4.1	8.1	1.6	0.9	5.5	1.1
Nuclei	115	241	68	149	4.2	12.6	2.3	1.7	0.8	0.6
Mitochondria	50	78	39	36	8.6	8.4	1.2	1.5	5.5	3.1
Endoplasmic reticulum	41	56	124	177	7.1	12.4	3.5	4.3	20.4	8.6
Plasma membrane	12	11	24	18	36.0	28.0	11.5	14.5	22.5	20.3
Cytosol	19	24	60	164	6.2	10.4	1.6	0.4	0.5	0.2

[a]Wet weight (g/rat) of liver 11 ± 1.9 and hepatoma after 30 days of transplant was 34.5 ± 5.4. The protein content (mg protein/g wet wt) of the liver homogenate was 143 ± 43 and 95.2 ± 18.3 for hepatoma homogenate. These data represent mean ± standard deviation of three rats.

[b]Data on the composition represent the mean of duplicate analysis of samples pooled from three rats.

[c]L, liver; H, hepatoma 7288CTC.

been reported for Novikoff hepatoma cells (31). Liver disrupted by nitrogen cavitation gave poor quality subcellular fractions and as a result was not used. Perhaps conditions could be found that would give comparable membranes from hepatocytes. It is possible that the elevated concentrations of spingomyelin and cholesterol may have contributed to the resistance to rupture of the hepatoma cells. After cell disruption, plasma membranes (PM), endoplasmic reticulum (ER), mitochondria and cytosol were isolated from liver and hepatoma by the same procedure, but a different procedure was required for hepatoma nuclei. This is in agreement with the work of Leroy-Houyet et al. (32) that indicated hepatoma nuclei occupy a larger fractional volume than hepatocyte nuclei.

The weight and protein concentrations of the livers and hepatomas are given in the footnote to Table 1. The cytosol contained ca. 50% of the total protein of both hepatic tissues. Hepatoma mitochondria accounted for 7.5% of the homogenate protein, approximately half the yield of mitochondria protein from normal liver. The low level of mitochondrial protein is consistent with earlier observations of fewer and smaller mitochondria in hepatomas relative to liver (31-33). Lower yields of PM and ER protein in the hepatoma may be attributable to different cell disruption procedures.

Composition of subcellular fractions. Subcellular fractions from both tissues were analyzed for the components whose relative increase served as a marker of enrichment and are given in Table 1. DNA content, a marker of nuclei and mitochondria, was the highest in both tissues, but hepatoma nuclei had double the concentration of liver DNA, an observation consistent with the higher nuclear protein yield and earlier data (32). RNA, a marker of ER, was elevated in this fraction of both tissues. Hepatoma nuclei and cytosol also contained elevated levels of RNA. This increased level is consistent with nuclear RNA and transfer RNA needed for rapid growth. Both protein- and lipid-bound sialic acid containing macromolecules were elevated in the plasma membrane fractions of both tissues. These concentrations agree with some reported values (34,35) but differ from others (34), suggesting that membrane sialic acid concentrations may be related to the tumor and method of isolation as proposed earlier (34,35). Phospholipid concentrations were elevated in PM and ER fractions of both tissues. A more detailed characterization of the lipids will be discussed later.

Marker enzymes. The purity of the subcellular fraction was also assessed by measuring the specific activity of marker enzymes (Table 2). NADH:cytochrome reductase, considered to be an ER and PM marker (30), exhibited two to three times higher activity in these liver fractions than hepatoma. The higher activity of this enzyme in hepatoma mitochondria might suggest contamination with ER, but low concentrations of RNA, sialic acid and reduced ATPase activities indicate this is not the case. As will be shown later, this represents an abnormal distribution of one of several enzymes in the hepatoma cell.

Cytochrome C oxidase, a mitochondrial marker enzyme, was found almost exclusively in this fraction from liver, whereas hepatoma ER contained some activity in addition to the mitochrondria. It is possible the cytochrome C oxidase activity found in the hepatoma ER may represent an abnormal distribution of this enzyme also.

The ATPases have been used routinely as a marker enzyme for liver and hepatoma PM (10,36). The data in Table 2 show that the PM of both tissues contained the highest relative specific activity. 5′-Nucleotidase, another accepted plasma membrane marker (16,37) was enriched in the liver PM fraction but not the hepatoma (Table 2). The specific activity of this enzyme in the hepatoma homogenate was fivefold liver values and, unlike liver, was found primarily in the ER and mitochondria. The low 5′-nucleotidase activity in hepatoma PM agrees with the data reported previously by Pezzino et al. (38) but differs from results obtained from other hepatomas (10,36). The enrichment of ATPases, sialic acid and phospholipids in the hepatoma PM fraction would appear to rule out the possibility of gross contamination.

In addition to the compositional and enzymatic characterization of the subcellular fractions, electron microscopy examinations were made. The results confirmed the subcellular fraction identifications.

Of the six marker enzymes examined, NADH:cytochrome C reductase, NADH:dihydrolipoamide reductase (data not shown) and 5′-nucleotidase exhibited abnormal

TABLE 2

Enzyme Activities in Subcellular Fractions from Hepatoma and Liver Relative to the Concentrations Found in Whole Cell Homogenates

	NADH: cytochrome C reductase		Cytochrome C oxidase		Mg ATPase[a]		NA-K ATPase[a]		5′-Nucleotidase[a]	
	L[b]	H[b]	L	H	L	H	L	H	L	H
Homogenate (n mol/min/ mg protein)	177.0 ± 30.0	48.9 ± 15.4	44.7 ± 7.7	16.1 ± 6.9	88.3 ± 5.8	40.0 ± 10.0	6.7 ± 2.1	10.0 ± 5.0	35.0 ± 1.7	181.7 ± 10.0
RSA[c]										
Nuclei	0.3	0.2	0.0	0.4	—	0.6	—	0.5	0.1	0.8
Mitochondria	1.9	4.7	4.5	12.2	1.7	2.0	1.2	2.4	0.14	2.1
Endoplasmic reticulum	5.8	2.9	0.5	5.0	0.3	2.9	5.0	3.4	0.18	2.2
Plasma membrane	6.0	1.4	0.0	0.0	2.4	10.8	14.5	8.1	5.8	0.6
Cytosol	0.1	0.2	0.0	0.2	0.0	0.5	0.0	0.3	0.2	0.4

[a]Specific activities represent n moles of pi released from ATP (in case of ATPases) and 5′AMP (for 5′-nucleotidase).

[b]L, liver; H, hepatoma 7288CTC.

[c]RSA (relative specific activity) = specific activity of a subcellular fraction/specific activity of homogenate.

distributions in the hepatoma subcellular fractions relative to liver. The abnormal distribution does not appear attributable to the different methods of cell disruption because different marker enzymes for the same organelle show different distributions (ATPases and 5′-nucleotidase). Because of the abnormal lipid composition of the hepatoma (discussed later) and reported effects of different lipid environments on 5′-nucleotidase activity (39,40), it is tempting to speculate that the low activity of 5′-nucleotidase activity in the PM is due to a lack of proper molecular species of lipids. However, the fivefold higher specific activity of 5′-nucleotidase in the hepatoma homogenate (Table 2) is difficult to explain unless one assumes the cell disruption makes the proper molecular species available to the enzyme. An alternate and more likely explanation for the abnormal distributions of some enzymes in the hepatoma is the abnormal transport of enzyme after synthesis (41).

LIPID ANALYSES

Class concentrations. Although the total neutral lipid concentration in the hepatoma homogenate was one-half liver levels (52–59 and 90–121 μg/mg protein, respectively), all hepatoma fractions except the cytosol contained higher concentrations of total neutral lipids than the corresponding liver fractions. A significant portion of the higher neutral lipid concentration in the hepatoma was due to elevated cholesterol levels. Free fatty acid, triglyceride and cholesterol ester concentrations in the hepatoma showed some differences from liver in various fractions (12), but cholesterol concentrations were elevated in all fractions. Hepatoma mitochondria contained 17 times more cholesterol than liver mitochondria. Elevated levels of cholesterol have been reported for Ehrlich ascites cell mitochondria (42). The lack of feedback inhibition of cholesterol biosynthesis (43,44) is probably responsible for the elevated levels in the subcellular fractions. The possible significance of the elevated cholesterol levels will be discussed later in conjunction with the elevated sphingomyelin levels.

As shown in Table 1 the hepatoma homogenate contained only 20–25% as much total phospholipid as the liver homogenate, which agrees with previous data (3,8,10,45). This lack of phospholipid or certain molecular species could affect the function of subcellular structures. Except for the plasma membrane fraction, all of the hepatoma subcellular fractions contained significantly less phospholipid than liver and should be taken into account when comparing class and fatty acid compositions. The phospholipid composition of the subcellular fractions obtained from liver and hepatoma are shown in Table 3. Three minor phospholipids, lysophosphatidylcholine, phosphatidylinositol and phosphatidylserine, not given in the table showed some tissue and organelle preferences but generally were not consistent (13). On the other hand, sphingomyelin, usually considered a minor component, was dramatically elevated in all subcellular fractions. Hepatoma nuclei and mitochondria contained approximately 20-fold more spingomyelin than the corresponding liver subcellular fractions. These increases in nuclei and mitochondria sphingomyelin of the hepatoma came primarily at the expense of phosphatidylethanolamine (PE), which was reduced to one-fourth and one-third of liver percentages. If one considers that the total phospholipids of the hepatoma cell were only 25% of liver concentrations, these additional reductions in PE percentages make this phospholipid class approximately eightfold less in these two organelles relative to liver. Such a dramatic change must affect membrane asymmetry, enzymatic activity of systems that require these phospholipids, transport properties, etc. After the fatty acid compositions have been examined, it will become more apparent that certain molecular species of PE are reduced

TABLE 3

Phospholipid Composition of Subcellular Fractions of Rat Liver and Hepatoma 7288CTC

Fractions	Hepatic tissues	Percentage of total PL[a,b]			
		SPH	PC	PE	SF + DPG
Homogenate	Liver	4.0-4.6	49.9-52.2	24.3-26.4	4.4-5.1
	Hepatoma	12.2-14.6	41.3-49.9	19.3-30.0	3.2-9.2
Nuclei	Liver	2.3	49.1	28.3	10.4
	Hepatoma	43.9	29.8	4.2	7.9
Mitochondria	Liver	T	44.1	34.8	14.5
	Hepatoma	20.5	31.9	10.4	15.8
Endoplasmic reticulum	Liver	6.3	53.6	13.2	3.0
	Hepatoma	14.7	38.7	28.6	1.5
Plasma membrane	Liver	9.8	52.3	14.8	2.4
	Hepatoma	22.2	39.1	24.4	1.2
Cytosol	Liver	4.6	57.6	19.7	6.3
	Hepatoma	11.1	49.8	26.9	11.2

[a]Percentages represent the mean of duplicate phosphorous analyses and are not corrected for differences in the molecular weights. Data for the homogenate represent determinations made on two independent pooled samples.

[b]Differences between the sum of any row and 100 represent the amounts of lysophosphatidylcholine, phosphatidylinositol and phosphatidylserine not given in the table. SPH, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SF + DPG, solvent front + diphosphatidylglycerol; T, trace amounts; PL, phospholipid.

even further. These reductions in nuclei and mitochondria of the hepatoma appear to represent a problem of distribution or transport rather than a lack of synthesis. This conclusion is supported by the fact that the ER, PM and cytosol fractions contained elevated percentages of PE. Phosphatidylcholine (PC), the major phospholipid class, was decreased in all hepatoma subcellular fractions, but the percentage change was usually less than the change in sphingomyelin and PE. The high percentage of solvent front (SF) plus diphosphatidylglycerol (DPG) (predominantly DPG as judged from the fatty acid compositions) from liver and hepatoma mitochondria (Table 3) supports the identity of this fraction, since DPG is synthesized primarily in the mitochondria (46).

There is real difficulty in attempting to evaluate the differences in the concentrations of lipid classes between liver and hepatoma subcellular fractions, but because cholesterol and sphingomyelin levels were dramatically elevated in all fractions one must believe these compounds affect cell function and properties. The parallel increases in sphingomyelin and cholesterol have been observed in other systems (47). Model systems composed of cholesterol and spingomyelin have been shown to form preferential strong complexes (48-50). In addition, the chemical nature of sphingomyelin may contribute to rigidity of the bilayer (51), decreased permeability (52) and osmotic fragility (53). The added stability of a membrane produced by increased levels of cholesterol and sphingomyelin may explain the difficulty encountered in the disruption of neoplastic cells. Elevated levels of spingomyelin have also been shown to inhibit the activities of phospholipid exchange proteins (54) and phospholipase C (55). This could cause a decrease in the transfer of phospholipids from the ER to other membranes, which could explain the increase of PE in the ER, PM and cytosol and much decreased levels in mitochondria and nuclei.

Fatty acid profiles. The fatty acid profiles of three neutral lipid and six phospholipid classes obtained from the subcellular fractions of liver and hepatoma are given in Tables 4 and 5. The nature of the acyl chains (length, unsaturation, double bond position, configuration of double bonds) plays a significant role in governing the properties of membranes (5,6), lipoprotein complex formation (57) and perhaps activities of enzymes. The three neutral lipid classes not considered to be membrane structural components may play an important role in membrane integrity by supplying acyl groups. The fatty acid profiles of the free fatty acids, triglycerides and sterol esters from all the hepatoma subcellular fractions differed from the corresponding liver fractions. Generally, palmitate percentages were lower and longer chain fatty acids represented a higher percentage in the hepatoma. Some organelle specificity was observed: stearate percentages were high in the free fatty acids from liver nuclei; liver mitochondria contained elevated percentages of 20:4 and 22:6 in the free fatty acid fraction; and hepatoma plasma membranes contained very low levels of 18:2 in the triglycerides. Although the full significance of these results is not readily apparent, it is possible that the composition of a neutral lipid class, unique to an organelle, may result from preferential lipid metabolism of that organelle.

In contrast to the neutral lipids, the phospholipids are membrane structural components whose compositions affect the membrane properties. Sphingomyelin, unique in composition, exhibited fatty acid compositions characteristic of the organelles in liver, but not in the hepatoma. As the percentage of stearate decreased in the various organelles, the percentage of 22:0, 23:0, 24:0 and 24:1 increased in liver. Hepatoma sphingomyelin, dramatically

TABLE 4

Comparison of the Fatty Acid Compositions of the Major Lipid Classes Isolated from Various Subcellular Fractions of Liver and Hepatoma[a]

Fatty acid[b]	FFA		TG		PC		PE		PI		PS		SF + DPG	
	L	H	L	H	L	H	L	H	L	H	L	H	L	H
							Homogenate[c]							
16:0	30	16	29	12	21	23	17	8	6	1	2	2	3	4
18:0	10	30	2	10	16	21	31	35	51	53	53	48	8	9
18:1	20	26	29	28	8	21	5	23	3	9	3	22	13	20
18:2	21	15	29	21	21	19	12	16	1	6	1	8	52	39
20:4	10	4	1	7	18	6	24	9	37	22	27	5	4	2
22:6	1	1	2	2	3	--	9	1	1	1	9	1	--	--
							Nuclei							
16:0	19	18	29	13	11	30	8	9	3	2	—	2	10	19
18:0	26	34	7	10	46	34	40	30	71	59	—	35	32	12
18:1	18	20	28	29	10	14	10	22	4	5	—	10	10	11
18:2	12	10	11	28	15	10	9	12	t	6	—	2	11	13
20:4	11	3	1	5	14	8	23	7	20	20	—	4	9	3
22:6	1	1	1	2	2	--	5	t	1	t	—	t	--	--
							Mitochondria							
16:0	19	16	23	13	25	41	19	17	2	2	9	2	4	11
18:0	10	30	2	11	22	22	26	32	53	58	49	54	2	11
18:1	10	20	28	27	9	19	5	25	1	10	19	22	13	18
18:2	19	19	32	28	21	10	10	15	1	11	9	8	76	41
20:4	25	4	1	9	17	2	27	5	31	18	12	5	2	3
22:6	4	1	1	1	2	--	8	1	1	t	3	1	--	--
							Endoplasmic reticulum							
16:0	32	17	35	20	25	31	21	10	7	1	6	2	12	27
18:0	9	25	2	22	22	21	26	36	47	58	54	53	22	27
18:1	24	23	30	28	10	20	6	24	2	9	4	25	9	22
18:2	19	20	20	12	23	18	14	19	2	5	3	9	19	6
20:4	4	3	1	2	12	3	22	8	37	21	15	3	11	2
22:6	1	1	t	t	2	--	7	1	1	t	7	1	--	--
							Plasma membrane							
16:0	41	20	31	21	34	33	16	10	13	2	17	2	20	19
18:0	12	25	2	35	38	22	29	35	77	54	70	53	19	28
18:1	20	24	32	24	9	20	7	26	2	9	7	25	5	25
18:2	15	15	20	1	10	17	16	18	t	7	t	8	10	10
20:4	4	2	1	1	4	2	23	8	2	22	t	4	11	7
22:6	1	1	t	t	1	--	6	1	t	t	t	1	--	--
							Cytosol							
16:0	30	13	22	16	21	29	20	18	6	23	9	22	32	23
18:0	9	15	1	8	23	19	22	23	46	53	53	28	15	16
18:1	17	23	22	20	8	20	11	27	2	15	5	16	12	21
18:2	20	26	29	26	22	20	18	15	t	5	3	5	10	13
20:4	9	11	1	9	16	6	20	5	39	7	22	6	9	3
22:6	2	2	2	6	2	--	5	t	1	--	7	--	--	--

[a]Only percentages of major fatty acids are given. The difference between the sum of any column and 100 represents the percentage of other fatty acids not reported here. See refs. 12 and 13 for a more complete listing.

[b]Fatty acids are identified by chain length (numbers before colon) and degree of unsaturation (number after colon). FFA, free fatty acids; TG, triglycerides; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SF, solvent front; DPG, diphosphatidylglycerol; L, liver; H, hepatoma; t, quantities less than 0.5%; --, detectable quantities of less than 0.1%; —, insufficient sample.

[c]Values given for the homogenate represent the mean of two independent analyses, whereas values for the other fractions represent a single determination from a pooled sample from three animals.

TABLE 5

Comparison of the Fatty Acid Profiles of Sterol Esters and Sphingomyelin Isolated from the Various Subcellular Fractions of Liver and Heptoma[a]

Subcellular fraction	Hepatic tissue	Fatty acid percentages[b]														
		16:0	16:1	18:0	18:1	18:2	20:4	22:0	23:0	24:0	24:1	24:2	22:5	22:6	26:0	26:1
									Sterol esters							
Homogenate	L	60	4	6	11	12	7				t		t	t	t	t
	H	7	3	7	19	11	15			1	11		5	4	1	4
Mitochondria	L	10	9	8	33	16	10			t	t		t	1	t	t
	H	7	2	6	18	21	26			1	3		1	3	t	t
Endoplasmic reticulum	L	10	7	9	41	13	5			1	1		t	1	t	t
	H	14	2	12	21	17	9			3	1		1	1	3	t
Plasma membrane	L	20	8	9	26	17	5			1	t		t	t	t	t
	H	7	4	12	16	8	6			10	10		4	2	8	1
Cytosol	L	52	6	8	13	10	8			t	t		t	t	t	t
	H	10	3	4	14	17	26			8	13		2	5	t	3
									Sphingomyelin							
Homogenate	L	10		15	2			11	10	29	11	t				
	H	20		8	t			5	t	26	31	6				
Mitochondria	L	8		41	15			6	1	5	1	t				
	H	30		5	t			3	t	25	27	7				
Endoplasmic reticulum	L	11		38	3			9	4	16	7	t				
	H	22		7	t			3	t	28	31	6				
Plasma membrane	L	9		21	2			11	9	30	12	t				
	H	40		6	t			3	t	20	24	5				
Cytosol	L	1		9	t			12	11	40	15	t				
	H	24		5	1			3	t	25	30	7				

[a]The difference between the sum of any row and 100 represents the amounts of other minor acids not given in the table. Because there were insufficient sterol esters and sphingomyelin in normal liver nuclei for analyses, hepatoma values were also deleted. See ref. 13 for these values.

[b]Fatty acids are identified by a number representing the chain length followed by a colon and a number that indicates the degree of unsaturation. L, liver; H, hepatoma; t, detectable quantities less than 0.5%.

elevated in all subcellular fractions (Table 3), exhibited the same approximate fatty acid composition in all subcellular fractions (Table 5). Palmitate, 24:0 and 24:1 predominated in the hepatoma. The occurrence of significant quantities of 24:2, but only in trace levels in liver, has been shown previously to be present in several neoplasma (58,59), probably due to the loss of enzymatic specificity in the hepatoma. Hepatoma palmitate percentages were more than double liver sphingomyelin palmitate percentages in all organelles, while stearate percentages were less than one-half liver values. Although the significance of the change in the ratio of 16:0 to 18:0 in biomembranes is unclear, it has been shown that sphingomyelin containing palmitate exhibits different interactions with cholesterol than sphingomyelin containing stearate (50). The possible relation between the altered sphingomyelin molecular species and elevated cholesterol concentrations in the hepatoma has been discussed earlier.

The hepatoma fatty acid profile of the major phospholipid class, PC, was distinctly different from liver in all fractions. Hepatoma PC contained a much decreased level of polyunsaturated fatty acids and an elevated percentage of 18:1 relative to liver subcellular fractions. Liver PC from nuclei and PM contained uniquely higher percentages of saturated fatty acids than the other fractions, which could produce different properties from the other fractions and from hepatoma membranes.

Liver PE, like PC, contained much higher percentages of polyunsaturated fatty acids in all fractions than the hepatoma fractions (Table 4). The fatty acid profiles of liver PE were similar to the profiles of PC in most fractions. We had previously concluded from studies with whole cells (5) that the fatty acids at the 1-position of PC and PE were of the same origin, whereas the origins of these fatty acids differed from hepatoma PC and PE. Except for nuclei, liver PE fatty acid profiles were not organelle-specific. In contrast to PC, PS and PI fatty acid compositions of liver PM, PE contained a high percentage of polyunsaturated fatty acids. This may be explained by the asymmetric distribution of PE in erythrocyte membranes (60,61). Polyunsaturated fatty acids in PE on the inner bilayer of the PM would not be exposed to enzymes and oxidants of the circulation as they would be on the outer bilayer of the membrane. In contrast to PE, PC and sphingomyelin have been shown to be located preferentially on the outer bilayer of the erythrocyte membrane (61). If the hepatocyte PM has a similar

asymmetric distribution of lipid classes as the erythrocyte, the low percentage of polyunsaturated fatty acids in PC of liver PM (Table 4) and the absence of polyunsaturated fatty acids in SPH (Table 1) supports the hypothesis of reduced unsaturation in the outer bilayer. If PI and PS are found to be located on the outer bilayer of the hepatocyte, the low levels of polyunsaturated fatty acids observed in the PM would lend further support to this hypothesis.

The presence of higher percentages of 20:4 in PI and PS of hepatoma PM than in liver is difficult to assess because the percentages are relatively low. Generally, the characteristic high percentage of stearate found in PS and PI was observed in most organelles from both liver and hepatoma (Table 4), but the organelle-specific fatty acid profiles observed in liver were much diminished or absent from the hepatoma fractions.

The mitochondrial fraction fatty acid profile of the solvent front plus DPG fraction is characterized by the high percentage of 18:2 in the liver and to a lesser degree in the hepatoma. The high 18:2 content of liver mitochondria, the site of DPG synthesis (62), has been shown to be characteristic of liver DPG (63). Because of the possible contamination of this fraction with other lipid classes, organelle characteristic fatty acid profiles and differences between liver and hepatoma profiles should be interpreted with care.

Examination of the fatty acid profiles of the subcellular fractions suggests a number of differences between the hepatoma and liver lipids, but two important differences warrant additional discussion. The much reduced percentages of palmitate in all the subcellular fractions of hepatoma free fatty acids, triglycerides, sterol esters and PE relative to liver might suggest a diminished supply of palmitate. This notion can be rejected after looking at the higher percentages of palmitate in sphingomyelin and PC. The abnormally higher percentage of palmitate in some lipid classes, while other classes have lower percentages than liver, has at least two possible explanations. The hepatoma acyltransferases may have lost some of their selectivity or preference for palmitate. Some enzymes in hepatoma fatty acid biosynthesis (58,59) and phosphoglyceride synthesis (6) have been reported to have lost some specificity. This does not appear to be very plausible because it would require separate acyltransferases for the biosynthesis of various lipid classes. A more attractive hypothesis involves compartmentalization of substrates. Workers in this laboratory have long held the idea, based upon mass measurements, that the 1-position fatty acids of liver PC and PE are derived from the same source, pool or compartment, whereas the 1-position fatty acids of hepatoma PC and PE are derived from different sources (63-65). Although these studies did not measure the fatty acids at the 1-position, the preferential esterification of palmitate at the 1-position of PC and PE permits the comparisons. The results from these subcellular fractions support the earlier conclusion and the idea of restricted substrate pools. The differences in the origins of palmitate from the biosynthesis of hepatoma and liver lipids appear to represent a basic difference in lipid metabolism between normal and neoplastic cells that may be exploited to control lipid synthesis in tumor cells.

The second issue to be discussed further is the trend to lipid class de-differentiation of membranes from hepatomas as proposed by Bergelson et al. (11). We did not see the loss of the organelle characteristic phospholipid class composition in this tumor. This may be partly overshadowed by the high percentages of sphingomyelin, but the high percentage of DPG in the hepatoma mitochondria was similar to the elevated percentages found in normal liver (Table 3). The present study has allowed de-differentiation to be examined in more detail. The characteristic fatty acid compositions of most liver lipid classes, especially PC, PE, PS and spingomyelin, were absent or reduced considerably in the hepatoma (Tables 4 and 5). Likewise, the fatty acid compositions unique to certain phospholipid classes of specific liver organelles, such as the 18:0 of sphingomyelin from mitochondria and ER, the 18:2 of DPG from mitochondria and the 20:4 of PS from mitochondria and ER were very much reduced in the hepatoma. Further, as will be shown later for the octadecenoate positional isomers, the distributions unique to liver neutral lipids and phospholipids, specific lipid classes, and specific lipid classes from specific cellular fractions are virtually absent in the hepatoma. Although the importance of the unique class compositions, fatty acid compositions and positional isomer distributions of cellular organelles and fractions is not known for normal liver, it must be assumed that they impart some special property to the system. The question of whether the absence of these unique compositions, distributions, etc., in the hepatoma membranes play any role in the neoplastic nature of the cells remains unanswered, but one must continue to assume that they do. This leads one to the central question of how the loss of specificity occurred in the hepatoma. Could this all be accounted for by changes in substrate pools, sources and compartments?

Octadecenoate positional isomers. The octadecenoates of liver and hepatoma lipid classes consist predominantly of *cis*-Δ9-octadecenoate (oleate) and *cis*-Δ11-octadecenoate (vaccenate) (5,6). The percentage distributions of oleate and vaccenate in the various lipid classes isolated from the membrane fractions of liver and hepatoma are given in Table 6. Except for PS, DPG and TG of liver mitochondria, there was no distribution of oleate and vaccenate that characterized any subcellular fraction. FFA and triglycerides from liver subcellular fractions contained ca. 70-75% and 80-85% oleate, respectively, whereas the phospholipid classes contained roughly equal amounts of oleate and vaccenate. In contrast, all hepatoma lipid classes contained roughly 70% oleate and 30% vaccenate. The exceptions were TG of PM, mitochondrial PI, PE of nuclei and DPG of hepatoma mitochondria and ER. These results clearly show that some of the lipid class characteristic distributions of oleate and vaccenate in liver subcellular fractions have been lost or nearly lost in the hepatoma. Although we do not yet know the significance of the increased percentage of vaccenate in the hepatoma phospholipid classes, it could affect membrane fluidity. It has been shown that fluidity decreases as the double bond moves from the center of the fatty acyl chain toward either end (67). Such changes in the ratio of oleate to vaccenate in the membrane phospholipids could be critical to membrane-mediated functions including insertion and transfer of integral membrane proteins (68) and receptor-mediated functions

TABLE 6

Percentage Distribution of Oleate and Vaccenate Isomers in the Various Lipid Classes Isolated from the Subcellular Fractions of Liver and Hepatoma[a]

Fraction	FFA	TG	PC	PI	PS	PE	SF + DPG
				Liver			
Homogenate[b]	64-77	82-84	52-57	58-68	66	48-50	27-32
Nuclei	72	88	55	64	—	67	64
Mitochondria	60	82	50	55	29	48	23
Endoplasmic reticulum	77	83	59	59	64	54	50
Plasma membrane	77	83	45	58	—	57	58
Cytosol	70	85	56	58	60	36	52
				Hepatoma			
Homogenate[b]	69-71	70-74	67-68	66-69	77-81	69-70	45-64
Nuclei	69	73	63	64	72	52	60
Mitochondria	69	77	68	55	77	62	47
Endoplasmic reticulum	70	78	68	67	82	73	46
Plasma membrane	70	49	66	67	82	71	73
Cytosol	70	76	70	—	—	58	70

[a]Only oleate percentages are given. Vaccenate percentages can be calculated by subtracting oleate percentage from 100.

[b]Analyses made on two independent pooled samples. FFA, free fatty acids; TG, triglycerides; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; SF, solvent front; DPG, diphosphatidylglycerol.

(69). At the present the relation between the altered lipids and neoplasia is not clear, but a detailed characterization of the asymmetric distribution of the lipid molecular species in the hepatoma membranes should give a clear insight.

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Ether Lipid Derivatives: Antineoplastic Activity In Vitro and the Structure-Activity Relationship

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The antineoplastic activity of two ether lipid derivatives, the alkyl-lysophospholipid derivative (ALP) ET-18-OCH_3 and the ether-linked lipoidal amine CP-46,665 was tested in a human tumor clonogenic assay (HTCA) in vitro. CP-46,665 suppressed the colony formation of various human tumors with a slight dose response relation after 1 hr incubation and with a clear optimum (85% response rate) after continuous exposure in the higher dose range tested (10 μg/ml). ET-18-OCH_3 did not have substantial activity after 1 hr of incubation. However, when continuous exposure to the compound was used, ET-18-OCH_3 seemed to have a modest dose response effect and yielded a response in about 60% of the tumor cell samples tested in the higher dose range (10 μg/ml). Thus, both compounds have in vitro antitumor activity in the HTCA within a dose range of 1–10 μg/ml, especially during continuous exposure. The tumor specific type activity was found in breast cancer, ovarian cancer, lung cancer and mesothelioma. Both compounds caused decreases in colony formation down to the 0%, 2% and 4% levels. In a comparison of specimens in which both compounds were used, only one of five times showed a discordance in sensitivity or resistance; therefore the compounds appear similar in their in vitro activity.

In a second set of experiments we tested the structure-activity relationship among a variety of ALP in the [^{3}H]thymidine incorporation assay after incubation with HL-60 leukemic blasts and other neoplastic cells from human origin. From these studies it can be concluded that in the ALP the alkyl linkage in the sn-1 position is a necessary prerequisite for cytotoxicity; furthermore, in the majority of tumors tested the substitution of the sn-2 position to prevent reacylation of the molecule is necessary for cytotoxicity.

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During recent years there has been increasing interest in the antitumor activity of certain ether lipids. ALPs represent a new class of biological response modifiers (1) which inhibit the growth (1,2) and metastasis (3) of syngeneic murine tumors and also have been used successfully in treating experimental rat tumors (4,5). This activity might be partially mediated by augmenting cytotoxic properties of macrophages (1–3) and direct effects on neoplastic cells, since some ALP destroy leukemic (6–8) and tumor cells (9,10) and others induce differentiation of leukemic blasts (11) or counteract tumor cell invasion (12). The first clinical pilot studies with the analogue rac 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine (ET-18-OCH_3) are in progress (13). However, other groups of ether lipids also have been shown to possess antitumor activity. They include glyceryl-ethers of fatty alcohols (14,15), halo-analogues of ALP (16), thioether-lysophospholipid derivatives (17), alkyl-ethyleneglycophospholipids (18,19), various analogues of platelet-activating factor (20) and certain alkyl-linked lipoidal amines such as CP-46,655 (21). Putative molecular mechanisms leading to the cytotoxicity of this material toward tumor cells have been reviewed recently (22).

In this report, we summarize data testing two different ether lipids, the ALP ET-18-OCH_3 and the alkyl-linked lipoidal amine CP-46,665, in the HTCA and testing the relationship between in vitro activity and structure of a variety of ALPs.

MATERIALS AND METHODS

Drugs. 4-Aminomethyl-1-[2,3-(di-n-decyloxy)-n-propyl]-4-phenyl-piperidine (CP-46,665) was supplied by K. E. Jensen, Pfizer Central Research (Groton, Connecticut). Details on chemistry, pharmacokinetics and toxicology have been published before (23–25). The ALP were synthesized in our laboratories or purchased from Medmark Chemicals (D-8022 Gruenwald bei Muenchen, Federal Republic of Germany). Figure 1 depicts chemical structures of CP-46,665 and 2-lysophosphatidylcholine (2-LPC). It furthermore explains the positions of the synthetic modifications of the 2-LPC leading to the class of ALP and the abbreviations used.

Cells. After surgery a variety of solid human tumors as well as malignant effusions were brought into single cell suspensions by methods described earlier (26–28)). Tumor samples obtained included breast cancers, colorectal cancers, kidney cancers, lung cancers, melanomas, ovarian carcinomas, mesotheliomas, soft tissue sarcomas, thymomas and various gynecological tumors. The cell suspensions of these tumor samples were incubated for 1 hr or continuously with one and 10 μg/ml of the compounds tested and directly assayed in the HTCA. Cells of the human leukemia HL-60 were continuously grown in vitro as monolayer cultures using serum-containing culture medium with a method described before (21).

In vitro assays for antineoplastic activity. The HTCA was performed using a method originally described by Hamburger and Salmon (26,27) and Von Hoff (28). Cells were incubated with the compounds for 1 hr or the compounds were continuously present in the double layer agar system throughout the test period. Antitumor activity of the test substances in this assay was tested as a suppression of colony formation of the tumor cells and a response was stated if a suppression of colony formation

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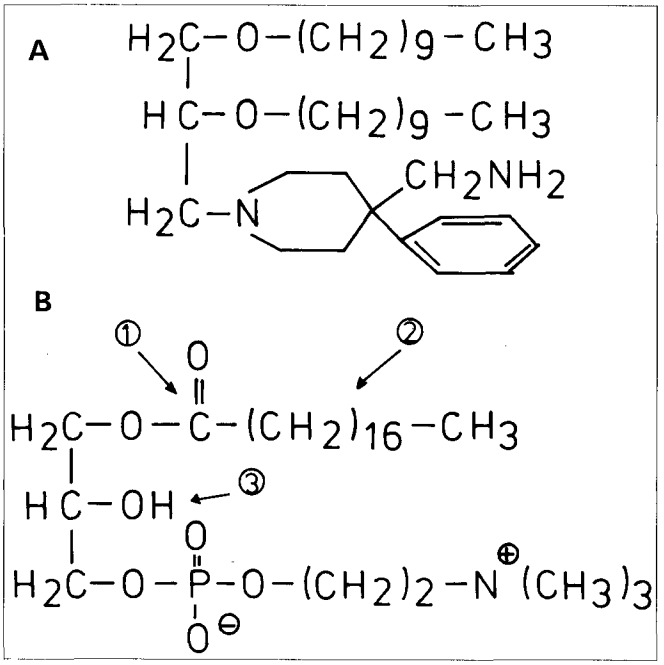

FIG. 1. Chemical structures of CP-46,665 (A) and 2-LPC (B). The structure of 2-LPC contains the positions of its synthetic modifications (1-3). ET: ether linkage in position 1 (ES: ester linkage); 18: number of carbon atoms in the aliphatic side chain (position 2); OCH_3: substitution of the sn-2 hydroxyl-group in position 3.

of more than 50% occurred in the drug test plates in comparison to the control plates.

The [^{3}H]thymidine incorporation assay was performed by a method described recently (21). In this assay HL-60 cells were incubated with the test compounds for various times in serum-containing medium, and antiproliferative, cytotoxic action of the ether lipids was measured as a decrease of [^{3}H]thymidine incorporation into the cells given as percentage of the controls.

RESULTS

In a first set of experiments, the antitumor activity of the ether lipid derivatives CP-46,665 and ET-18-OCH_3 at both 1 hr and continuous exposure was examined in the HTCA. Table 1 summarizes these data. Compound CP-46,665 suppressed the colony formation of various human tumors with a slight dose response relation after 1 hr incubation and with a clear optimum after continuous exposure in the higher dose range tested (10 μg/ml). ET-18-OCH_3 did not have substantial activity after 1 hr of incubation. However, with continuous exposure to the compound, ET-18-OCH_3 seemed to have a modest dose response effect and yielded a response in about 60% of the tumor cell samples tested in the higher dose range (10 μg/ml). In summary both compounds have in vitro antitumor activity in the HTCA within a dose range of 1-10 μg/ml, especially during continuous exposure. The tumor-specific type activity was found in breast cancer, ovarian cancer, lung cancer and one mesothelioma. It was of particular note that these ether lipid derivatives caused impressive decreases in colony formation down to the 0%, 2% and 4% levels, which was comparable to a group of other cytotoxics including doxorubicin tested against the same tumors in parallel. This was particularly true for the compound CP-46,665. However, in a comparison of specimens in which both CP-46,665 and ET-18-OCH_3 were used, only one of five times showed a discordance in sensitivity or resistance (data not shown). The compounds thus appear similar in their in vitro activity.

TABLE 1

Summary of Data on CP-46,665 and ET-18-OCH_3 in a Human Tumor Cloning System

Compound	No. responses/no. evaluated	
	1 Hr exposure (%)	Continuous exposure (%)
CP-46,665		
1.0 μg/ml	3/7 (42)	1/7 (14)
10.0 μg/ml	3/6 (50)	6/7 (85)
ET-18-OCH_3		
1.0 μg/ml	2/6 (33)	3/6 (50)
10.0 μg/ml	0/4 (0)	3/5 (60)

In a second set of experiments we tested the structure-activity relationship among a variety of ALP using ES-16/18-OH (2-LPC), ET-18-OH (racemic), the isomers in sn-2 D-ET-16-OH and L-ET-16-OH, ET-12-H, ET-18-H and ET-18-OCH_3 in the [^{3}H]thymidine incorporation assay after incubation with HL-60 leukemic blasts. Figures 2 and 3 summarize these results. As can be seen in Figure 2, the compound with an ester linkage in the sn-1 position, ES-16/18-OH, is ineffective in the dose range tested. Activity starts to occur after introduction of an ether bond in the sn-1 position replacing the ester linkage as in the compound ET-18-OH (racemic). Activity increases considerably when, furthermore, in the sn-2 position the lyso-configuration is replaced as in the compounds ET-12-H, ET-18-H and ET-18-OCH_3. Comparing these ALP, modified in the sn-1 and the sn-2 position the length of the aliphatic side chain in sn-1 seems to be of importance, as compounds with a number of carbon atoms in the sn-1 side chain between 16 and 18 seemed to be more active than short chain analogues, e.g., ET-12-H. As Figure 3 interestingly depicts, the activity of a racemic compound with an alkyl bond in the sn-1 position and the sn-2 hydroxyl group seems to be partially based on the activity of its D-isomer in sn-2.

DISCUSSION

The data presented here indicate that certain ether lipids and derivatives with a high metabolic stability (22) exert a clear antineoplastic activity toward cells of a variety of human tumors when assayed as suppression of colony formation in the HTCA in vitro (see Table 1). Particularly in the experiments in which the cells were continuously exposed to the drugs, there was a clear dose response relationship and both compounds were active within a dose range of 1-10 μg/ml. This activity was comparable to a group of other cytotoxics of known clinical activity including doxorubicin, which was tested against the same tumors in parallel. Furthermore, in vitro activity in HTCA in ≥20% of the tumors tested may be roughly predictive for activity in the clinic. A tumor-specific type

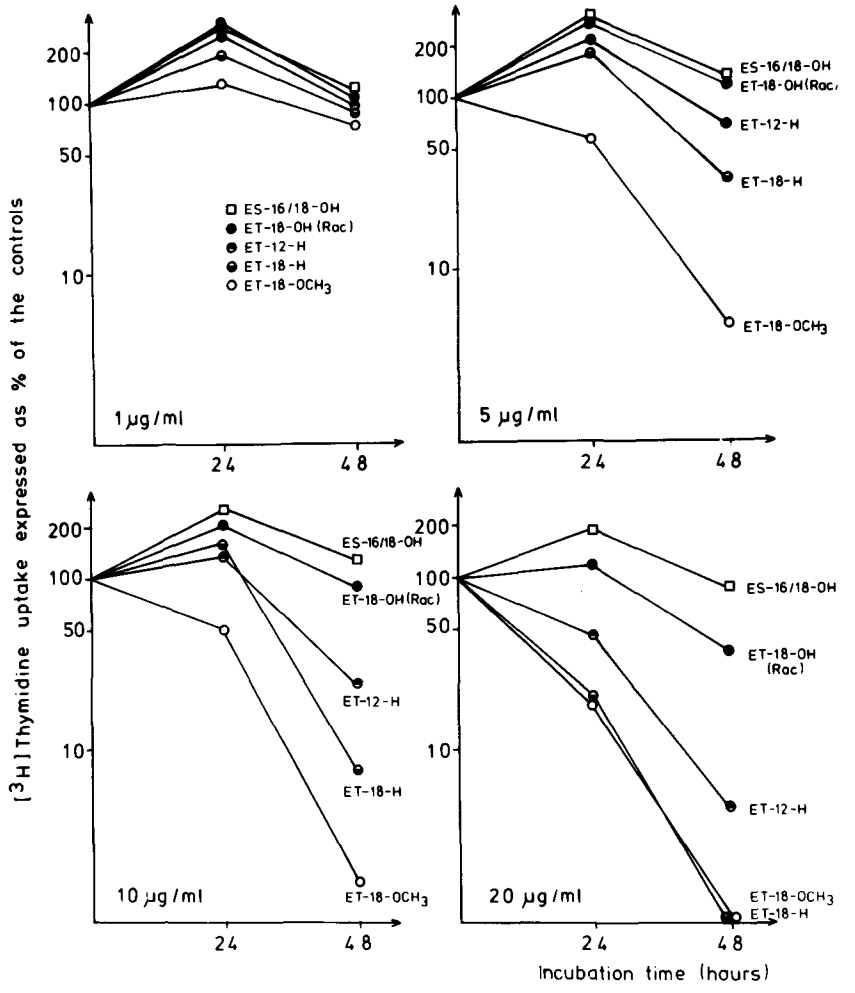

FIG. 2. Structure-cytotoxicity relationship of different ALP compounds as tested in the [^{3}H]thymidine incorporation assay after incubation of the ALP with HL-60 leukemia cells for the times indicated.

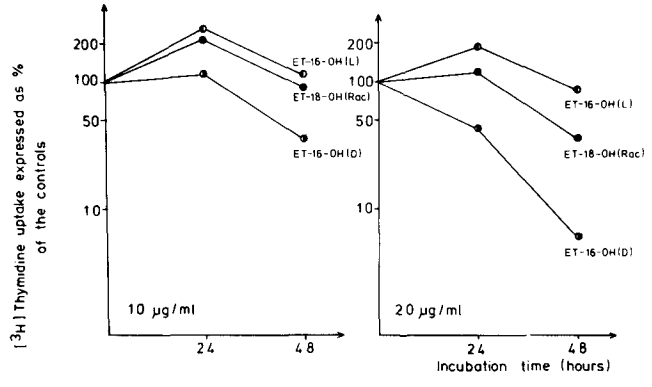

FIG. 3. Structure-cytotoxicity relationship between the racemic ET-18-OH and the D- and L-forms of ET-16-OH as tested in the [^{3}H]thymidine incorporation assay after incubation of the compound with HL-60 leukemia cells for the times indicated.

activity was found in breast cancer, ovarian cancer, lung cancer and mesothelioma. However, the precise relevance of this in vitro activity for the importance of these drugs in human cancer therapy is uncertain unless we know the concentrations of these compounds that could be achieved in man. Pharmacokinetic studies in man have not yet been performed. However, ALP-type compounds have already shown therapeutic activity in various animal tumor systems in vivo (22) and also have yielded first tumor responses in clinical pilot studies (13). But also here critical evaluation of the results is necessary since it is not yet completely understood whether this in vivo activity of the compounds is based on biological response modification, e.g., augmentation of macrophage cytotoxicity, or on direct cytotoxic properties of the material, as has been studied in this report (for further discussion see [22]). Furthermore, first in vivo experiments in mouse and rat tumors indicate a small therapeutic range for CP-46,665 (unpublished data).

The molecular mechanisms on which the cytotoxicity of the lipoidal amine CP-46,665 is based could be related to the affinity of this material to cellular membranes and its high metabolic stability, but have not yet been studied in detail. There is already data available on the molecular mechanism of the cytotoxicity of the ALP-type compounds (29–31). ALP-type compounds, e.g., ET-18-OCH_3 or ET-18-H, are not metabolizable by lysophospholipase or acyltransferase; furthermore, many neoplastic cells in contrast to normal tissues lack a 1-*O*-alkyl-cleavage enzyme (32,33). Thus, the material or its metabolites accumulate in tumor cells and might then act as an antimetabolite for vital pathways of the phospholipid

metabolism and thereby lead to destruction of the cellular membranes (29), or might after accumulation consecutively inhibit certain enzyme systems as a phospholipid-sensitive, calcium-dependent protein kinase (31). This might then lead to cell death. However, other yet unknown mechanisms also might add to the toxicity of these compounds. Our studies concerning the structure-activity relationship of various ALP (see Figs. 2 and 3) are in accordance with the previous findings reported. It could be demonstrated that the smallest synthetic modification necessary to render 2-LPC a cytotoxic molecule was the replacement of the ester bond in sn-1 position by an ether linkage. However, the cytotoxicity of the compound could be considerably increased by substituting the sn-2 hydroxyl group, resulting in compounds which cannot necessarily be reacylated (see Fig. 2). Furthermore, the effectiveness of the 1-alkyl-2-lyso compound ET-18-OH if tested as a racemic compound seems to be based on the activity of its D-isomer (see Fig. 3). The experiments regarding the comparison of the activity of the D- and the L-forms of the ET-16-OH could be reproduced using freshly explanted blasts of two different acute myeloid leukemias and cells of a human kidney carcinoma and a colorectal adenocarcinoma (data not shown). However, we also have performed experiments with other solid tumors, e.g., a human astrocytoma, in which both isomers of the compound were of the same activity. On the other hand, compounds with an ester bond in the sn-1 position and a modification in the sn-2 position such as ES-18-OCH_3 do not seem to have cytotoxic activity when tested in leukemias within the same dose range (6; details not shown).

The necessity of a length of the aliphatic side chain in the sn-1 position between 16 and 18 carbon atoms to yield optimum activity might be caused by the comparably high binding of these structures to cell surfaces (34).

We conclude from these studies that in ALP the alkyl linkage in the sn-1 position is a necessary prerequisite for cytotoxicity and, furthermore, that in the majority of tumors tested the substitution of the sn-2 position to prevent reacylation of the molecule is necessary for cytotoxicity.

Final conclusions concerning the molecular mechanism of the cytotoxicity of ALP and related compounds can probably be drawn from studies following the metabolic pathways of this material inside viable cells. In this respect metabolites resulting from activity of phospholipase C deserve more interest (35).

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The Metabolism of Malondialdehyde

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Interest in malondialdehyde (MDA) metabolism stems from its formation as a product of lipid peroxidation in the diet and in the tissues; its reactivity with functional groups of nucleic acid bases, proteins and phospholipids; its mutagenicity in bacteria, and its reported skin and liver carcinogenicity in animals. Administration of the Na enol salt of MDA in the drinking water of mice over a range of 0.1–10.0 μg/g/day for 12 mo produced dose-dependent hyperplastic and neoplastic changes in liver nuclei and increased mortality at the highest level but produced no gross hepatic tumors. Addition of MDA to the medium of rat skin fibroblasts grown in culture caused nuclear abnormalities at concentrations as low as 10^{-6} M despite an uptake of only 4%. [1,3-^{14}C]MDA was rapidly oxidized to [^{14}C]acetate in rat liver mitochondria and to $^{14}CO_2$ in vivo; however, ~10% of the radioactivity was recovered in the urine. Chromatographic analysis of rat urine revealed the presence of several compounds which yield MDA on acid hydrolysis. Total MDA excretion increased in response to conditions which stimulate lipid peroxidation in vivo, including vitamin E deficiency, Fe or CCl_4 administration, and enrichment of the tissues with PUFA. N-acetyl-e-(2-propenal)lysine was identified as a major urinary metabolite of MDA in rat and human urine. This compound is derived primarily from N-α-(2-propenal)lysine released in digestion as a product of reactions between MDA and the ε-amino groups of N-terminal lysine residues in food proteins. However, its presence in the urine of animals fasted or fed MDA-free diets indicates that it is also formed in vivo. Identification of the metabolites of MDA excreted in the urine may provide clues to the mechanisms of cellular damage caused by this compound in the tissues.
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Interest in the metabolism of malondialdehyde (MDA) stems from its reactivity with the functional groups of a variety of cellular compounds, including the amino groups of proteins (1) and nucleic acid bases (2), the N bases of phospholipids (3), and the SH groups of sulfhydryl compounds (4). Its affinity for the N atoms of DNA bases, crosslinking action, nucleotoxicity at low concentrations in animal cells grown in culture and mutagenic activity in bacteria also have created an interest in its potential carcinogenicity.

MDA occurs in foods in amounts ranging from <0.1 to about 10 ppm, depending upon their fatty acid composition and conditions of storage (5,6). It arises predominantly from the oxidation of polyunsaturated fatty acids (PUFA) with three or more double bonds, and therefore generally occurs at higher concentrations in foods of animal origin. It is also formed in vivo, both nonenzymatically as a product of lipid peroxidation and enzymatically as a product of the cyclooxygenase reaction in prostaglandin metabolism.

The following is a summary of recent work on the occurrence, toxicity and metabolism of MDA conducted in this and other laboratories.

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CHRONIC TOXICITY OF MDA IN ANIMALS

Although application of a large dose of MDA to the skin of mice followed by daily treatment with croton oil has been reported to produce a high incidence of skin tumors, and daily application of MDA alone to produce internal tumors (7), chronic oral administration has failed to produce clear evidence of tumorigenesis (8,9). Nevertheless, administration of MDA as the Na enol salt in the drinking water at a level of 10 μg/g body weight/day for 12 mo significantly increased mortality from other causes (9). There was also a dose-dependent increase in hyperplastic and neoplastic changes in the liver from 0.1 to 10.0 μg/g/day (anisokaryosis, changes in cytoplasmic volume, architectural derangements, necrosis and neoplastic changes). Three animals (6%) given the highest dose developed the only stomach neoplasms observed. A further 22-mo study again yielded an increase in liver lesions but no increase in tumor incidence.

While these studies are reassuring with respect to the carcinogenicity of dietary MDA, the occurrence of hepatic nucleotoxicity in mice at an intake of free MDA as low as one μg/g/day is noteworthy. The results also do not necessarily reflect the carcinogenicity of MDA which may be formed in proximity to DNA in nuclear membranes.

MDA TOXICITY FOR CELLS GROWN IN CULTURE

Addition of MDA to the medium of rat skin fibroblasts grown in culture causes concentration-dependent abnormalities over a range of 10^{-6} M to 10^{-3} M (10) (Table 1). The lesions are predominantly nuclear: micro- and multinucleation, karyorrhexis, chromosomal fragments, chromatid breaks and increased DNA repair synthesis. Small and irregular nuclei are observed at 10^{-5} and 10^{-6} M MDA. The results imply that MDA formed in the nuclear

TABLE 1

Effects of Malondialdehyde on Rat Skin Fibroblasts Grown in Culture[a]

Conditions	Effects
10^{-3} M, 120 hr	Altered morphology, cytoplasmic vacuolization, karyorrhexis, micro- and multinucleation, < mitotic index, < DNA, RNA and protein synthesis
10^{-4} M, 12 hr	Chromosomal fragments, achromatic lesions, chromatid breaks
10^{-4} M, 120 hr	Mitotic aberrations, micronucleation, < mitotic index, < DNA synthesis
10^{-5} M, 10^{-6} M, 3 hr	> DNA repair synthesis
10^{-5} M, 10^{-6} M, 120 hr	Small and irregular nuclei

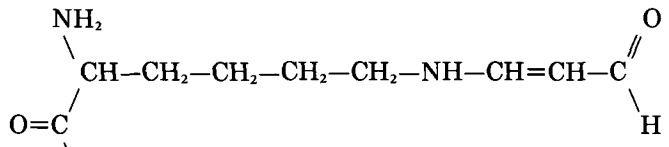
[a]Bird and Draper (10).

membranes of mammalian cells in vivo may be genotoxic. Studies on bacterial cells have demonstrated that MDA is a weak mutagen (11). Quantitative evaluation of MDA toxicity for cells grown in culture is complicated by its rapid binding to proteins and other ingredients of the medium.

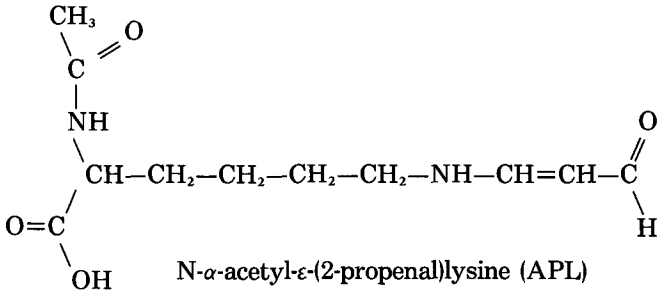
FORMS OF MDA IN THE DIET

A controversy which has persisted for many years over the specificity of the spectrophotometric method for the estimation of MDA in foods and other biological materials as the thiobarbituric acid (TBA) derivative (12) appears to have been satisfactorily resolved by the development of HPLC methods which clearly separate the TBA-MDA complex from other TBA reaction products. There remains a problem of determining the optimal conditions for release of MDA from its "bound forms," which differ from one material to another and require different conditions for hydrolysis. It is difficult, for example, to hydrolyze all the MDA bound to a meat sample without using strongly acidic conditions which endanger the stability of the TBA-MDA complex. Also, MDA may be formed from polyunsaturated fatty acids, endoperoxides and other compounds in the course of the TBA assay procedure. Thus, although MDA can be estimated satisfactorily as the TBA-MDA complex by HPLC, this method gives no indication of the forms in which MDA originally was present in the sample or how much may have been formed from precursors during the procedure.

To evaluate the possible pathogenicity of MDA in the diet, it is necessary first to determine the form(s) in which it occurs in foods and is absorbed from the gastrointestinal tract. Based on the observation that MDA reacts with the ε-amino group of terminal lysine residues in proteins (13), beef muscle protein was exposed to MDA in solution and then digested sequentially with pepsin and hog intestinal juice. Thin layer chromatography of the digesta revealed the presence of a TBA-reactive compound which was purified and shown to be the lysine-MDA adduct N-ε-(2-propenal)lysine (Scheme 1) (Piche, L. A., and Draper, H. H., unpublished results). If this adduct is the main form of dietary MDA released during digestion and



N-ε-(2-propenal)lysine



N-α-acetyl-ε-(2-propenal)lysine (APL)

SCHEME 1

absorbed into the blood stream, its metabolic fate, including the extent, if any, to which it undergoes scission to release free MDA, is of obvious toxicological interest.

OXIDATION OF MDA IN VITRO AND IN VIVO

MDA is rapidly oxidized by rat liver mitochondria through the action of the low specificity mitochondrial aldehyde dehydrogenase. In vitro experiments using [1,3-^{14}C]MDA yielded an apparent K_m and V_{max} of 0.5 mM and 9.3 nmol/min/mg protein for O_2 uptake, respectively, and 2.0 mM and 2.4 nmol/min/mg protein for $^{14}CO_2$ production (14). The biochemical sequence involves oxidation to malonic semialdehyde and decarboxylation to form acetaldehyde which is converted to acetate by the same dehydrogenase enzyme.

[1,3-^{14}C]MDA administered to rats by stomach intubation was extensively oxidized to $^{14}CO_2$, but at a slower initial rate than that for ^{14}C-acetate (14) (Table 2). About 65% of the ^{14}C administered was recovered in expired $^{14}CO_2$ within 12 hr, ca. 13% in the urine and 10% in the feces. The amounts of ^{14}C recovered in the urine and feces were greater than those for ^{14}C-acetate and were present in reaction products formed either in the intestinal lumen or in the tissues.

URINARY MDA AS AN INDICATOR OF LIPID PEROXIDATION IN THE DIET AND TISSUES

Based on reports of the presence of MDA in urine, confirmation of these reports by an HPLC procedure, and the finding of labeled metabolites in rat urine after oral administration of ^{14}C-MDA, the urinary excretion of MDA was evaluated as a possible reflection of lipid peroxidation in the diet and in the tissues (15).

MDA excretion was found to be responsive to its oral administration as the Na enol salt, ingestion of MDA-treated serum albumin and feeding a diet containing a highly peroxidizable oil (cod liver oil) (Table 3). Urinary MDA also increased in response to the increase in lipid peroxidation in vivo produced by vitamin E deficiency and administration of iron nitrilotriacetate. Administration of DPPD, a biologically active lipid antioxidant, but not BHA, a nonbiologically active antioxidant, reversed the increase in MDA excretion by vitamin E-deficient animals. However, urinary MDA was not responsive to the Se- and vitamin E-deficient hepatonecrogenic Torula yeast diet.

TABLE 2

Percent Recovery of Radioactivity from Rats 12 hr after Intubation with [1,3-^{14}C]MDA or [1,2-^{14}C]Acetate[a]

	^{14}C-MDA	^{14}C-Acetate
$^{14}CO_2$	75	65
Urine	2.5	13
Feces	1.5	10
Plasma	0.1	0.1
Total	79.1	88.1

[a]Siu and Draper (14).

TABLE 3

Influence of Various Conditions on Urinary Malondialdehyde in Rats[a]

Condition[b]	Urinary MDA μg/24 hr[c]
+E diet	1.2 ± 0.2
−E diet	3.8 ± 0.7
−E diet + 0.1% DPPD	1.2 ± 0.1
10% Coconut oil + 5% corn oil diet	1.1 ± 0.1
10% Corn oil + 5% cod liver oil diet	10.7 ± 1.8
Low PUFA diet + 48 hr fast	1.3 ± 0.2
High PUFA diet + 48 hr fast	2.9 ± 0.2
NTA injection	1.4 ± 0.2
FeNTA injection (9 mg Fe/Kg)	7.4 ± 0.8

[a]See ref. 15 for details.

[b]E, vitamin E; DPPD, N,N'-diphenyl-p-phenylene diamine; PUFA, polyunsaturated fatty acid; NTA, nitrilotriacetate; FeNTA, iron nitrilotriacetate.

[c]Mean ± SEM.

It is noteworthy that rats chronically fed a high PUFA diet exhibit an increase in urinary MDA when subsequently fasted or fed an MDA-free diet (Table 3), indicating that enrichment of the tissues with highly unsaturated fatty acids results in an increase in lipid peroxidation in vivo even in the presence of normal concentrations of vitamin E. Fasting for more than 24 hr also results in an increase in MDA excretion, implying that lipolysis is associated with peroxidation of the fatty acids released.

The diet appears to be the main source of urinary MDA under most conditions, and it is therefore necessary to employ fasting or an MDA-free diet to evaluate MDA excretion as an indicator of lipid peroxidation in vivo. While urinary MDA may be useful as an index of generalized lipid peroxidation in the tissues caused by such factors as vitamin E deficiency or iron administration, it is probably of little value in detecting peroxidative effects on specific target organs, such as the effect of adriamycin on cardiac tissue.

IDENTITY OF MDA COMPOUNDS IN URINE

Identification of the excretory forms of MDA is of interest as a possible clue to its reaction products in the diet and in the body. Ion exchange chromatography of rat urine revealed the presence of several compounds which yield MDA under the acidic conditions of the TBA reaction. There is a notable absence of free MDA. The main metabolite in the urine of rats fed Purina Chow® has been isolated and identified as N-α-acetyl-ε-(2-propenal)-lysine (APL) (Scheme 1) (McGirr, L. G., Hadley, M., and Draper, H. H., unpublished results). This compound appears to be derived mainly from MDA bound to the lysine residues of protein in the diet, from which it is released as a lysine-MDA adduct during digestion and acetylated prior to excretion in the urine. N-acetylation in the liver is a well-known detoxification reaction. Oral administration of lysine-MDA led to excretion of 28% of the dose in the urine as the acetylated derivative, indicating that only a portion of the adduct undergoes acetylation in the tissues.

The presence of APL in the urine of fasting animals and animals fed a saturated fat diet and its excretion in an increased amount after MDA injection indicates that it also is synthesized from MDA formed in vivo. However, its excretion does not increase following the intraperitoneal administration of iron nitrilotriacetate (Hadley, M., and Draper, H. H., unpublished data), despite the fact that there is a several-fold increase in total urinary MDA (Table 3). The varied responses in MDA excretion to different peroxidative stimuli, including the ability of CCl_4 to increase total MDA excretion and the failure of iron to increase APL excretion while increasing the excretion of other metabolites, is evidence for the presence of multiple pathways for the degradation of lipid peroxides. Identification of the MDA derivatives excreted in the urine under various conditions of peroxidative stress may provide a better understanding of the mechanisms and pathophysiology of lipid peroxidation in vivo.

ACKNOWLEDGMENTS

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Stereospecific Analysis of Fatty Acid Esters of Chloropropanediol Isolated from Fresh Goat Milk

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The fatty acid esters of chloropropanediol isolated from goat milk fat in small quantities were subjected to a stereospecific analysis via phospholipase C and phosphocholine esters as intermediates. Synthetic rac-1-chloro-2,3-dioleoyl-propanediol was prepared by standard methods and was used as a control. The stereospecific analyses were performed following a release of the fatty acids from the primary positions of each chloropropanediol diester with pancreatic lipase. The resulting X-1-chloro-2-acylpropanediols were then converted into the corresponding phosphocholine derivatives by a stepwise reaction with phosphorus oxychloride and choline chloride. The X-1-chloro-2-acyl-3-phosphocholinepropanediols were subjected to hydrolysis with phospholipase C *(C. perfringens)*, which hydrolyzed 50% of the phosphatide within two min and the rest of it in two hr. From previous experience with glycerol esters, it was assumed that the more rapidly hydrolyzed molecules were the sn-1-chloro-2-acyl-propanediol derivatives and the more slowly hydrolyzed ones the sn-2-acyl-3-chloropropanediol derivatives. A hydrolysis with phospholipase A_2 *(Crotalus adamanteus)* released 50% of the total fatty acid along with the corresponding lyso compound within 10 min, after which there was no further reaction. The hydrolysis products were assayed directly by gas liquid chromatography (GLC) or were isolated by thin layer chromatography (TLC) prior to quantitation by GLC. Both naturally occurring and synthetic chloropropanediol diesters behaved similarly on stereospecific analysis and were therefore concluded to be racemic.

Lipids 21, 309–314 (1986).

Fatty acid esters of chloropropanediol have now been isolated from natural sources in several laboratories. Valisek et al. (1,2) and Davidek et al. (3) identified the mono- and diesters of chloropropanediol as components of HCl hydrolysates of proteins from vegetable meals and flours containing triacylglycerols, while Gardner et al. (4) reported the isolation of the chloropropanediol esters from toxic Spanish cooking oils believed to have been treated with HCl. In both instances the chemical structure of the esters was established beyond doubt by mass spectrometry (MS), while the assumed chemical genesis left little doubt about the racemic nature of the product. Stereospecific analyses, however, were not carried out and the relationship of the fatty acid composition in the chloropropanediol esters and in the parent glycerol esters was not investigated.

Recently Cerbulis et al. (5) identified chloropropanediol esters in raw milk from several herds of goats not known to have been exposed to halogenating agents during either milk production or processing. It was demonstrated that the composition and molecular association of the fatty acids in the chloropropanediol diesters corresponded to that found in the long chain diacylglycerol moieties of goat milk triacylglycerols (6), but the stereochemical configuration of the chloropropanediol esters was not determined and their origin in the milk fat was not established. Since a stereospecific analysis could potentially distinguish between enantiomeric biosynthesis from chloropropanediol and a nonspecific acylation of a racemic monoacyl derivative of chloropropanediol, we have now determined the stereochemical configuration of the chloropropanediol diesters of goat milk. The results show that the chloropropanediol diesters of goat milk fat are racemic. The implications of these findings are discussed.

MATERIALS AND METHODS

The chloropropanediol diester fractions of raw goat milk were prepared as described by Cerbulis et al. (5). 3-Chloro-1,2-propanediol and 1,3-propanediol were from Aldrich Chemical Co. (Milwaukee, Wisconsin). Synthetic rac-1-chloro-2,3-propanediol dioleate was prepared by acylation of rac-1-chloropropanediol in benzene with oleic acid anhydride in the presence of dimethylaminopyridine. The synthetic ester was isolated by TLC using petroleum ether/diethyl ether (88:12, v/v) as the developing solvent. It gave a single peak on capillary GLC corresponding to a C_{36} acyl carbon number ester of chloropropanediol identified in the goat milk fat (6). Liquid chromatography (LC)/MS yielded a single peak for an 18:1 18:1 species of chloropropanediol corresponding to a similar peak identified in goat milk fat.

Preparation of X-1-chloro-2-acylpropanediols. The synthetic and natural chloropropanediol diesters (1–2 mg) were dissolved in diethyl ether (0.5 ml) and separately hydrolyzed nearly to completion with pancreatic lipase as described previously (7). The reaction mixture was extracted with diethyl ether and the free fatty acids, X-1-chloro-2-acylpropanediols and any unhydrolyzed chloropropanediol diesters were resolved by TLC using chloroform/acetone (97:3, v/v) as the solvent. The X-1-chloro-2-acylpropanediols ran just ahead of the free fatty acids and were located by spraying with a solution of dichlorofluorescein. The X-1-chloro-2-acylpropanediols and the free fatty acids were recovered by extracting the scrapings of appropriate silica gel areas with chloroform/methanol (2:1, v/v).

Preparation of X-1-chloro-2-acylpropanediol-3-phosphocholines. The method of synthesis was modeled on the preparation of rac-phosphatidylcholines described earlier (7). A chilled solution (0.65 ml) of chloroform/pyridine/phosphorus oxychloride (47.5:47.5:5, v/v/v) was added to 0.5–1 mg X-1-chloro-2-acylpropanediols contained in a 15 ml tube with a Teflon-lined screw cap. The contents were mixed and allowed to stand for one hr at 0 C and one hr at 25 C. The solution was then transferred to a similar tube containing 200 mg dry powdered choline chloride and a small magnetic stirring bar. The mixture was vigorously stirred for 15 hr at 30 C and then for

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30 min more after addition of 20 μl of water. The reaction products were worked up as described for the phosphatidylcholines (7). The X-1-chloro-2-acylpropanediol-3-phosphocholines were purified and isolated by TLC using silica gel H and chloroform/methanol/acetic acid/water (75:45:12:6, v/v/v) as the solvent (8) and chloroform/methanol/water/acetic acid (50:39:10:1, v/v/v/v) as the extracting solvent (9). An identical procedure was employed for the preparation of the rac-1-chloro-2-oleoylpropanediol-3-phosphocholine.

Stereospecific hydrolysis with phospholipase C. The chloropropanediol phosphocholines were digested with phospholipase C under the general conditions described previously for the stereospecific analysis of X-1,2-diacylphosphatidylcholines (7). The X-1-chloro-2-acylpropanediolphosphocholine (100 μg) was vortexed for 15 sec with 0.5 ml diethyl ether and 0.5 ml of buffer (17.5 mM tris(hydroxymethyl)aminomethane, pH 7.3, containing 1 mM $CaCl_2$ and 0.5 units phospholipase C *(C. perfringens).* The mixture was then shaken at 37 C on a Buchler rotary Evapo-mix for various periods of time. At selected time intervals the digestion was stopped by chilling the reaction mixture in an ice bath and the lipids were extracted with chloroform/methanol (2:1, v/v). The extent of hydrolysis of the chloropropanediol phosphocholines was monitored by TLC and GLC of the transmethylated reaction products, as described for the phospholipase C digestion of *rac*-phosphatidylcholines (7).

Hydrolysis with phospholipase A_2. The monoacyl esters of chloropropanediolphosphocholine were hydrolyzed with phospholipase A_2 using a scaled-down version of the procedure described previously (10). To 100 μg substrate were added 0.5 ml diethyl ether and 0.5 ml buffer with $CaCl_2$ (as described above for phospholipase C) containing 10 units phospholipase A_2 *(Crotalus adamanteus).* The mixture was vortexed for 15 sec and then shaken at 37 C for periods up to two hr on a Buchler rotary Evapo-Mix. The reaction products were isolated by TLC using a double development. The silica gel H plate was first developed to a height of 9 cm with chloroform/methanol/acetic acid/water (75:45:12:6, v/v/v/v) and after a brief period of drying was rechromatographed to a height of about 15 cm with heptane/isopropyl ether/acetic acid (60:40:4, v/v/v). The free fatty acid and residual acylchloropropanediolphosphocholine were quantitated by GLC of the fatty acid methyl esters following methylation in the presence of heptadecanoic acid as internal standard (7).

GLC. Capillary GLC of the acylchloropropanediols was performed on a Hewlett-Packard Model 5880A gas chromatograph (Hewlett-Packard Co., Palo Alto, California) as previously described (11). The chromatograph was equipped with an on-column injector and a glass capillary column (8 m × 0.32 mm I.D.) wall-coated with bonded SE-54 (Hewlett-Packard). Hydrogen was used as the carrier gas. The column temperature was programmed linearly from 40 to 150 C at 30 C/min, then to 230 C at 20 C/min, to 280 C at 10 C/min and finally to 340 C at 5 C/min. Capillary GLC of the fatty acid methyl esters was performed on glass columns (10 m × 0.25 mm I.D.) coated with a polar SP-2330 liquid phase and supplied by Supelco (Bellefonte, Pennsylvania). The columns were installed and operated as previously described (7).

LC/MS. High performance liquid chromatography (HPLC) analyses were performed with a Hewlett-Packard Model 1084B liquid chromatograph equipped with a Supelcosil LC-18 reversed phase column (Supelco) using a gradient of 30–90% (by vol) propionitrile in acetonitrile. The columns were operated at a flow rate of 1.5 ml/min and 30 C oven temperature. MS was done on a Hewlett-Packard Model 5985B quadrupole mass spectrometer equipped with a Hewlett-Packard direct liquid inlet interface and positive and negative ion detectors as previously described (13). Full mass spectra were taken every seven sec in the 200–800 mass range over the entire HPLC elution profile. Single ion chromatograms were constructed by extracting appropriate intensities from the total mass spectra stored on magnetic discs.

RESULTS

Chromatography of X-1-chloro-2,3-diacylpropanediols. The natural and synthetic diacylchloropropanediols were characterized by TLC, GLC and HPLC. The natural compounds and the synthetic X-1-chloro-2,3-dioleoylpropanediol showed identical Rf values on TLC in the solvent systems tested. On GLC the natural chloropropanediol diesters gave a series of peaks ranging in acyl carbon number from 26 to 38. The synthetic dioleate gave a single peak, which overlapped with acyl carbon 36 in capillary GLC of the natural compounds. Figure 1 shows a capillary GLC run as obtained for the chloropropanediol diesters and for the triacylglycerols of goat milk fat. The identity of the synthetic dioleoyl with the natural dioleoylchloropropanediol is further indicated in Figure 2, which compares the mass spectra of the synthetic and natural compounds following emergence from the HPLC column. The mass spectra show identical intensities for the M + 1 and the MH-RCOOH ions at m/z 639 and 357, respectively.

Table 1 gives the total and positional distribution of the fatty acids in the natural chloropropanediol diesters as obtained by analysis and by calculation. There was a reasonably close agreement between the calculated

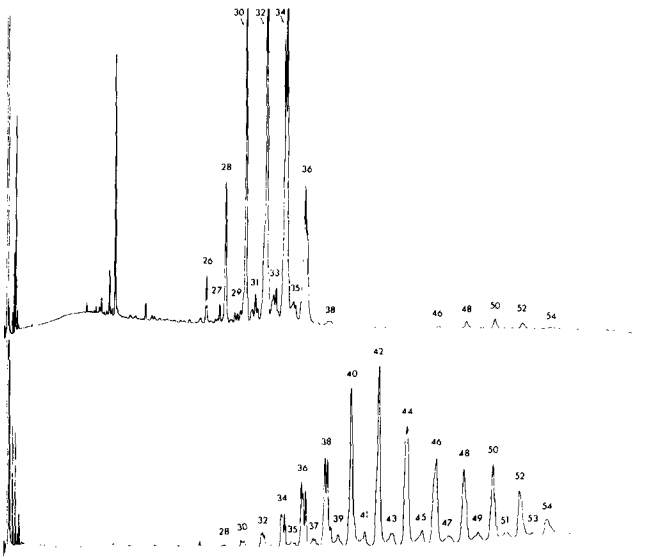

FIG. 1. Carbon number profiles of the chloropropanediol diesters (upper panel) and triacylglycerols (lower panel) of goat milk fat. Peaks are identified by number of total acyl carbons per acyl ester molecule. Capillary GLC conditions as given in text.

composition of the primary positions and the composition of the free fatty acids released by pancreatic lipase. The essential correctness of the composition and positional distribution of the fatty acids in the natural chloropropanediol diesters is indicated in Table 2, which compares the experimentally determined and calculated compositions of the X-1-chloro-2,3-diacylpropanediols. The theoretical values were obtained by 1(3)-random-2-random calculation (14).

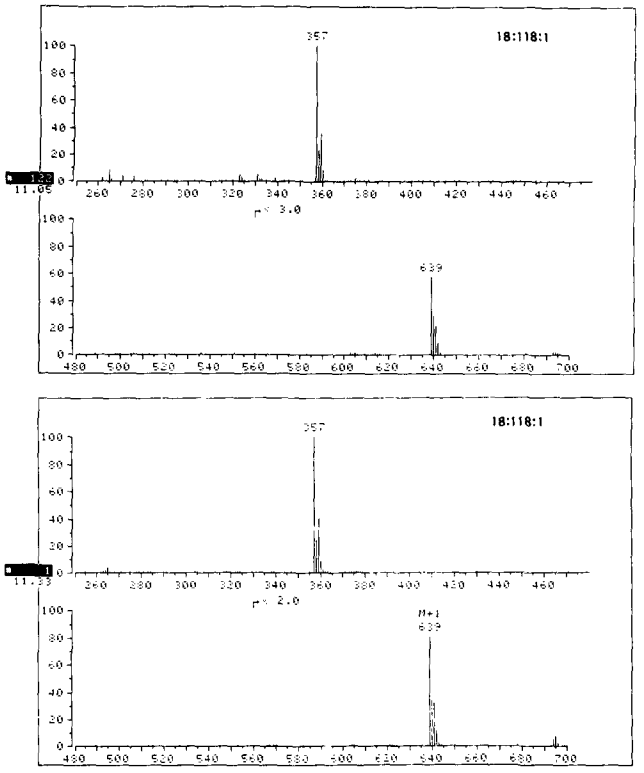

FIG. 2. Positive chemical ionization spectra of natural dioleoylchloropropanediol (upper panel) and synthetic dioleoylchloropropanediol (lower panel). Both spectra were obtained in the LC/MS mode using a gradient of propionitrile in acetonitrile as described in text.

Structure of X-1-chloro-2-acylpropanediol-3-phosphocholine. The chemical structure of the phosphocholine derivatives of the chloropropanediols isolated from the goat milk fat was implied on the basis of the known attack of pancreatic lipase on the primary ester bonds (15) and chemical synthesis. This was consistent with hydrolysis by phospholipases A_2 and C. Capillary GLC of the products of pancreatic lipolysis of the diacylchloropropanediol isolated from goat milk and of that synthesized in the laboratory showed chloropropanediol monoesters and free fatty acids.

The two-step conversion of the X-1-chloro-2-acylpropanediol into phosphocholine derivative took place under conditions that had previously given high yields of phosphatidylcholine. The new phosphocholine derivative on TLC migrated more slowly (Rf 0.25) than egg phosphatidylcholine (Rf 0.45). It was clearly resolved from the original monoacyl ester of chloropropanediol (solvent front), as well as from chloropropanediolphosphocholine resulting from hydrolysis of the product with phospholipase A_2, which remained in the water phase.

Stereochemistry of X-1-chloro-2-acylpropanediol. The stereochemical configuration of the X-1-chloro-2-acylpropanediol-3-phosphocholine was demonstrated by means of the stereospecific enzymes, phospholipase A_2 and phospholipase C, using the synthetic rac-1-chloro-2-acylpropanediol-3-phosphocholine as a reference compound. Table 3 shows that the hydrolysis of the synthetic racemate by phospholipase A_2 was complete within 10 min, at which time about 50% of the total fatty acid had been released. There was no further hydrolysis when the incubation time was extended to 2 hr. TLC demonstrated the release of free fatty acid. The deacylated chloropropanediolphosphocholine was retained in the aqueous phase. Thus, the racemic nature of the chloropropanediol diester was established on the basis of the stereospecificity of the phospholipase A_2. The synthetic rac-1-chloro-2-oleoylpropanediolphosphocholine was hydrolyzed by phospholipase C to 50% of total within 2–3 min with a liberation of a chlorooleoylpropanediol. A total hydrolysis of the compound was obtained within two hr, with the chlorooleoylpropanediol being the only lipid-soluble

TABLE 1

Composition of Fatty Acids of Diacylchloropropanediols Isolated from Goat Milk Fat (Mol %)

Fatty acid	Diacylchloropropanediols		Total
	Position 1(3)[a]	Position 2[b]	
10:0	3.0	—	1.5
12:0	5.0	1.0	3.0
14:0	15.6	10.2	12.9
15:0	2.1	1.2	1.7
16:0	35.9	43.5	39.7
16:1	0.7	1.5	1.1
17:0	3.7	2.5	3.1
18:0	17.5	23.2	20.4
18:1	15.5	16.5	16.0
18:2	0.05	0.09	0.07
20:0	0.7	0.3	0.5

[a](2 × Total) − position 2.

[b]X-2-monoacyl-3-chloro-sn-propanediol.

TABLE 2

Composition of X-1-Chloro-2,3-Diacylpropanediols from Goat Milk Fat (Mol %)

Carbon number	Experimental[a]	Calculated[b]
26	1.8	2.0
27	0.6	0.2
28	6.1	5.4
29	1.0	0.7
30	14.0	13.1
31	3.0	2.2
32	25.4	26.2
33	4.0	3.8
34	28.1	29.7
35	2.7	2.3
36	13.3	13.6

[a]Capillary GLC on a nonpolar liquid phase; average of two determinations.

[b]1(3) Random-2-random calculation.

TABLE 3

Relative Rates of Hydrolysis of Rac-1-Chloro-2-Oleoylpropanediol-3-Phosphocholine by Phospholipase C and Phospholipase A_2

	Percent hydrolysis[a]	
Time (min)	Phospholipase C[b]	Phospholipase A_2[c]
1	43	
2	47	
3	50	
5	—	41
10	57	
20	65	
90	95	
120	99	50

[a]Average of two determinations.
[b]*Clostridium perfringens.*
[c]*Crotalus adamanteus.*

TABLE 4

Composition of Stereoisomers of X-1-Chloro-2,3-Diacylpropanediols from Goat Milk Fat

	Percent composition	
Method	sn-1-chloro-	sn-3-chloro-
Phospholipase C (3 min)	55	45
Phospholipase C (2 min)[a]	54	46
Phospholipase A_2 (2 hr)	52	48
Average	54	46

[a]Codigested with rac-1-chloro-2-oleoylpropanediol-3-phosphocholine as internal standard.

TABLE 5

Composition of Fatty Acids of Position 2 of the sn-1-Chloro-2,3-Diacyl- and sn-3-Chloro-1,2-Diacylpropanediols of Goat Milk Fat (Mol %)[a]

	Stereoisomer	
Fatty acid	sn-1-chloro-	sn-3-chloro-
12:0	1.2 ± 0.2	0.8 ± 0.1
14:0	10.2 ± 0.2	10.3 ± 0.1
15:0	1.2 ± 0.1	1.2 ± 0.1
16:0	45.0 ± 0.3	42.0 ± 0.2
16:1	1.5 ± 0.1	1.5 ± 0.1
17:0	2.5 ± 0.1	2.5 ± 0.1
18:0	23.1 ± 0.8	23.3 ± 0.4
18:1	15.3 ± 0.1	17.7 ± 0.5
18:2	0.1 ± 0.0	0.1 ± 0.0
20:0	0.3 ± 0.1	0.3 ± 0.1

[a]Average of phospholipase C and phospholipase A_2 procedures ± range/2.

product. Since phospholipase C has been shown to attack the sn-1,2-diacylphosphatidylcholines much more readily than the sn-2,3-diacylphosphatidylcholines, it was concluded that the enzyme initially released sn-1-chloro-2-oleoylpropanediol, with sn-2-oleoyl-3-chloropropanediol being recovered upon extended hydrolysis. Thus the racemic nature of the synthetic chlorooleoylpropanediol-phosphocholine was confirmed by the stereospecificity of phospholipase C.

Likewise, the results in Table 4 show that phospholipase A_2 was capable of only 52% hydrolysis of the phosphocholine derivative of the chloropropanediol ester derived from raw goat milk. About 54% of the total phosphocholine derivative was quickly hydrolyzed by phospholipase C. Apparently the original natural ester was also racemic.

Table 5 compares the composition of the fatty acids in the 2-position of the sn-1- and sn-3-chloropropanediolphosphocholines. There is good agreement between the two fatty acid distributions. When compared to the composition of the fatty acids in the combined sn-1- and sn-3-positions (Table 1), there were significant differences in the content of the 14:0, 16:0 and 18:0 fatty acids. The primary positions contained more of the 14:0 and less of the 16:0 and 18:0 acids. Because the fatty acids in the primary positions of the chloropropanediol diesters were removed together during pancreatic lipolysis, it was not possible to obtain the fatty acid composition of the sn-1- and the sn-3-positions separately by direct measurement. There was reasonably close agreement between the composition of molecular species of chloropropanediol diesters determined by LC/MS and that calculated assuming identical composition for the sn-1- and sn-3-positions of the diester (data not shown). A close agreement was also found between the calculated carbon number composition and that determined by GLC (Table 2). On the basis of the present data we cannot claim a complete identity in the fatty acid composition of the sn-1- and sn-3-positions in the natural chloropropanediol diesters.

DISCUSSION

Reliability of reference standards. The commercial 3-chloropropanediol was a racemate of the sn-1- and sn-3-chloropropanediols. The chemical synthesis of the racemic chloropropanediol diesters used an acylation method commonly employed for the preparation of glycerol esters. The chlorine was retained by the chloropropanediol backbone during the acylation reaction as shown by LC/MS. The preparation of the phosphocholine derivatives of the chloropropanediol monoesters was modeled on routines established for the derivatization of enantiomeric glycerol esters (7).

Validity of the stereospecific assay. Figure 3 shows the structural configuration of the chloropropanediol derivatives in relation to the corresponding glyceryl analogs. Phospholipase A_2 requires an acyl group in the sn-(n-1)-position with respect to the phosphocholine group. Thus, in addition to the 2-position in sn-3-phosphocholines, the enzyme also hydrolyzes the sn-1-position in 2-phosphocholines (16). An attack on the 2-position is therefore logical and specific in sn-1-chloro-2-acylpropanediol-3-phosphocholine. The enzyme does not attack the 2-position in sn-1-phosphocholine derivatives of glycerol

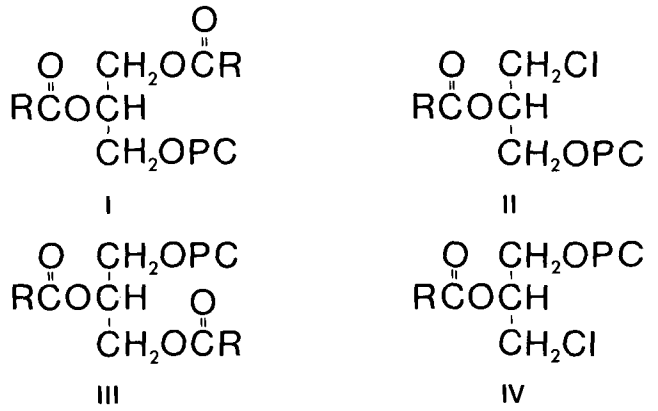

FIG. 3. Stereochemical relationships of diacylglycerophosphocholine and the acylchloropropanediol analogues. I, 1,2-diacyl-sn-glycerol-3-phosphocholine; II, 1-chloro-2-acyl-sn-propanediol-3-phosphocholine; III, 2,3-diacyl-sn-glycerol-1-phosphocholine; and IV, 2-acyl-3-chloro-sn-propanediol-1-phosphocholine.

(16) and would not be expected to attack the corresponding position in the chloropropanediol derivative. A 50% hydrolysis of the phosphocholine derivative of the rac-1-chloro-2-oleoylpropanediol confirms this reasoning and the validity of the phospholipase A_2 assay of the stereospecificity of the chloropropanediol esters.

The phospholipase A_2 hydrolysis of rac-1-chloro-2-oleoylpropanediol-3-phosphocholine was in full agreement with the results of phospholipase C hydrolysis of this compound. The present study shows that the method of preparation of pure enantiomeric diacylglycerols via racemic phosphatidylcholines (7) is also effective for the preparation of the sn-1-chloro-2-oleoyl- and sn-3-chloro-2-oleoylpropanediols and can be used to obtain enantiomeric chloropropanediol diesters and acylphosphocholines. Apparently, the presence of chlorine instead of a fatty ester group had no great effect on the stereospecificity or rate of hydrolysis of the 2-oleoylchloropropanediolphosphocholine when compared to the dioleoylglycerophosphocholine, as enantiomers were hydrolyzed at their characteristic rates. In view of the results with the two different stereospecific phospholipases and the method of chemical synthesis of the reference standards, it follows that the chemical derivatizations and enzymic stereospecific analyses of the goat milk chloropropanediol diesters are also valid.

Origin of fatty esters of chloropropanediol in milk fat. The racemic nature of the chloropropanediol diesters of goat milk would appear to exclude their biosynthesis via the phosphatidic acid pathway, as implied previously (6) on the basis of similarities in composition of molecular species of the chloropropanediol diesters and of the long chain sn-1,2-diacylglycerol moieties of goat milk triacylglycerols. Jones (17) has concluded that chloropropanediol cannot be phosphorylated by glycerokinase.

Alternatively, the chloropropanediol might have entered the mammary gland from the bloodstream in the form of a monoacyl ester, generated by lipoprotein lipase hydrolysis of chloropropanediol diesters in chylomicrons. The monoacyl ester of the chloropropanediol might possibly resemble a monoacylglycerol (single fatty chain), although it has only one hydroxyl group. Both enantiomers of the monoacyl ester then could be further acylated by the mammary gland to yield the racemic diacylchloropropanediol. In such a case the origin of the racemic chloropropanediol esters would have to be sought at the level of the intestinal cell or in the food of the animals. If chloropropanediol diesters were ingested in the food, they would likely become hydrolyzed by pancreatic lipases to the monoacyl esters, which then could be absorbed and acylated by the intestinal cell before incorporation into chylomicrons and secretion into lymph. It is not known, however, whether or not monoacylchloropropanediols are absorbed by the intestine. Although glyceryl esters are usually broken down completely in the rumen (18), the monoacylglycerol pathway has been inferred for ruminants because of the preponderance of palmitate in the sn-2-position in blood and milk lipids (19).

This leaves the possibility that the chloropropanediol diesters were formed following the secretion of milk through exposure to chlorinating agents in the sterilized and disinfected equipment used in milk collection and handling (see, however, ref. 5). In such a case both primary ester groups of the triacylglycerol molecules would have reacted and a racemate of diacylchloropropanediols would have been obtained. The short chain species of the chloropropanediol diesters, the formation of which would have been anticipated but were not detected in the present work, might have remained buried in the mass of the triacylglycerol during the TLC isolation. Although an LC/MS search failed to locate such species, the lack of well-resolved characteristic masses may have prevented their sensitive detection. According to all accounts the various goat herds were not exposed to chlorinating agents and presumably did not receive chlorinated oils in their food.

Finally, it can be noted that ethylene glycol as well as long chain alkyl diol esters (20) have been identified in lipid extracts of animal tissues, but their origin has not been established. The present study demonstrates that the chloropropanediol diesters in milk fat are racemic and indicates a need for biochemical experiments to settle their origin.

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Mitochondrial Membrane Fatty Acid Composition in the Marmoset Monkey Following Dietary Lipid Supplementation

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Diets supplemented with high levels of saturated fatty acids derived from sheep kidney (perirenal) fat or unsaturated fatty acids derived from sunflowerseed oil were fed to marmoset monkeys for 22 wk. The effect of such diets on plasma, red blood cell phospholipids, and liver, heart, kidney and brain mitochondrial phospholipid fatty acids was determined. Despite large differences in the level and type of lipid present in the experimental diets, there was little effect on the proportion of saturated to unsaturated fatty acids in the phospholipids of the membranes examined. The diets did, however, alter the proportion of the various classes of polyunsaturated fatty acids in the membrane phospholipids, with the sunflowerseed oil diet elevating and the sheep kidney fat diet reducing the n-6/n-3 unsaturated fatty acid ratio, relative to a low (mixed fat) reference diet. This change occurred in all membranes except brain, in which only a small response to altered dietary lipid intake was observed. Elevation of dietary linoleic acid led to an increase in membrane linoleic acid and a marked decrease in membrane arachidonic acid, such that the membranes from animals fed the sunflowerseed oil diet exhibited the lowest proportion of arachidonic acid. In this latter respect, the response of the marmoset monkey to dietary lipid supplementation differs markedly from the rat. Our inability to alter significantly membrane lipid saturation/unsaturation supports the notion that a homeostatic mechanism is in some way responsible for buffering membranes from the effects of significant changes in the nature of the dietary lipid intake.
Lipids 21, 315-323 (1986).

The association between lipids and proteins in biological membranes allows potential for modulation of many membrane-associated enzymes by changes in the physical properties of the surrounding membrane lipids (1-3). This has been demonstrated in experiments where the physical properties of membrane lipids have been altered by changes in their composition (3) or in temperature (4,5). Changes in membrane lipid composition are a convenient means of changing membrane physical properties; for membrane phospholipids this can be achieved by a variety of means, such as changing acyl fatty acid composition or membrane cholesterol content (3).

Changes in lipid composition have been observed in mitochondrial and other membranes upon feeding animals diets supplemented with elevated levels of saturated or unsaturated fatty acids (see review in [3]). For example, although the ratio of saturated to unsaturated fatty acids in rat liver and heart mitochondrial membrane lipids is not greatly influenced by extensive differences in that ratio in diets enriched in sunflowerseed oil or sheep kidney fat, such diets do result in significant changes in the proportion of various types of unsaturated fatty acids, particularly those of the n-6 (linoleic acid) and n-3 (linolenic acid) families (6). Sunflowerseed oil-supplemented diets elevate the n-6/n-3 unsaturated fatty acid ratio, while the opposite has been observed for animals fed diets supplemented with sheep kidney fat as a natural source of saturated fat. As a result of these compositional changes, various properties associated with the thermotropic behavior of the membrane lipids are affected (7), as are the activities of some of the respiratory enzymes associated with the inner mitochondrial membrane (8-10).

The fact that large variations in the level of dietary lipid saturation do not significantly alter the degree of saturation/unsaturation in the membrane lipids suggests that a homeostatic mechanism may buffer the membrane from the effects of changes in the nature of the dietary lipid intake (6,11). Such a mechanism could diminish possible dietary lipid effects on membrane physical properties and membrane-associated enzyme and receptor function.

Considerable study on the effect of dietary lipids on the modification of membrane lipid composition from various tissues has been undertaken using nonprimate models, particularly the laboratory rat (5-9,11-14). Given the increasing awareness of the association of dietary lipids in many human disease states (15) including coronary heart disease (16-18), it is relevant to expand such studies to include nonhuman primate models. In the present study we have analyzed the fatty acid composition of mitochondrial and other membranes isolated from a variety of tissues of marmoset monkeys fed diets enriched in either saturated fatty acids provided by sheep kidney (perirenal) fat or unsaturated fatty acids provided by sunflowerseed oil.

MATERIALS AND METHODS

Marmosets. Common cotton-eared marmosets *(Callithrix jacchus jacchus)* were approximately 12 mo of age at the start of the experiment and weighed between 307 and 349 g. Nineteen marmosets of both sexes were used. They were divided into three dietary groups: reference (n = 4; 3 males, 1 female); sheep kidney fat (n = 8; 6 males, 2 females); and sunflowerseed oil (n = 7; 5 males, 2 females). The marmosets were maintained on the various dietary lipid regimes for 22 wk, at which time their body weights were between 314 and 362 g. There was no significant difference in weight among the three groups, nor were there any significant weight gains as a result of the fat-supplemented diets. Marmosets were paired to prevent pining and wasting and kept in aluminum alloy marmoset cages in a room with natural light supplemented by fluorescent light and 30 min of UV irradiation daily. The temperature was maintained at 26 C and the humidity at approximately 50%. At the end of the feeding period, marmosets were anesthetized and killed with an intramuscular injection of ketamine hydrochloride (Parke Davis, Adelaide, Australia).

Marmoset diets. One group of marmosets was fed a 1:1 mixture of Harper's greyhound chow and marmoset chow

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(Milling Industries, Australia, Ltd., Adelaide). The overall composition of this diet (REF) has been described previously (19), and on analysis contained 6% (w/w) total fat. This diet had first been crushed and repelleted, as were the two lipid-supplemented diets described below. A second group was fed the above diet supplemented at the time of pelletting with sunflowerseed oil (Nuttelex Pty. Ltd., Melbourne, Australia) to give a total of 14% (w/w) fat (SSO). A third group was fed the reference diet supplemented with sheep kidney (perirenal) fat, which was added and mixed with the reference diet prior to repelletting. On analysis, this diet (SKF) contained 13.6% (w/w) fat. Combustion calorimetry of the reference, sunflowerseed oil and sheep kidney fat diets gave energy values of 17.9, 20.0, 20.2 kJ/g, respectively. Fatty acid compositions of the diets are shown in Table 1. All diets were supplied ad libitum. A daily portion of 10 g fresh peeled banana was fed to each animal in addition to the pellets as described previously (19,20).

Blood and plasma collection. Immediately after the animals were killed, blood was collected and centrifuged to obtain plasma and red blood cells. The red blood cells were washed three times by centrifugation in phosphate buffered saline and finally resuspended in glass-distilled water (GDW).

Liver, kidney and brain mitochondrial fractions. For preparation of mitochondria from the above tissues, ca. 4 g from one liver, 1.2 g from both kidneys or 4 g from brain was chopped and rinsed in ice-cold medium containing 250 mM sucrose, 2 mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (Hepes), 0.5 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA) and 0.05% (w/v) delipidated bovine serum albumin (BSA), pH 7.4, and then homogenized in 40 ml of the above medium using a Polytron tissue homogenizer (Kinematica, Lucerne, Switzerland) at setting 3.5 for two bursts of six sec each. The brei was filtered through cheesecloth and centrifuged at 500 g for 12 min and the supernatant saved. The pellet was resuspended in the above medium to the original volume and recentrifuged at 500 g for 12 min. The supernatants from this and the previous centrifugation were combined and centrifuged at 6000 g for 15 min. The resulting mitochondrial pellet was then washed twice by centrifugation in the above medium at 6000 g for 15 min, and the pellet was finally resuspended in the above medium.

Heart mitochondrial fraction. Ventricular tissue from one animal was chopped and rinsed in ice-cold medium containing 100 mM KCl, 50 mM 4-morpholinepropane-sulphonic acid (Mops), 2 mM EGTA and 0.2% (w/v) delipidated BSA, pH 7.2, and then homogenized in 40 ml of the above medium in the same manner as already described. The procedure for the subsequent isolation of the heart mitochondrial fraction by differential centrifugation was as described for the other tissues, except that the first mitochondrial pellet was resuspended in the above medium without BSA. After washing the mitochondrial pellet by centrifugation in heart-resuspending medium, the pellet was finally resuspended in this buffer.

Lipid analysis. Prior to lipid analysis, the mitochondrial preparations were diluted in 50 vol of 20 mM Tris, 2 mM ethylene-diaminetetraacetic acid (EDTA), pH 7.2, and centrifuged at 250,000 g for 60 min to remove sucrose or KCl. The resulting membrane pellet was resuspended in GDW. To one vol of plasma, washed red blood cells or washed mitochondrial preparations, four vol of 2-propanol were added and the mixture was boiled for 30 sec. After the mixture cooled, eight vol of chloroform containing the antioxidant butylated hydroxyanisole (0.1% of the estimated lipid weight) were added. Following the addition of one vol of GDW, the samples were shaken and centrifuged and the organic phase was collected. After re-extracting the aqueous phase with a further four vol of chloroform, the organic phases were combined and dried using anhydrous sodium sulphate. For all samples except the plasma lipids, phospholipids were separated from the total lipid extract by thin layer chromatography on silica gel H plates developed in petroleum ether/diethyl ether/acetic acid (90:15:1, v/v/v). The phospholipids remaining at the origin were eluted from the silica gel and methylated in 1% (v/v) H_2SO_4 in methanol by heating at 70 C for three hr. For plasma, the fatty acids of the total lipids were analyzed. Fatty acid methyl esters were extracted, identified and analyzed by gas liquid chromatography using the method of Gibson and Kneebone (21). Columns were packed with 5% SP-2310 on 100/120 chromosorb WAW (Supelco, Bellefonte, Pennsylvania) with the column temperature programmed from 125 C to 225 C at 4 C/min. For analysis of the fatty acids present in the various dietary lipid supplements, the diet pellets were extracted and fatty acid analysis was performed on the total lipid extract as described above.

Statistical analysis. Student's t-test was used for determining the significance between differences in fatty acid compositions.

RESULTS

Fatty acid compositions of the fabricated marmoset diets are shown in Table 1. The proportion of unsaturated to saturated fatty acids was highest in SSO and lowest in SKF. Differences in the proportions of major fatty acids

TABLE 1

Fatty Acid Composition of Fabricated Marmoset Diets

Fatty acid	REF	SKF	SSO
14:0	2.0	3.0	0.6
16:0	23.1	24.3	11.9
16:1(n-7)	3.0	2.7	1.4
17:0	0.9	1.1	0.3
18:0	14.6	21.9	8.3
18:1(n-9)	35.4	35.4	27.1
18:2(n-6)	19.3	10.3	49.8
18:3(n-3)	1.6	1.4	0.5
Unsaturated	59.3	49.5	78.8
Saturated	40.7	50.4	21.1
Unsaturated/saturated	1.5	1.0	3.7
n-6/n-3	12.1	7.3	99.6

Fatty acids are designated by the number of carbon atoms, followed by the number of double bonds, and are expressed as the percentage (w/w) of the total fatty acids. REF, reference, standard laboratory diet; SKF, sheep kidney fat-supplemented diet; SSO, sunflowerseed oil diet. Trace amounts (less than 0.2%) were detected for the fatty acids 20:0, 20:1, 20:5, 22:0, 22:1 and 22:6. No 20:4 was detected in any of the diets.

among the diets were apparent for palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acids. No arachidonic acid (20:4) was detected in any of the diets.

Although each dietary group comprised animals of both sexes, there were insufficient numbers to treat the data separately according to sex. For the limited number of animals available, inspection of the fatty acid data did not reveal significant differences between males and females within each dietary group. Thus, all data has been combined for each group.

The fatty acid composition of the total plasma lipids of marmosets maintained on the diets for 22 wk is shown in Table 2. Changes were evident both in the proportion of individual fatty acids and in the various computational parameters associated with these fatty acid analyses. The proportion of saturated to unsaturated fatty acids was elevated in the sheep kidney fat-supplemented animals and reduced in the sunflowerseed oil-supplemented animals in comparison to the animals on reference diet. In addition, there was a marked change in the proportion of the n-6 to n-3 series of unsaturated fatty acids, with SSO elevating and SKF reducing this ratio in comparison to REF. The proportion of n-9 fatty acids, principally 18:1, was also influenced by the dietary lipid treatments. In comparison to animals fed REF, there was a reduction in the proportion of this fatty acid in SKF and SSO animals, and a significantly lower proportion of this fatty acid in the SSO compared to the SKF animals.

In contrast to the results obtained for the plasma fatty acids, in which the ratio of saturated to unsaturated fatty acids reflected to some extent the relative proportion present in the respective diets, no such correspondence was observed in the phospholipid fatty acids of red blood cell membranes (Table 3). Indeed, in comparison to the

TABLE 2

Fatty Acid Composition of Plasma Lipids from Marmosets after Dietary Lipid Supplementation

Fatty acid	REF	SKF	SSO	Significance SKF vs SSO
DMA 16:0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	n.d.
16:0	17.7 ± 0.7	15.1 ± 0.7	13.3 ± 0.7	N.S.
16:1(n-7)	3.3 ± 0.2	1.9 ± 0.1	0.8 ± 0.3	P < 0.01
17:0	0.4 ± 0.04	0.8 ± 0	0.4 ± 0	n.d.
17:1	0.6 ± 0.05	0.6 ± 0	0.3 ± 0	n.d.
DMA 18:0	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0	n.d.
18:0	6.8 ± 0.4	10.4 ± 0.6	9.5 ± 0.4	N.S.
18:1(n-9)	36.7 ± 0.3	30.6 ± 0.1	19.8 ± 0.9	P < 0.001
18:2(n-6)	23.8 ± 2.2	27.3 ± 0.8	45.7 ± 1.7	P < 0.001
γ18:3(n-6)	0.3 ± 0.2	0.3 ± 0.1	tr.	n.d.
α18:3(n-3)	0.9 ± 0.1	1.3 ± 0.1	0.6 ± 0.04	P < 0.001
20:0	0.5 ± 0.04	0.8 ± 0.1	0.4 ± 0	n.d.
20:1	1.0 ± 0.1	0.9 ± 0.3	0.9 ± 0.2	n.d.
20:2(n-6)	0.6 ± 0.1	0.5 ± 0.1	1.2 ± 0.1	P < 0.01
20:3(n-6)	1.7 ± 0.1	1.3 ± 0.2	1.4 ± 0.3	N.S.
20:4(n-6)	2.8 ± 0.4	3.4 ± 0.3	2.7 ± 0.3	N.S.
22:0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	n.d.
20:5(n-3) + 22:1	0.4 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	n.d.
22:4(n-6)	tr.	0.4 ± 0.2	0.3 ± 0.1	n.d.
24:0	0.4 ± 0.02	0.4 ± 0.1	0.5 ± 0.1	n.d.
22:5(n-6) + 24:1	0.3 ± 0.04	0.4 ± 0.1	0.3 ± 0.1	n.d.
22:5(n-3)	tr.	0.5 ± 0.1	0.3 ± 0.1	n.d.
22:6(n-3)	0.8 ± 0.1	1.4 ± 0.2	0.7 ± 0.2	P < 0.05
Σ Saturated	26.6 ± 1.2	28.5 ± 1.2	24.8 ± 0.8	P < 0.05
Σ Unsaturated	73.2 ± 1.0	71.5 ± 1.2	75.3 ± 0.6	P < 0.02
Σ n-6	29.5 ± 2.3	33.6 ± 0.9	51.6 ± 1.4	P < 0.001
Σ n-3	1.7 ± 0.2	3.2 ± 0.2	1.6 ± 0.2	P < 0.001
n-6/n-3	17.3	10.5	32.2	
UI	117	125	140	
Σ DMA	0.6	0.8	0.5	

Fatty acids are designated as described in Table 1, with the designated fatty acid series for particular unsaturated fatty acids also indicated. Major fatty acids are presented as the mean relative percentage ±S.E. for n = 4 animals in the REF group, n = 8 in the SKF group and n = 7 in the SSO group. DMA, dimethyl acetal derivative. The unsaturation index (UI) is $\Sigma[(a)(b)]$ where a is the relative percentage of each unsaturated fatty acid and b is the number of double bonds for that particular fatty acid. Significance between the major fatty acids of the sheep kidney fat and sunflowerseed oil groups was determined by Student's t-test and is shown in the significance column. N.S., not significant; n.d., not determined.

REF and SKF animals, a slight increase in the proportion of saturated fatty acids was observed upon feeding the more unsaturated SSO; however, this was not statistically significant. A reduction in the unsaturation index (UI) occurred in the SSO animals. The n-6/n-3 unsaturated fatty acid ratio was elevated in the SSO group in comparison to the other groups, mainly due to an elevation in 18:2 and a slight decrease in the proportion of the n-3 unsaturated fatty acids, docosapentaenoic (22:5) and docosahexaenoic (22:6). Although no significant difference was observed in the proportion of 22:6(n-3), the sum of the total n-3 unsaturated fatty acids was significantly different for the SKF and SSO animals (Table 3). Furthermore the sum of 22:5(n-3) and 22:6(n-3) was also significantly different for the two groups (data not shown). Although the proportion of 18:2 was elevated in the SSO group, the proportion of arachidonic acid [20:4(n-6)] was decreased in comparison to the other dietary groups.

In liver mitochondrial membrane phospholipids, no significant change in the proportion of total saturated and unsaturated fatty acids was evident; however, the n-6/n-3 ratio was elevated in the SSO group and reduced in the SKF group relative to the REF group. These changes were brought about by an increase in 18:2(n-6) [but not 20:4(n-6)] and a decrease in 22:6(n-3) (Table 4). The proportions of a number of other fatty acids were also altered upon feeding the different lipid-supplemented diets.

The proportion of saturated fatty acids in heart mitochondrial membrane phospholipids was actually elevated in the SSO group in comparison to the other dietary groups, and this was due in part to increases in 16:0 and 18:0 (Table 5). The n-6/n-3 ratio showed changes in the same direction as was evident for liver mitochondria; this was mainly due to changes in the proportions of 18:2(n-6), 22:5(n-3) and 22:6(n-3). While 18:2(n-6) increased in the SSO group, the proportion of 20:4(n-6) in this group was significantly lower compared to the other dietary groups. The UI value was also reduced in the SSO group.

In kidney mitochondrial phospholipids, no significant effect on the ratio of saturated to unsaturated fatty acids was observed as a result of the dietary treatments (Table 6). The n-6/n-3 ratio underwent the same change as in liver and heart mitochondria, i.e., elevation in the SSO group and reduction in the SKF group in comparison

TABLE 3

Fatty Acid Composition of Red Blood Cell Phospholipids from Marmosets after Dietary Lipid Supplementation

Fatty acid	REF	SKF	SSO	Significance SKF vs SSO
DMA 16:0	0.6 ± 0.1	0.4 ± 0	0.4 ± 0	n.d.
16:0	17.0 ± 0.2	14.2 ± 0.3	14.3 ± 0.7	N.S.
16:1(n-7)	0.7 ± 0.3	0.3 ± 0.1	tr.	n.d.
17:0	0.4 ± 0	0.7 ± 0	0.5 ± 0	n.d.
17:1	tr.	tr.	tr.	n.d.
DMA 18:0	0.8 ± 0.2	0.9 ± 0.1	0.6 ± 0	n.d.
18:0	14.7 ± 0.7	18.6 ± 0.3	18.9 ± 0.8	N.S.
18:1(n-9)	24.3 ± 1.5	22.9 ± 0.2	18.5 ± 0.6	$P < 0.001$
18:2(n-6)	10.8 ± 0.7	12.6 ± 0.5	18.8 ± 1.2	$P < 0.001$
γ18:3(n-6)	0.4 ± 0.2	0.2 ± 0.1	tr.	n.d.
α18:3(n-3)	0.3 ± 0	0.5 ± 0	tr.	n.d.
20:0	0.4 ± 0.1	0.6 ± 0	0.5 ± 0	n.d.
20:1	1.5 ± 0.4	0.9 ± 0.2	0.7 ± 0.1	n.d.
20:2(n-6)	0.7 ± 0.1	0.5 ± 0	0.9 ± 0	n.d.
20:3(n-6)	1.5 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	N.S.
20:4(n-6)	11.4 ± 0.6	11.9 ± 0.3	9.7 ± 0.5	$P < 0.01$
22:0	tr.	tr.	0.5 ± 0.1	n.d.
20:5(n-3) + 22:1	0.7 ± 0.2	0.8 ± 0.1	0.4 ± 0.2	n.d.
22:4(n-6)	2.6 ± 0.3	2.3 ± 0.05	4.9 ± 0.3	$P < 0.001$
24:0	3.2 ± 0.1	2.2 ± 0.1	3.4 ± 0.1	$P < 0.001$
22:5(n-6) + 24:1	0.4 ± 0.1	tr.	0.5 ± 0	n.d.
22:5(n-3	2.4 ± 0.2	3.7 ± 0.1	1.7 ± 0.2	$P < 0.001$
22:6(n-3)	5.1 ± 0.6	4.4 ± 0.2	3.5 ± 0.7	N.S.
Σ Saturated	37.1 ± 1.0	37.7 ± 0.4	39.1 ± 0.7	N.S.
Σ Unsaturated	62.9 ± 0.9	62.3 ± 0.4	60.9 ± 0.6	N.S.
Σ n-6	27.8 ± 1.1	28.7 ± 0.3	35.9 ± 1.0	$P < 0.001$
Σ n-3	8.5 ± 1.0	9.4 ± 0.4	5.6 ± 1.1	$P < 0.005$
n-6/n-3	3.3	3.0	6.4	
UI	156	159	151	
Σ DMA	1.4	1.3	0.9	

Data are as presented in Tables 1 and 2 for n = 4 animals in the REF group, n = 8 in the SKF group and n = 7 in the SSO group.

to the REF group. The proportion of 20:4(n-6) was again reduced in the SSO group compared to animals in the other two dietary groups.

The overall fatty acid profile of brain mitochondrial phospholipids was quite distinct from that observed for the other tissues, being characterized by a relatively low proportion of 18:2(n-6) and a high proportion of 22:6(n-3) (Table 7). Dietary-induced changes in the fatty acid composition of brain mitochondrial phospholipids were far less pronounced than those observed in the other tissues. Significant changes in the proportion of 18:2(n-6) between the SKF and SSO dietary groups resulted in a small change in the n-6/n-3 ratio, with the direction of change being the same as for the other tissues. The proportion of saturated fatty acids and the value of the UI were not significantly affected by the dietary lipid treatments.

DISCUSSION

A large number of studies has documented the types of changes which occur in tissue and membrane lipids as a result of feeding a variety of lipid supplements (6,11, 22-28). For mammalian membrane lipids, it is generally observed that large changes in the level of dietary lipid saturation have little or no effect on the level of saturation present in membrane lipids (3,5,6). This may reflect some homeostatic mechanism which maintains a fairly constant proportion of saturated to unsaturated fatty acids in the membrane despite large variations in the level of dietary lipid saturation. Whether such homeostasis would lead to the maintenance of a constant level of membrane lipid fluidity is not known. However, for those organisms which exhibit homeoviscous adaptation of their membrane lipids in response to some form of exogenous perturbation, it is believed that the main purpose of this mechanism is to preserve a relatively constant level of membrane fluidity (29).

Using dietary lipid supplements similar to those in the present study, we have previously reported that no significant change in membrane saturation/unsaturation occurred in the phospholipids isolated from rat mitochondrial or microsomal membrane fractions. This result was also apparent after 12 mo of dietary lipid supplementation (6). The present results with the marmoset monkey extend the concept of membrane lipid homeostasis to a nonhuman primate model.

TABLE 4

Fatty Acid Composition of Liver Mitochondrial Phospholipids from Marmosets after Dietary Lipid Supplementation

Fatty acid	REF	SKF	SSO	Significance SKF vs SSO
DMA 16:0	tr.	tr.	tr.	n.d.
16:0	13.6 ± 0.4	11.5 ± 0.5	10.1 ± 0.5	N.S.
16:1(n-7)	2.2 ± 0.2	1.3 ± 0.1	0.8 ± 0.1	P < 0.01
17:0	0.5 ± 0.1	0.7 ± 0	0.5 ± 0	n.d.
17:1	0.3 ± 0	0.3 ± 0	0.3 ± 0	n.d.
DMA 18:0	tr.	0.2 ± 0	tr.	n.d.
18:0	15.0 ± 0.9	16.6 ± 0.4	17.5 ± 1.1	N.S.
18:1(n-9)	25.3 ± 2.1	21.8 ± 0.7	16.1 ± 0.5	P < 0.001
18:2(n-6)	19.7 ± 1.0	21.4 ± 0.5	33.3 ± 0.7	P < 0.001
γ18:3(n-6)	0.5 ± 0	0.3 ± 0.1	tr.	n.d.
α18:3(n-3)	0.5 ± 0	0.8 ± 0	0.3 ± 0	n.d.
20:0	0.3 ± 0.1	0.7 ± 0	0.3 ± 0	n.d.
20:1	0.7 ± 0.1	0.7 ± 0	0.5 ± 0	n.d.
20:2(n-6)	0.9 ± 0.1	0.8 ± 0	2.3 ± 0.2	P < 0.001
20:3(n-6)	3.0 ± 0.5	2.4 ± 0.2	2.6 ± 0.3	N.S.
20:4(n-6)	12.4 ± 0.7	12.9 ± 0.3	11.9 ± 0.2	P < 0.02
22:0	0.4 ± 0.1	0.4 ± 0.1	tr.	n.d.
20:5(n-3) + 22:1	0.5 ± 0.1	1.0 ± 0.1	0.3 ± 0.1	P < 0.001
22:4(n-6)	tr.	0.2 ± 0	tr.	n.d.
24:0	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	n.d.
22:5(n-6) + 24:1	tr.	tr.	tr.	n.d.
22:5(n-3)	0.3 ± 0	0.6 ± 0.1	0.3 ± 0	n.d.
22:6(n-3)	3.0 ± 0.1	4.5 ± 0.2	2.0 ± 0.2	P < 0.001
Σ Saturated	30.6 ± 1.3	30.8 ± 0.7	29.1 ± 1.1	N.S.
Σ Unsaturated	69.3 ± 1.3	69.1 ± 0.7	70.7 ± 1.1	N.S.
Σ n-6	36.5 ± 1.3	38.0 ± 0.4	50.1 ± 0.9	P < 0.001
Σ n-3	4.3 ± 0.1	6.9 ± 0.4	2.9 ± 0.3	P < 0.001
n-6/n-3	8.5	5.5	17.3	
UI	151	163	159	
Σ DMA	tr.	0.2	tr.	

Data are as presented in Tables 1 and 2 for n = 4 animals in the REF group, n = 8 in the SKF group and n = 7 in the SSO group.

TABLE 5

Fatty Acid Composition of Heart Mitochondrial Phospholipids from Marmosets after Dietary Lipid Supplementation

Fatty acid	REF	SKF	SSO	Significance SKF vs SSO
DMA 16:0	1.5 ± 0.2	1.2 ± 0.1	1.0 ± 0.2	N.S.
16:0	13.3 ± 0.3	10.5 ± 0.6	13.2 ± 0.9	N.S.
16:1(n-7)	0.3 ± 0.3	0.5 ± 0.2	1.6 ± 0.8	N.S.
17:0	0.2 ± 0	0.5 ± 0	0.5 ± 0.1	n.d.
17:1	tr.	tr.	0.2 ± 0	n.d.
DMA 18:0	1.4 ± 0.4	2.8 ± 0.7	1.1 ± 0.2	N.S.
18:0	15.9 ± 0.6	16.8 ± 0.7	19.0 ± 0.5	N.S.
18:1(n-9)	12.8 ± 0.4	13.4 ± 0.4	11.2 ± 0.9	N.S.
DMA 18:1	1.4 ± 0.1	1.2 ± 0.3	1.3 ± 0.5	N.S.
18:2(n-6)	23.4 ± 0.9	22.4 ± 0.8	30.1 ± 2.1	$P < 0.05$
γ18:3(n-6)	0.4 ± 0.2	0.7 ± 0.3	tr.	n.d.
α18:3(n-3)	0.3 ± 0.1	0.4 ± 0.1	tr.	n.d.
20:0	tr.	0.3 ± 0.1	tr.	n.d.
20:1	0.9 ± 0.4	1.2 ± 0.5	0.7 ± 0.3	N.S.
20:2(n-6)	0.7 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	n.d.
20:3(n-6)	1.5 ± 0.1	1.5 ± 0.2	0.8 ± 0.1	$P < 0.05$
20:4(n-6)	17.0 ± 0.4	14.4 ± 0.4	10.9 ± 1.0	$P < 0.05$
22:0	tr.	tr.	0.2	n.d.
20:5(n-3) + 22:1	0.9 ± 0.1	1.2 ± 0.1	0.4 ± 0.2	n.d.
22:4(n-6)	0.8 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	$P < 0.05$
24:0	2.0 ± 0.1	1.2 ± 0.2	1.7 ± 0.1	N.S.
22:5(n-6) + 24:1	tr.	0.7 ± 0.4	tr.	n.d.
22:5(n-3)	1.5 ± 0.2	3.5 ± 0.7	1.3 ± 0.2	$P < 0.05$
22:6(n-3)	3.6 ± 0.2	4.0 ± 0.4	1.9 ± 0.2	$P < 0.01$
Σ Saturated	34.3 ± 0.4	33.2 ± 0.6	36.8 ± 1.0	$P < 0.05$
Σ Unsaturated	65.6 ± 0.4	66.6 ± 0.6	63.0 ± 1.0	$P < 0.05$
Σ n-6	43.8 ± 0.4	41.2 ± 0.5	44.0 ± 2.3	N.S.
Σ n-3	6.3 ± 0.4	9.1 ± 0.4	3.6 ± 0.5	$P < 0.001$
n-6/n-3	6.9	4.5	12.2	
UI	177	180	153	
Σ DMA	4.3	5.2	3.4	

Data are as presented in Tables 1 and 2 for n = 3 animals in the REF group, n = 3 in the SKF group and n = 3 in the SSO group.

TABLE 6

Fatty Acid Composition of Kidney Mitochondrial Phospholipids from Marmosets after Dietary Lipid Supplementation

Fatty Acid	REF	SKF	SSO	Significance SKF vs SSO
DMA 16:0	0.8 ± 0.5	0.7 ± 0	0.6 ± 0	n.d.
16:0	18.9 ± 0.4	16.0 ± 0.2	15.7 ± 0.5	N.S.
16:1(n-7)	1.0 ± 0.3	0.4 ± 0.2	tr.	n.d.
17:0	0.3 ± 0	0.5 ± 0	0.4 ± 0	n.d.
17:1	0.2 ± 0	0.2 ± 0	0.2 ± 0	n.d.
DMA 18:0	0.4 ± 0	0.5 ± 0	0.4 ± 0.1	n.d.
18:0	10.0 ± 0.6	11.8 ± 0.2	11.6 ± 0.5	N.S.
18:1(n-9)	21.0 ± 0.6	19.5 ± 0.7	15.6 ± 0.5	$P < 0.01$
18:2(n-6)	21.8 ± 1.0	21.5 ± 0.3	29.6 ± 0.7	$P < 0.001$
γ18:3(n-6)	tr.	tr.	tr.	n.d.
α18:3(n-3)	0.3 ± 0.1	0.5 ± 0	tr.	n.d.
20:0	0.4 ± 0.1	0.5 ± 0	0.3 ± 0	n.d.
20:1	0.6 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	n.d.
20:2(n-6)	0.9 ± 0.2	1.0 ± 0.1	3.0 ± 0.2	$P < 0.001$
20:3(n-6)	1.6 ± 0.1	1.5 ± 0.1	1.9 ± 0.1	$P < 0.02$
20:4(n-6)	16.4 ± 1.1	16.3 ± 0.3	13.6 ± 0.5	$P < 0.001$
22:0	0.2 ± 0	0.2 ± 0	0.2 ± 0	n.d.

(Continued)

TABLE 6 *(Continued)*

Fatty acid	REF	SKF	SSO	Significance SKF vs SSO
20:5(n-3) + 22:1	1.1 ± 0.6	1.8 ± 0.4	0.6 + 0.1	N.S.
22:4(n-6)	0.3 ± 0.1	0.6 ± 0.1	0.7 ± 0	n.d.
24:0	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	n.d.
22:5(n-6) + 24:1	0.3 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	n.d.
22:5(n-3)	0.5 ± 0.2	0.7 ± 0.1	0.4 ± 0	n.d.
22:6(n-3)	2.0 ± 0.3	3.5 ± 0.5	2.9 ± 0.3	N.S.
Σ Saturated	31.8 ± 0.4	31.1 ± 0.4	30.1 ± 0.5	N.S.
Σ Unsaturated	68.0 ± 0.4	68.9 ± 0.4	69.7 ± 0.5	N.S.
Σ n-6	41.3 ± 0.1	41.5 ± 0.3	49.3 ± 0.8	$P < 0.001$
Σ n-3	3.9 ± 0.1	6.5 ± 0.8	3.9 ± 0.4	$P < 0.02$
n-6/n-3	10.6	6.4	12.6	
UI	157	167	168	
Σ DMA	1.2	1.2	1.0	

Data are as presented in Tables 1 and 2 for n = 4 animals in the REF group, n = 7 in the SKF group and n = 6 in the SSO group.

TABLE 7

Fatty Acid Composition of Brain Mitochondrial Phospholipids from Marmosets after Dietary Lipid Supplementation

Fatty acid	REF	SKF	SSO	Significance SKF vs SSO
DMA 16:0	2.2 ± 0.3	2.1 ± 0.2	2.0 ± 0.2	N.S.
16:0	17.3 ± 0.7	18.1 ± 0.5	18.3 ± 0.6	N.S.
16:1(n-7)	1.4 ± 0.5	0.7 ± 0.3	0.7 ± 0.3	n.d.
17:0	0.3 ± 0	0.3 ± 0	0.3 ± 0	n.d.
17:1	0.3 ± 0	0.2 ± 0	tr.	n.d.
DMA 18:0	3.0 ± 0.2	3.3 ± 0.1	3.2 ± 0.2	N.S.
18:0	22.8 ± 0.5	22.9 ± 0.2	22.4 ± 0.2	N.S.
18:1(n-9)	20.8 ± 1.1	19.8 ± 0.5	18.9 ± 1.1	N.S.
18:2(n-6)	1.5 ± 1.4	1.6 ± 0	3.1 ± 0.1	$P < 0.001$
γ18:3(n-6)	0.3 ± 0	0.3 ± 0	0.3 ± 0	n.d.
α18:3(n-3)	tr.	tr.	tr.	n.d.
20:0	tr.	0.4 ± 0.2	0.2 ± 0	n.d.
20:1	1.7 ± 0.3	1.5 ± 0.2	1.5 ± 0.2	N.S.
20:2(n-6)	0.4 ± 0	0.4 ± 0	0.6 ± 0	n.d.
20:3(n-6)	0.5 ± 0	0.5 ± 0	0.6 ± 0	n.d.
20:4(n-6)	7.1 ± 0.4	7.4 ± 0.2	7.6 ± 0.1	N.S.
22:0	tr.	0.3 ± 0.1	0.3 ± 0.1	n.d.
20:5(n-3) + 22:1	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	n.d.
22:4(n-6)	4.6 ± 0.4	4.1 ± 0.2	4.8 ± 0.3	N.S.
24:0	2.9 ± 0.5	2.4 ± 0.2	3.4 ± 0.3	$P < 0.02$
22:5(n-6) + 24:1	1.1 ± 0.4	0.9 ± 0.2	0.3 ± 0.1	n.d.
22:5(n-3)	tr.	tr.	0.2 ± 0.1	n.d.
22:6(n-3)	11.0 ± 1.4	12.2 ± 0.6	10.8 ± 1.1	N.S.
Σ Saturated	48.5 ± 0.6	49.8 ± 0.7	50.1 ± 1.0	N.S.
Σ Unsaturated	51.3 ± 0.6	50.0 ± 0.7	50.0 ± 1.0	N.S.
Σ n-6	15.5 ± 0.4	15.2 ± 0.3	17.3 ± 0.6	$P < 0.01$
Σ n-3	11.6 ± 1.6	12.6 ± 0.6	11.3 ± 1.1	N.S.
n-6/n-3	1.3	1.2	1.5	
UI	145	149	148	
Σ DMA	5.2	5.4	5.2	

Data are as presented in Tables 1 and 2 for n = 4 animals in the REF group, n = 8 in the SKF group and n = 7 in the SSO group.

In regard to membrane lipid saturation, we have previously reported that the proportions of saturated fatty acids in rat liver, kidney and particularly heart mitochondrial and microsomal fraction phospholipids actually undergo a small change opposite to that expected from the proportion of saturated fatty acids in the dietary lipid supplement. Thus in rat heart mitochondrial phospholipids, the feeding of sunflowerseed oil supplements for 20 wk actually increased the proportion of saturated fatty acids relative to that obtained after feeding a sheep kidney fat supplement (6). This paradox is also observed in the present study, particularly for the fatty acids of heart mitochondria and, to a lesser extent, red blood cell phospholipids. Therefore, as far as the influence of dietary lipid supplements on the proportion of saturated fatty acids in membrane lipids is concerned, the major effect appears to be that the proportion of saturated fatty acids is unaltered, or on occasion is changed in a direction opposite to that expected from the saturation of the dietary lipid supplement. The latter phenomenon may also be the result of a homeostatic mechanism in the membrane related to the nature of the dietary-induced changes in the homeoviscous adaptation of their membrane lipids in response to some form of membrane polyunsaturated fatty acids, as discussed below.

Although these experimental diets were designed primarily to provide large differences in their saturated to unsaturated fatty acid ratios, the fact that all diets contained small amounts of linolenic acid [18:3(n-3)] meant that upon changing the proportion of saturated fatty acids primarily at the expense of linoleic acid (an n-6 polyunsaturated fatty acid), the value of the n-6/n-3 unsaturated fatty acid ratio was markedly altered. Thus the n-6/n-3 ratio of the SSO diet was about 13-fold greater than that of the SKF diet. The two lipid-supplemented diets did bring about changes in the type of unsaturated fatty acids present in the membrane phospholipids. However, only a relatively small effect was observed in brain mitochondria, consistent with its reported lack of response to dietary manipulation in other animal models (6,11,30). In terms of the n-6/n-3 unsaturated fatty acid ratio of the membrane lipids, the diet with the highest n-6/n-3 ratio, i.e., SSO, increased the value of this ratio relative to that observed for animals in the REF group. The diet characterized by the lower n-6/n-3 ratio, i.e., SKF, had the opposite effect. Changes in this ratio in the membrane lipids were, in most instances, induced by a concomitant change in the proportion of both total n-6 and n-3 polyunsaturated fatty acids. These results confirm the results of other workers using nonprimate models, who have reported that the composition of membrane lipids is modulated by dietary long chain fatty acids, even when the diet is adequate in all nutrients (11,22–28).

The major n-6 polyunsaturated fatty acid to undergo change in the marmoset was linoleic acid [18:2(n-6)], while docosahexaenoic [22:6(n-3)] and docosapentaenoic [22:5(n-3)] were the major n-3 unsaturated fatty acids to change. (The changes in arachidonic acid [20:4(n-6)] are opposite to those expected from the dietary lipid supplementation and will be discussed later.) The compensatory variation in the proportions of 22:6(n-3) in response to oscillating levels of n-6 fatty acids has been reported by others (6,28,31). The above result is consistent with a change in the flux of the various families of polyunsaturated fatty acids through their particular metabolic pathways induced either by changes in precursor levels and/or by changes in the activity of the various fatty acid desaturases and/or elongases. This latter effect could be brought about by changes in the concentration of those polyunsaturated fatty acids which act as competitive inhibitors of these enzymes (31,32). Studies both in vivo and in vitro (23,24,33–35) have demonstrated that the competition of 18-carbon unsaturated fatty acids for further desaturation is in the order of n-3 > n-6 > n-9 series unsaturated fatty acids (36,37). This would account for the increased synthesis of the n-3 class of polyunsaturated fatty acids from the relatively low level of 18:3(n-3) present in SKF. These dietary-induced effects on membrane fatty acid composition are qualitatively similar to those previously reported for cardiac tissue (7,8,11–13,20,27, 38–40) and in other mammalian tissues after similar types of dietary lipid supplementation (5,7,9,11).

Despite the dietary-induced changes in membrane polyunsaturated fatty acids noted above, the value of the UI, which is still a popular way of expressing membrane unsaturation (3), is only slightly affected by the nature of the dietary lipid intake. Given the major difference in the level of unsaturation of SKF compared to SSO, the only significant effect on the UI is a raised plasma lipid value and an unexpected decrease in the heart mitochondrial phospholipids. With these exceptions, the UI remains fairly constant. This also may reflect a degree of homeostasis in the final composition of polyunsaturated fatty acids in the membrane lipids. Although monounsaturates are involved in the maintenance of the UI, by far the greatest contributors to the UI are the n-6 and n-3 polyunsaturated fatty acids. In heart mitochondrial phospholipids, the marked decrease in the UI observed in the SSO animals has probably been primarily the result of decreased arachidonic acid [20:4(n-6)] and to a lesser extent decreased 22:5(n-3) and 22:6(n-3).

The response of the membrane phospholipid fatty acids to the SSO diet is particularly interesting in regard to the changes in 20:4(n-6). In rat membrane phospholipids, this diet elevated both 18:2(n-6) and 20:4(n-6), albeit to different extents (7,8,12,13). However, in the marmoset the proportion of 20:4(n-6) in the SSO animals actually decreased in comparison to the other dietary groups. This decrease occurred despite a significant increase in the proportion of 18:2(n-6) in both the dietary supplement and the membrane lipids as a result of feeding the sunflowerseed oil supplement. This was apparent in the total fatty acids of plasma, red blood cell phospholipids, liver and kidney mitochondrial phospholipids, but was most prominent in heart mitochondrial phospholipids. This phenomenon has also been observed in platelet membrane phospholipids of the marmoset monkey upon feeding a sunflowerseed oil supplement (results not shown). This result points to a very distinct species difference in the control of the interconversion of the various n-6 polyunsaturated fatty acids in the marmoset relative to the rat. It would appear that in the marmoset, conditions which induce an elevation in 18:2(n-6) may also lead to an inhibition of Δ6 or Δ5 desaturase and/or elongase activity and a subsequent decrease in 20:4(n-6). Dietary polyunsaturated fatty acids, particularly those of the n-6 series, have been shown to inhibit both Δ9 and Δ6 desaturase activity by the possible formation of a product interfering

with the synthesis of desaturase proteins at the level of transcription (41). Our studies therefore suggest that desaturase activity and its control by dietary lipids differ in the marmoset and the more commonly studied laboratory rat. This observation also has relevance for the availability of 20:4(n-6) for prostaglandin production in the marmoset and suggests that dietary lipid influences on prostaglandin production may also be quite different from those operating in the rat.

The above changes in mitochondrial membrane lipid composition in the marmoset may have effects on the functioning of various membrane-associated biochemical and physiological processes. Indeed, we have previously reported that the rate of oxidation of a variety of respiratory substrates in liver and heart mitochondria of the marmoset is decreased when the dietary lipid content is increased from 4 to 16%, irrespective of whether a predominantly saturated or unsaturated supplement is used (42). If the effects of dietary lipid supplementation extend to membranes other than the mitochondrial membrane in the marmoset, as has been shown for various tissue membranes of the rat (6,43), it is conceivable that dietary-induced alterations in cellular membrane lipid composition may have profound effects on many membrane-associated physiological processes in this animal.

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Acylation of Docosahexaenoic Acid into Phospholipids by Intact Human Neutrophils

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Docosahexaenoic acid was not only acylated into phospholipids but also into triacylglycerols by intact human neutrophils. The distribution of radiolabeled docosahexaenoic acid among individual phospholipids was dependent on the incubation time. [1-^{14}C]Docosahexaenoic acid at all concentrations (1 to 8 μM) was acylated mainly into phosphatidic acid after 1–2 min incubation, and the radioactivity of phosphatidic acid started to decline after a longer period of incubation, suggesting the participation of docosahexaenoyl-phosphatidic acid in the synthesis of other glycerolipids. It was acylated primarily into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) after a 2-hr incubation. The labeled phosphatidic acid may be rapidly deacylated and the 22:6(n-3) moiety is then reacylated into other lysophospholipids. The low levels of [^{14}C]22:6(n-3) in 1,2-diacylglycerol suggest that the deacylation-reacylation cycle may be a major pathway in the formation of [^{14}C]22:6(n-3)-PC and -PE in intact neutrophils. This n-3 fatty acid was a relatively poor substrate for acylation into phosphatidylinositol as compared to arachidonic acid and eicosapentaenoic acid. However, the patterns of distribution of all three polyunsaturated fatty acids among the diacyl- and ether-linked class compositions of PC and PE were similar. These data suggest the potential of increasing the content of docosahexaenoic acid of membrane lipids in neutrophils by dietary supplement of this fatty acid.
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Polyunsaturated fatty acids appear to participate in the regulation of neutrophil functions. In response to physiological or chemical stimuli, leukotriene B_4 (LTB_4; 5,12-dihydroxyeicosatetraenoic acid) and 5-hydroxyeicosatetraenoic acid (5-HETE) are formed by the action of 5-lipoxygenase from either exogenous arachidonic acid (20:4[n-6]) (1,2) or endogenous 20:4(n-6) released from neutrophil phospholipids by activation of phospholipase A_2 (3). LTB_4 and 5-HETE are not only chemotactic stimuli toward neutrophils (4,5) but also modulate the formation of another potent chemotactic agent, platelet-activating factor (PAF; 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine), by enhancing the expression of phospholipase A_2 (6).

Eicosapentaenoic acid (20:5[n-3]), a major polyunsaturated acid in fish oils, is anti-inflammatory by inhibiting LTB_4 formation in human neutrophils (7,8). This fatty acid is metabolized to leukotriene B_5 (LTB_5; 5,12-dihydroxyeicosapentaenoic acid) which is a much weaker chemotactic agent for human neutrophils (9). In vitro studies have demonstrated that the patterns of the incorporation of 20:4(n-6) and 20:5(n-3) into individual phospholipids by neutrophils are similar (10), suggesting the potential of modification of fatty acid composition in neutrophil phospholipids by dietary supplements of 20:5(n-3).

Docosahexaenoic acid (22:6[n-3]) is also a major polyunsaturated fatty acid in fish oils. It is a poor substrate for the leukotriene-synthesizing system (11). In response to ionophore A23187, human neutrophils metabolize exogenous 22:6(n-3) to 7-hydroxydocosahexaenoic acid (8,12), which is not chemotactic for neutrophils (8). The present study was undertaken to examine whether human neutrophils can acylate 22:6(n-3) into cellular lipids. The results show that resting human neutrophils can acylate [1-^{14}C]22:6(n-3) into phospholipids and triacylglycerols.

MATERIALS AND METHODS

Preparation of human neutrophils. Human neutrophils were prepared according to the method of Lee et al. (13). Thirty-ml portions of venous blood from normal donors were each mixed with 4 ml of 0.15 M sodium citrate, pH 5.2, and 5 ml of 5% dextran T500 (Pharmacia Fine Chemicals, Piscataway, New Jersey) in 0.15 M NaCl and allowed to sediment at room temperature for 30 min. The supernatants containing leukocyte-rich plasma were removed and centrifuged at 250 × g for 10 min at 25 C. After hypotonic lysis of contaminating erythrocytes, leukocytes were washed once and resuspended in Krebs-Ringer phosphate buffer modified to contain 1.3 mM $CaCl_2$ and 5 mM glucose at 1–2 × 10^8 cells/ml. Three-ml cell suspensions were layered on 3 ml Ficoll-Hypaque (Pharmacia) cushions and centrifuged at 400 × g for 20 min at 25 C to yield a neutrophil pellet which was washed twice and suspended at a concentration of 20 × 10^6 cells/ml in Krebs-Ringer phosphate buffer. Cell counts were made in a hemocytometer, and cell viability was measured by trypan blue exclusion. Cell preparations contained more than 95% neutrophils.

Incubation of cells with [1-^{14}C]docosahexaenoic acid. [1-^{14}C]Docosahexaenoic acid (55.0 Ci/mol, New England Nuclear Corp., Boston, Massachusetts) was dissolved in dimethylsulfoxide (DMSO) and mixed with fatty acid-free bovine serum albumin (Miles Laboratories, Elkhart, Indiana, 4 mg/ml 0.9% NaCl). In a final volume of 2 ml, each tube contained 2.44 × 10^5 dpm (1 μM) to 1.95 × 10^6 dpm (8 μM) [1-^{14}C]22:6(n-3) and 20 × 10^6 neutrophils. The system was incubated at 37 C for various periods of time (1 to 120 min). The highest concentration of DMSO in the incubation medium was 0.1% and had no adverse effect on cell viability. Incubations were terminated by the addition of 10 ml methanol to each tube.

Lipid extraction and analysis. Neutrophil lipids were extracted (14) and dissolved in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene.

Triacylglycerols, 1,2-diacylglycerols and total phospholipids were separated by one-dimensional thin layer chromatography (TLC) on silica gel H (Analtech, Newark, Delaware) developed with petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v) (15). The radioactivity in each fraction was counted by liquid scintillation and expressed as a percentage of the total radioactivity in each incubation system.

Individual phospholipids were resolved by two-dimensional TLC on silica gel H and analyzed as described

previously (16). The chromatogram was developed with chloroform/methanol/28% aqueous ammonia (65:25:5, v/v/v) for the first dimension and with chloroform/methanol/acetic acid/water (95:10:30:3, v/v/v/v) for the second dimension.

PC and PE were each scraped and extracted from the silica gel with 10 ml chloroform/methanol/water (2:2:0.5, v/v/v) by vigorous shaking. The extract was washed once with 3 ml distilled water and centrifuged at 1,000 × g for 10 min. The lower chloroform layer was evaporated to dryness and redissolved in diethyl ether for subsequent analyses.

Treatment of the purified PC and PE with phospholipase C and acetylation of the resulting 1-radyl-2-acylglycerols were performed according to the method of Waku et al. (17). Each incubation tube contained 0.5 ml of 0.1 M Tris-HCl buffer, pH 7.4, containing 10 mM $CaCl_2$, 10 μl (16.7 units, 25 μg protein) *Bacillus cereus* phospholipase C (Sigma, St. Louis, Missouri) and 2 ml diethyl ether containing 2 to 2.5 μg phosphorus of PC or PE. The mixture was incubated for 16 hr at room temperature with constant stirring. The ether layer containing 1-radyl-2-acylglycerols was separated from the aqueous layer after a brief centrifugation, and the aqueous layer was extracted once more with 2 ml diethyl ether. After evaporation of the combined ether solutions to dryness, 0.2 ml of acetic anhydride/pyridine (10:1, v/v) was added to each tube. Acetylation was performed at room temperature for 16 hr, and it was terminated by the addition of 1 ml distilled water to each tube. The mixture was extracted three times each with 2 ml hexane. After evaporation of the combined hexane solutions to dryness, the residue was redissolved in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. The resulting 1-radyl-2-acyl-3-acetylglycerols were resolved into 1-alkenyl-2-acyl-3-acetylglycerol, 1-alkyl-2-acyl-3-acetylglycerol and 1,2-diacyl-3-acetylglycerol by TLC as described by Rendonen and Luukkonen (18) on silica gel H. The radioactivity of each lipid class was measured by liquid scintillation, and the amount of radioactivity in each class of the resulting diglyceride acetates was expressed as a percent of the total radioactivity recovered from the thin layer plate.

RESULTS AND DISCUSSION

The results in Table 1 show that [1-^{14}C]22:6(n-3) was acylated not only into phospholipids but also into triacylglycerols by intact human neutrophils. The rate of acylation of the fatty acid was a rapid process and reached maximum acylation into triacylglycerols and total phospholipids in 20 min under the experimental conditions in which the concentration of bovine serum albumin in the incubation medium was 0.1 mg/ml. This process was slower in the presence of a higher ratio of albumin to [1-^{14}C]22:6(n-3) in the incubation medium (data not shown). After a 2-hr incubation of 20 × 10^6 cells with 1 μM [1-^{14}C]22:6(n-3), 69.2% labeled fatty acid was acylated into triacylglycerols and 10.7% into phospholipids. The radioactivity of 1,2-diacylglycerol (1,2-DG) at all time intervals was less than 1% of the total radioactivity in the incubation system and reached a maximum after a 5-min incubation. It started to decline after longer periods of incubation, suggesting its participation in the formation of other lipids.

TABLE 1

Incorporation of [1-^{14}C]22:6(n-3) into Neutrophil Triacylglycerols and Phospholipids[a]

Time (min)	Triacylglycerols	1,2 Diacylglycerols (% of total radioactivity)	Phospholipids
1	5.09 ± 0.8	0.281 ± 0.02	2.67 ± 0.22
2	14.6 ± 1.2	0.587 ± 0.03	4.50 ± 0.17
5	41.0 ± 4.4	0.832 ± 0.03	9.54 ± 0.51
10	66.2 ± 6.8	0.448 ± 0.02	9.70 ± 0.44
20	68.9 ± 7.3	0.398 ± 0.01	10.3 ± 0.72
30	69.1 ± 6.7	0.323 ± 0.01	10.3 ± 0.81
60	69.5 ± 7.5	0.215 ± 0.01	10.4 ± 0.95
120	69.2 ± 8.4	0.212 ± 0.02	10.7 ± 1.0

[a]Neutrophils (20 × 10^6 cells) were incubated with [1-^{14}C]22:6(n-3) (2.44 × 10^5 dpm, 1 μM) at 37 C at indicated time. Lipids were extracted and resolved as described in Materials and Methods. Each value is mean ± SD from three separate experiments and is expressed as a percentage of the total radioactivity in each incubation system.

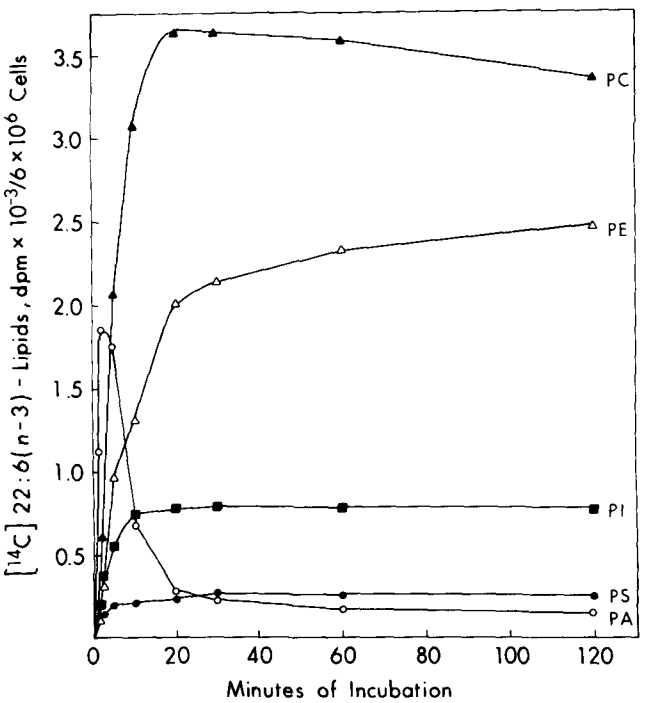

FIG. 1. Time-dependent acylation of [1-^{14}C]22:6(n-3) into individual neutrophil phospholipids. Human neutrophils (20 × 10^6 cells) were incubated at 37 C at the indicated time with 1 μM [1-^{14}C]22:6(n-3) (2.44 × 10^5 dpm) as described in Materials and Methods. Each point represents the average value from three separate experiments.

In human neutrophils the average molar phospholipid composition measured in the present study from five separate cell preparations was PC, 39.0%; PE, 27.9%; phosphatidylserine (PS), 10.9%; phosphatidylinositol (PI), 6.8%; and sphingomyelin, 15.4%. There were 1030 nmol of lipid phosphorus/10^8 cells. These values are in general agreement with published values (19). The phosphorus content of phosphatidic acid (PA) cannot be accurately measured; thus the distribution of radioactivity among individual phospholipids is expressed as dpm/6 × 10^6 cells. As shown in Figure 1, [1-^{14}C]22:6(n-3) was acylated

mainly into PA after 1-2 min of incubation, and the radioactivity of PA started to decline after a 5-min incubation. The labeled PA may be rapidly deacylated and the 22:6(n-3) moiety is then reacylated into other lysophospholipids. The low levels of [^{14}C]22:6(n-3) in 1,2-DG suggest that the deacylation-reacylation cycle may be a major pathway in the formation of [^{14}C]22:6(n-3)-PC and -PE in intact neutrophils. Also shown in Figure 1 is that 22:6(n-3) was a relatively poor substrate for acylation into PI as compared to 20:4(n-6) and 20:5(n-3) (10). However, the time course of the acylation of [1-^{14}C]-22:6(n-3) into PC and PE resembled those of [1-^{14}C]-20:4(n-6) and [1-^{14}C]20:5(n-3) (10). The radioactivity of PC reached a maximum after a 20-min incubation with 1 μM [1-^{14}C]22:6(n-3) and began to decline thereafter with increasing incubation time, whereas that of PE continued to increase. These data suggest a transfer of the [1-^{14}C]22:6(n-3) from PC to PE in neutrophils. There is evidence for the presence of a CoA-mediated, ATP-independent acyltransferase catalyzing the transfer of 20:4(n-6) from PC to lysoPE in mouse thymocytes (20), and a CoA-independent transacylase catalyzing the transfer of 20:4(n-6) from diacyl-PC to 1-alkenyl-lysoPE in human platelets (21) and to 1-acyl-lysoPE in dog heart membranes (22). PS contained the lowest radioactivity among the phospholipids, and sphingomyelin was not labeled by [1-^{14}C]22:6(n-3).

Figure 2 illustrates a time-dependent incorporation of [1-^{14}C]22:6(n-3) into individual phospholipids by neutrophils in the presence of various concentrations of [1-^{14}C]22:6(n-3) in the incubation medium. After a 2-min incubation, 6 μM of [1-^{14}C]22:6(n-3) was approximately substrate-saturating for acylation of this fatty acid into all phospholipids, and PA exhibited the highest radioactivity at all concentrations of [1-^{14}C]22:6(n-3) tested. After a 20-min incubation of cells with 1 to 8 μM [1-^{14}C]22:6(n-3), the radioactivity of PC became the highest among the phospholipids and it was markedly increased with increasing [1-^{14}C]22:6(n-3) concentrations in the incubation medium. These data suggest that in intact neutrophils lysoPA serves as a better acceptor than lysoPC for 22:6(n-3)-CoA under the experimental conditions. These data also indicate that [1-^{14}C]22:6(n-3) was acylated into PE at a slower rate than into PC and PI after a shorter period of incubation.

Human neutrophils have a high content of alkylacyl-PC and alkenylacyl-PE, and these ether-linked species are rich in 20:4(n-6) (23). Previous studies showed that exogenous 20:4(n-6) was acylated more rapidly into the diacyl-linked PC and PE than into the corresponding ether-linked class after a shorter period of incubation (1-20 min); however, more 20:4(n-6) appeared in alkylacyl-PC and in alkenylacyl-PE after a longer period of incubation (2 hr) (10). These studies were compatible with the distribution of 20:4(n-6) in vivo (23). Similarly, as shown in Table 2, the rate of acylation of [1-^{14}C]22:6(n-3) into the diacyl-linked and ether-linked PC and PE differed. The acylation of [1-^{14}C]22:6(n-3) into diacyl-linked PC and PE appears to precede that into the corresponding ether-linked class. With increasing incubation time a decrease in the radioactivity in the diacyl-linked class was accompanied by an increase in the radioactivity in alkylacyl-PC and alkenylacyl-PE, suggesting that part of the 22:6(n-3) moiety in alkylacyl-PC and alkenylacyl-PE was

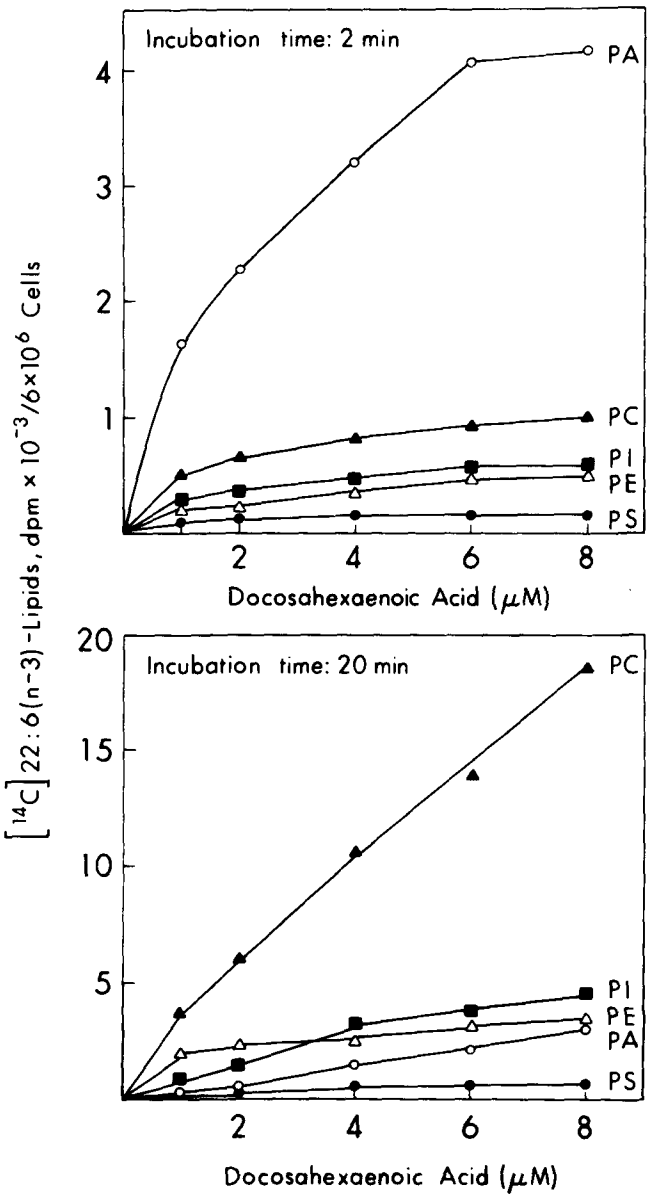

FIG. 2. Substrate-dependent acylation of [1-^{14}C]22:6(n-3) into individual neutrophil phospholipids. Human neutrophils (20×10^6 cells) were incubated at 37 C at the indicated time with 1 to 8 μM [1-^{14}C]22:6(n-3) (2.44×10^5 to 1.95×10^6 dpm) as described in Materials and Methods. Each point represents the average value from three separate experiments.

derived from the respective diacyl-linked class. While an enzymatic transfer of 22:6(n-3) or 20:4(n-6) from diacyl-PE to alkenyl-lysoPE has not been determined, a coenzyme A-independent transacylase catalyzing the transfer of 20:4(n-6) moiety from the sn-2-position of diacyl-linked PC to 1-alkyl-2-lysoPC has been demonstrated in human platelets (24) and in rabbit macrophages (25,26). It seems likely that a similar transacylase is present in human neutrophils. This enzyme probably exhibits a preference for 20:4(n-6) over 22:6(n-3), if one compares the data in Table 2 with that in previous studies which were performed under identical incubation conditions (10). After a 2-hr incubation about 43% of total labeled 20:4(n-6) in PC fraction was recovered in alkylacyl-PC (10), whereas

TABLE 2

Distribution of [1-^{14}C]22:6(n-3) in Diacyl-, Alkylacyl- and Alkenylacylphosphatidylcholine and -phosphatidylethanolamine[a]

	Phosphatidylcholine			Phosphatidylethanolamine		
Time (min)	Diacyl	Alkylacyl (%)	Alkenylacyl	Diacyl	Alkylacyl (%)	Alkenylacyl
10	88.7	10.1	1.20	70.5	20.0	9.5
20	85.6	12.7	1.70	62.4	24.8	12.8
30	83.3	15.0	1.70	58.2	22.1	19.7
60	75.3	21.7	3.07	43.0	24.0	33.0
120	72.8	23.8	3.43	38.2	23.3	38.5

[a]Neutrophils (20×10^6 cells) were incubated with [1-^{14}C]22:6(n-3) (2.44×10^5 dpm, 1 μM) at 37 C at indicated time. PC and PE were purified and treated with phospholipase C as described in Materials and Methods. The amount of radioactivity in each class of the resulting diglyceride acetates is the average value from two separate experiments and is expressed as a percentage of the total radioactivity recovered from the thin layer plate.

about 24% of total labeled 22:6(n-3) in PC fraction appeared in alkylacyl-PC (Table 2).

The present study demonstrates that human neutrophils can acylate exogenous 22:6(n-3) into PC and PE, suggesting the potential of increasing the content of 22:6(n-3) of membrane lipids in neutrophils by dietary supplements of this fatty acid. When neutrophils respond to physiological or chemical stimuli, free 22:6(n-3) derived either from phospholipids or from extracellular medium could be oxygenated to form 7-hydroxy-22:6(n-3) which has no chemotactic activity for neutrophils; it could also attenuate the production of PAF, thereby modifying the process of inflammation.

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Change of Substrate Specificity of Rat Liver Microsomal Fatty Acyl-CoA Synthetase Activity by Triton X-100

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The effect of Triton X-100 on the activities and apparent molecular size of fatty acyl-CoA synthetase, solubilized and partially purified from rat liver microsomes, was studied. In the presence of Triton X-100, the activity for lignoceroyl-CoA synthesis was decreased, but activity was restored when the detergent was removed. The appearance and disappearance of lignoceroyl-CoA synthesis appeared related to the size of the aggregated form of the enzyme. On the other hand, activity for palmitoyl-CoA synthesis was not significantly affected by the detergent. Because available evidence suggests that both fatty acids are converted to CoA esters by the same enzyme, it seems likely that the substrate specificity of the enzyme is influenced by changes in the aggregation state and that the microenvironment of the enzyme in membranes may determine the substrate specificity of acyl-CoA synthetase.
Lipids 21, 328–332 (1986).

Cerebrosides, sulfatides and sphingomyelin in the nervous system are characterized by enrichment of fatty acids with very long carbon chains (C_{22}–C_{26}) (1). These fatty acids are converted by condensation with sphingoid bases into ceramides, which form the common backbone structure of the sphingolipids. This process requires the formation of fatty acyl-CoA esters (2–5), although conversion of free fatty acids directly to ceramide without participation of CoA occurs in other subcellular preparations (6–9).

Because of our interest in the mechanism of incorporation of very long chain fatty acids into brain sphingolipids, we recently purified acyl-CoA synthetase solubilized by Triton X-100 from microsomes of young rat brain and compared its activity for lignoceroyl (24:0)-CoA synthesis with that of palmitoyl (16:0)-CoA synthesis as well as the activity of liver microsomal enzyme (10). The results indicated that the brain and liver enzymes may be identical (11,12). Although lignoceroyl- and palmitoyl-CoA synthetase activities in both tissues were not separated from each other during several purification steps, a significantly different distribution of these activities was found in two fractions obtained by Sephadex G-200 gel filtration, one with significantly higher molecular weight than the other. Most of the activity for lignoceroyl-CoA synthesis was found in the fraction with apparently higher molecular weight, whereas the activity converting the more common palmitic acid was distributed almost equally in these two fractions. This difference was possibly caused by residual Triton X-100. The present study was designed to elucidate the mechanism of the effects of Triton X-100 on fatty acyl-CoA synthetase. In particular, we wished to determine whether lignoceric and palmitic acids were activated by different enzymes or by a single enzyme with multiple substrate specificity. The results reported here suggest that fatty acid substrate specificity of CoA synthetase may depend on the degree of aggregation of the enzyme. Preliminary accounts of this investigation were previously presented (13).

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Lignoceric acid was synthesized in this laboratory as described previously (10) and [1-^{14}C]palmitic acid was purchased from New England Nuclear (Boston, Massachusetts). Sepharose 6B-CL was from Pharmacia (Piscataway, New Jersey) and Extracti-Gel D from Pierce (Rockford, Illinois). CoA was supplied by P.L. Biochemicals (Milwaukee, Wisconsin) and other chemicals were from Sigma (St. Louis, Missouri). Sprague-Dawley rats (CD strain) were obtained from Charles River Breeding Labs (Wilmington, Massachusetts).

Enzyme preparation. Acyl-CoA synthetase activity was solubilized by treating microsomes prepared from 20 livers of 30-day-old rats with 120 ml of 5 mM Triton X-100 (average mol wt 647) as described previously (10) and partially purified by ammonium sulfate precipitation as described by Bar-Tana et al. (11). The enzyme preparation had a specific activity of 74 pmol of lignoceroyl-CoA synthesized/mg protein/min and 26 nmol of palmitoyl-CoA synthesized/mg protein/min. In some experiments, the ammonium sulfate precipitation was replaced by Blue-Sepharose 6B-CL column chromatography, performed as described previously (10). The specific activity of this preparation was 23 pmol of lignoceroyl-CoA synthesized/mg protein/min and 9 nmol of palmitoyl-CoA synthesized/mg of protein/min. All operations were performed at 2–4 C.

Removal of detergent. Triton X-100 was removed by passing the enzyme preparations through a column containing Extracti-Gel D. After the column was rinsed, the enzyme was eluted with 2 column volumes of 50 mM Bicine, pH 7.8, containing 1 mM dithiothreitol and 1 mM EGTA. The eluent and rinses were combined and concentrated by use of an Amicon filter PM10. Over 90% of the Triton X-100 was removed by this procedure.

Assay of acyl-CoA synthetase activity. Activities were assayed by measuring the formation of CoA thioesters from [1-^{14}C]lignoceric acid (56 mCi/mmol) or [1-^{14}C]palmitic acid (0.96 mCi/mmol) as described (10). For the synthesis of lignoceroyl-CoA, the assay mixture contained 10 mM ATP, 10 mM α-cyclodextrin, 50 mM Bicine-Na^+, pH 7.8, 0.2 mM CoA Li, 1 mM $MgCl_2$ and 4 μM lignoceric acid coated on Celite. After adding the enzyme preparation, the tubes were incubated at 37 C for 20 min. Lipids were extracted from the incubation mixture by chloroform/methanol (2:1, v/v) and acyl-CoA formed was extracted from the total lipids by Folch partition. The formation of palmitoyl-CoA was assayed similarly except

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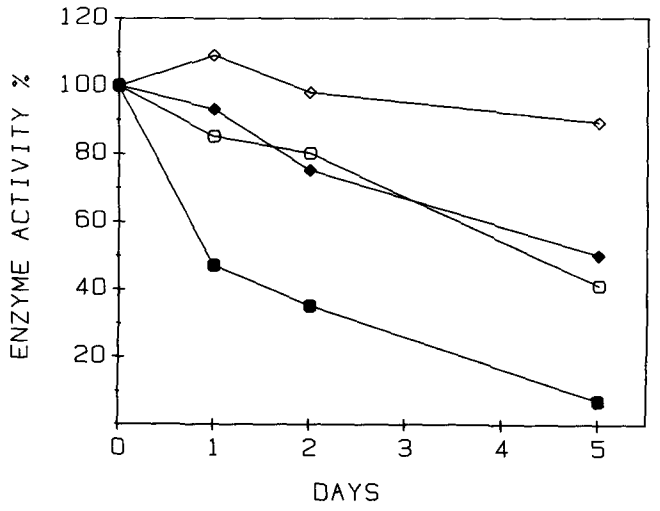

FIG. 1. Effect of Triton X-100 on activity of fatty acyl-CoA synthetase as a function of time. The enzyme preparation partially purified by ammonium sulfate precipitation with or without removal of Triton X-100 was maintained at 4 C for the time indicated and then assayed for enzymatic activity. Lignoceroyl-CoA synthetase activity is shown after treatment with Extracti-Gel D (□) or without treatment (■). Palmitoyl-CoA synthetase activity is shown after treatment with Extracti-Gel D (◇) or without treatment (◆).

that 0.4 mM CoA Li and 0.2 mM palmitic acid were used.

Protein concentration was measured with a Bio-Rad protein kit by following manufacturer's instructions. The amount of Triton X-100 was determined as described by Horigome and Sugano (14).

RESULTS

Effect of Triton X-100 on storage of acyl-CoA synthetase. The enzyme preparation partially purified from rat liver microsomes by ammonium sulfate precipitation contained 0.22 μmol of Triton X-100/mg of protein (10). An aliquot of this solution was treated with Extracti-Gel D to remove residual detergent and stored at 4 C together with an aliquot of untreated enzyme preparation. As shown in Figure 1, after five days, lignoceroyl-CoA synthetase activity in the untreated preparation had decreased to 8% of its original level, whereas if the detergent was removed before storage, 41% of the original activity remained. Palmitoyl-CoA synthetase activities were less affected by the same treatment; after five days of storage, about 50% of the original activity remained in the untreated preparations, and about 90% in the Extracti-Gel D-treated preparation.

We further examined the differential effects of Triton X-100 by treating a partially purified preparation of the synthetase with Extracti-Gel D, then adding Triton X-100 to a final concentration of 2 mM. The preparation was stored at 4 C and at specified times portions of the preparation were fractionated by Sepharose 6B gel filtration with use of an eluting buffer containing 0.5 mM Triton X-100. Figure 2 shows that when the preparation was fractionated immediately after the addition of detergent, lignoceroyl-CoA synthetase activity was detected only in the fractions of higher mol wt. Activity in these fractions progressively decreased upon storage and was barely detectable after eight days. On the other

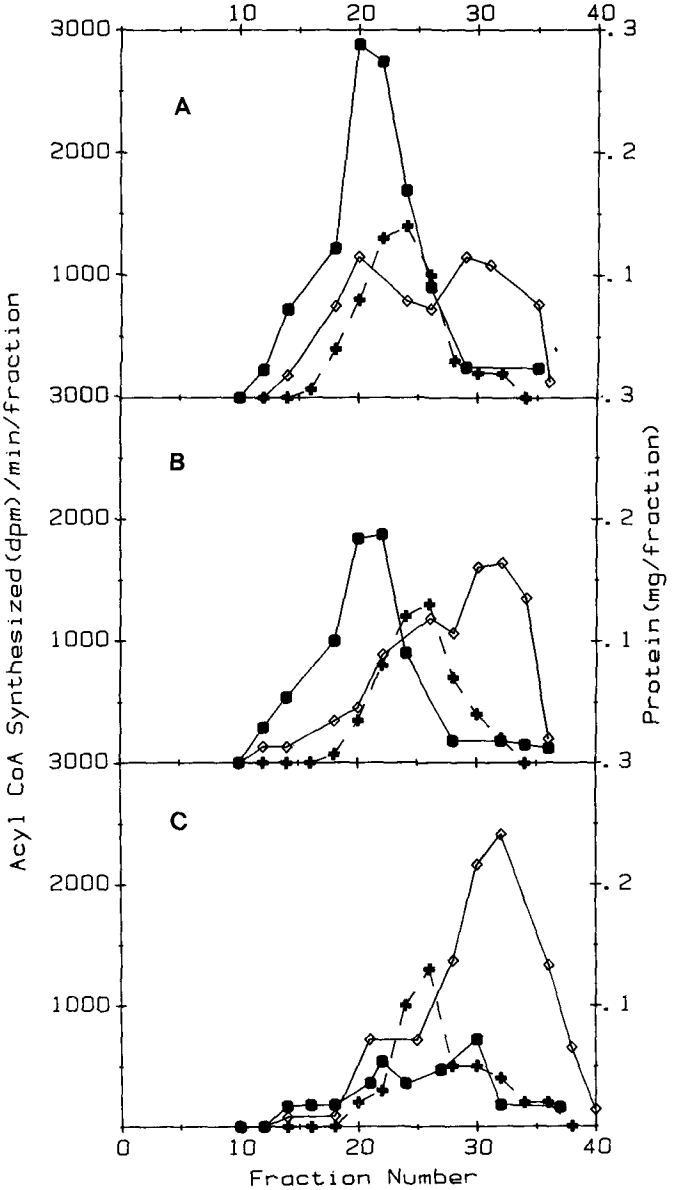

FIG. 2. Effect of Triton X-100 on apparent size of fatty acyl-CoA synthetase as a function of time. Triton X-100 was added to the enzyme preparation, partially purified by Blue Sepharose chromatography to a final concentration of 2 mM and allowed to stand at 4 C. Portions of this preparation were fractionated on Sepharose 6B gel column (1.5 × 6.0 cm) after 0 (A), 4 (B) and 8 (C) days storage. A 50 mM Bicine buffer, pH 7.8, containing 1 mM dithiothreitol and 0.25 M NaCl containing 0.5 mM Triton X-100 were used for elution. The flow rate was 1.2 ml/hr and fractions of 3.6 ml were collected. ■, Activity for lignoceroyl-CoA synthetase; ◇, activity for palmitoyl-CoA synthetase; +, protein concentration.

hand, the activity for palmitoyl-CoA synthetase was initially distributed evenly between fractions of higher and lower mol wt. Similar to lignoceroyl-CoA synthetase activity, palmitoyl-CoA synthetase activity in the fractions of higher mol wt was almost completely lost after storage for eight days. In contrast to lignoceroyl-CoA synthetase activity, however, palmitoyl-CoA synthetase activity in the fractions of lower mol wt increased progressively during this period.

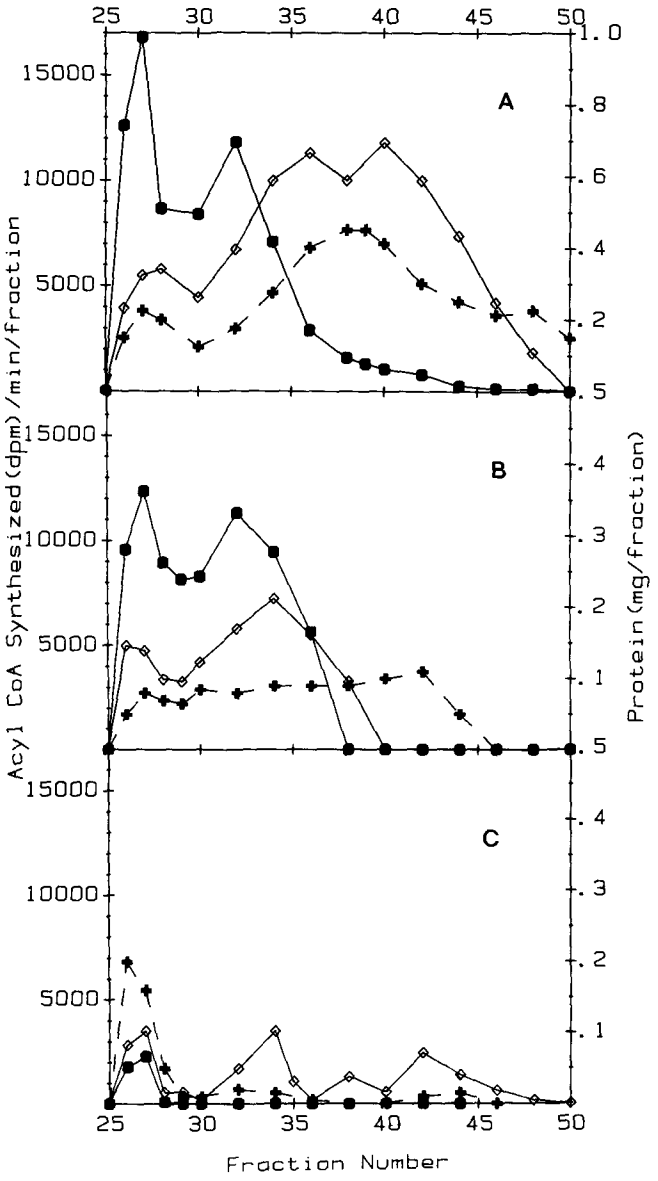

FIG. 3. Effect of Triton X-100 on distribution of fatty acyl-CoA synthetase activity fractionated by Sepharose 6B gel filtration. (A) The enzyme preparation solubilized from rat liver microsomes with 5 mM Triton X-100 and partially purified by ammonium sulfate precipitation was fractionated on a column (2.6 cm i.d. × 100 cm) containing Sepharose 6B-CL. The column was eluted with 50 mM Bicine buffer, pH 7.8, containing 1 mM dithiothreitol and 1 mM EGTA, at a flow rate of 1.12 ml/hr. Fractions of 5.6 ml were collected and assayed for lignoceroyl-CoA synthetase (■), palmitoyl-CoA synthetase (◇) and protein (+). (B) Portions of fractions 38–44 (see [A]) were pooled, treated with Extracti-Gel D and reapplied to the same column of Sepharose 6B under the same conditions as described for (A). (C) Portions of fractions 26–28 (see [A]) were pooled, treated with Triton X-100 to 5 mM, stored for three days and reapplied to the same column of Sepharose 6B under the same conditions as described for (A).

Effect of Triton X-100 concentration on acyl-CoA synthetase. The experiments described above suggested that Triton X-100 might be exerting its effects by altering the degree of aggregation of the enzyme. To examine this possibility in greater detail, we subjected the partially purified enzyme preparation to gel filtration similar to that described above, except that Triton X-100 was omitted from the eluting buffer. As shown in Figure 3A, the distribution of enzyme activity was essentially the same as that shown in Figure 2A. Fractions 38–44 (smaller molecular size) were then combined, treated with Extracti-Gel D to remove Triton X-100 (see Experimental Procedures) and applied to the same Sepharose 6B column. In this case, not only were the peaks of the enzyme activities eluted earlier (Fig. 3B), but activity for lignoceroyl-CoA synthesis increased 14-fold after the detergent was removed. On the other hand, the total activity recovered for palmitoyl-CoA synthesis increased only 10%.

A marked effect was also obtained when Triton X-100 was added to the fraction containing higher mol wt protein. Pooled fractions 26–28 (Fig. 3A) were treated with Triton X-100 to a final concentration of 5 mM and stored for 3 days at 4 C, after which the solution was fractionated by Sepharose 6B gel filtration as described above. The activity for lignoceroyl-CoA synthesis was reduced to 16% of that of the original activity applied to the column and activity was confined to fractions 25–28 (Fig. 3C). On the other hand, almost all the original activity for palmitoyl-CoA synthesis was recovered in fractions 25 through 50.

Effect of Triton X-100 on kinetics of acyl-CoA synthetase. We next examined the kinetics of the effect of Triton X-100 on the activity of acyl-CoA synthetase by determining the values for Km and Vmax for lignoceric and palmitic acids in the presence and absence of the detergent. With use of pooled fractions 38 and 39 (Fig. 3A), we found that in the presence of the detergent the Km value for lignoceric acid was about 11 μM and Vmax was 175 pmol of lignoceroyl-CoA synthesized/mg of protein/min. When the detergent was removed by Extracti-Gel D treatment, the Km value was decreased to 3 μM and Vmax increased to 350 pmol. On the other hand, the same preparation, in the presence of the detergent, showed Km and Vmax values for palmitic acid of 78.6 μM and 27.6 nmol of palmitoyl-CoA synthesized/mg protein/min, respectively. After Extracti-Gel D treatment, the Km value was also decreased to 33.3 μM, but Vmax remained unchanged at 30.3 nmol.

Since lignoceroyl-CoA synthetase activity was increased by removing Triton X-100, it is possible that the detergent had an inhibitory effect on this activity. We therefore included various amounts of Triton X-100 in the assay system. We found that Triton X-100 was slightly stimulatory to both lignoceroyl- and palmitoyl-CoA synthetase activities up to 0.5 mM (data not shown). A further increase of Triton X-100 inhibited lignoceroyl-CoA synthesis but continued to stimulate palmitoyl-CoA synthesis. Similar effects of Triton X-100 were obtained in each fraction obtained by Sepharose gel filtration as described above.

DISCUSSION

The results obtained showed that the activity and apparent molecular size of acyl-CoA synthetase were reversibly modulated by Triton X-100 and that the effect was much more marked for lignoceroyl-CoA synthetase than for palmitoyl-CoA synthetase. The enzyme preparation which had an apparent molecular size of about 250,000 daltons shifted to about 500,000 daltons when Triton

X-100 was removed. With the shift of apparent molecular size, the activity of lignoceroyl-CoA increased over 10-fold, whereas the activity of palmitoyl-CoA synthetase remained almost unchanged. On the other hand, the enzyme preparation with an apparently high mol wt shifted to a lower mol wt after being treated with Triton X-100. In this instance, most lignoceroyl-CoA synthetase activity was lost, but, again, palmitoyl-CoA synthetase activity was unchanged. Kinetic studies showed that Triton X-100 increased the apparent Km value for lignoceric acid and decreased the Vmax value for lignoceroyl-CoA synthesis. The detergent also increased the Km value for palmitic acid but did not change the Vmax value significantly. We also showed that small amounts of Triton X-100 in the assay mixture stimulated rather than inhibited the enzyme activity.

These observations were in accord with the view that the substrate specificity of acyl-CoA synthetase changes with its apparent molecular size, possibly because of aggregation and deaggregation of the enzyme resulting from removal or addition of Triton X-100. However, they did not rule out the possibility that the synthesis of CoA esters of lignoceric and palmitic acids may be catalyzed by separate enzymes. Indeed, from substrate competition studies with use of a microsomal preparation from rat brain, Bhushan et al. (15) concluded that the enzyme for lignoceroyl-CoA ester synthesis is different from that for palmitoyl-CoA synthesis. Nevertheless, our previous study established that the activities with the two fatty acids could not be separated during purification which included ammonium sulfate precipitation, and Blue-Sepharose and DEAE-Sepharose column chromatography. In addition, we found that the two activities were eluted identically by hydroxylapatite and phosphocellulose chromatography (Soeda, S., and Kishimoto, Y., unpublished results). Bar-Tana et al. (11) and Tanaka et al. (12) showed that their highly purified acyl-CoA synthetase from rat liver catalyzed the conversion of lignoceric acid and behenic acid (22:0), respectively, to their CoA esters to a small extent. In recent experiments, a highly purified enzyme obtained from rat liver microsomes showed a single spot on SDS-polyacrylamide gel electrophoresis and was also active with both substrates; furthermore, antibodies raised against this enzyme preparation inhibited CoA ester formation from both lignoceric and palmitic acids (16). Although more unequivocal evidence may be desirable, these observations suggest that a single enzyme acts on both palmitic and lignoceric acid.

It was also possible that the different activities resulted from the difference in concentration of two substrates. In routine assays, we used concentration of palmitic acid (0.4 mM) 100-fold higher than that of lignoceric acid (4 μM), which may have contributed to the different effects of Triton X-100 on synthesis of the two fatty acyl-CoA derivatives. In our kinetic study, however, specific activity remained the same over a wide range of substrate concentrations (Fig. 4). In fact, the lowest concentration of palmitic acid tested was similar to that of the highest concentration of lignoceric acid tested. This observation suggests that the difference in substrate concentration may not be responsible for the Triton X-100 effects.

These observations indicate that dissociation and reassociation of acyl-CoA synthetase have a significant

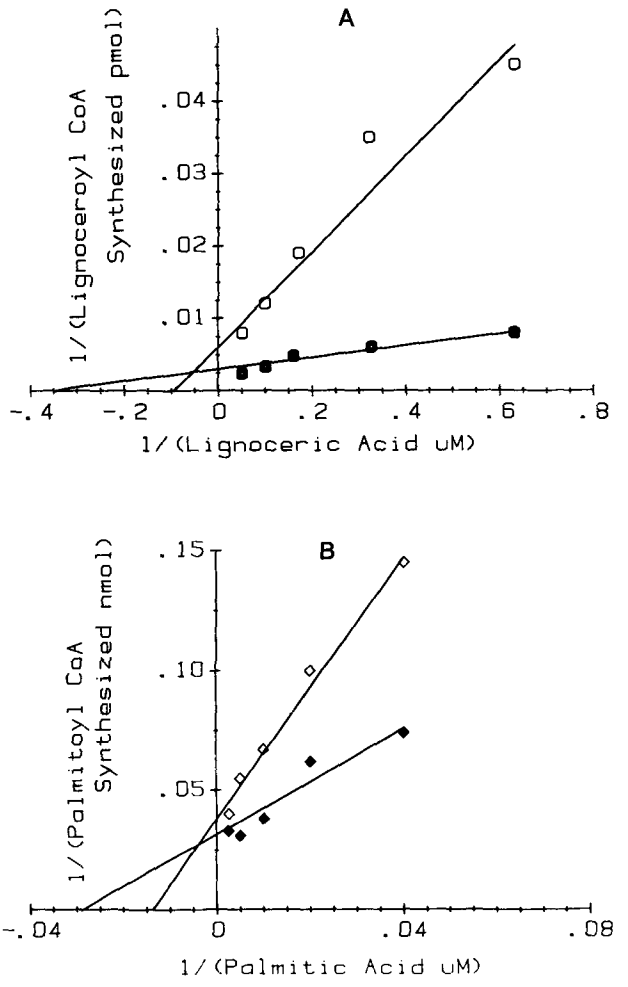

FIG. 4. Effect of Triton X-100 on double reciprocal plots of fatty acyl-CoA synthetase activity as a function of fatty acid concentration. (A) Synthesis of lignoceroyl-CoA. Pooled fractions 38 and 39 from Sepharose 6B gel filtration (Fig. 3A) were used as enzyme source before (□) and after (■) removal of Triton X-100. Conditions of assays were as described under Experimental Procedures, except that concentrations of lignoceric acid were varied as indicated. (B) Synthesis of palmitoyl-CoA. All conditions were identical to those described in (A), except that palmitic acid was used as substrate. ◇, Activity obtained with the Sepharose 6B fractions; ◆, activity with the same fractions after the Extracti-Gel D treatment.

effect on the synthesis of lignoceroyl-CoA but not palmitoyl-CoA synthesis. The enzyme apparently requires a larger area of interaction with lignoceric acid (C_{24}) than with palmitic acid (C_{16}). Reaggregation resulting from the removal of Triton X-100 may make the configuration more suitable for interaction with lignoceric acid. Membrane enzymes, such as acyl-CoA synthetase studied in this investigation, interact with surrounding lipids and proteins in the membrane. Therefore, it is conceivable that the configuration of the enzyme is altered as its microenvironment changes. Since acyl-CoA synthetase in different subcellular organelles such as microsomes, mitochondria and peroxisomes was found to be identical (12,17), and the enzymes in different tissues such as liver and brain appear to be the same (13), the substrate specificity of this enzyme in different tissues or subcellular organelles may well be affected by different environments in these membranes.

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Takashi Hashimoto provided antibodies against fatty acyl-CoA synthetase from rat liver microsomes. Pamela Talalay gave editorial assistance and Janice White provided secretarial support. This study was supported by Grants NS 13569 and NS 13559 from the National Institutes of Health and Grant BNS-83-14337 from the National Science Foundation.

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Chemical Ionization-Mass Spectrometry of Secondary Oxidation Products from Methyl Linoleate and Linolenate

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Chemical ionization-mass spectrometry (CI-MS) with a direct exposure probe was used to analyze a series of hydroperoxy cyclic peroxides and dihydroperoxides obtained from methyl linoleate and linolenate by either autoxidation or photosensitized oxidation. The mass spectra obtained with isobutane and ammonia as reacting gases showed fragmentation patterns similar to those previously observed by thermal decomposition under conditions of gas chromatography (GC)-electron impact (EI)-MS. Because the fragmentation patterns obtained under either CI-MS with a direct exposure probe or GC-(EI)-MS conditions are sufficiently predictable, these techniques are powerful analytical tools for the structural characterization of lipid oxidation products. These techniques are also useful to elucidate the cleavage pathways to volatile lipid oxidation products of flavor and biological significance.
Lipids 21, 333-337 (1986).

Intact monohydroperoxide isomers of methyl linoleate were previously analyzed by chemical ionization-mass spectrometry (CI-MS) (1). By using a direct exposure probe, structurally useful fragmentation patterns were obtained with isobutane CI and ammonia CI, which were explained by both heterolytic and homolytic cleavages around the hydroperoxide group (2). The same pathways were previously established by analyzing the volatile thermal and acid decomposition products of monohydroperoxides (3-6) and secondary products of lipid oxidation (4,7,8) using conventional gas chromatography (GC) followed by electron ionization-mass spectrometry (EI-MS). With hydroperoxy cyclic peroxides (epidioxides), the thermal fragmentation observed was sufficiently predictable that GC-MS was recommended as a tool for the structural characterization of these types of lipid oxidation products (8).

We now report the application of CI-MS for the structural analysis of hydroperoxy cyclic peroxides and dihydroperoxides obtained from linoleate and linolenate by either free radical autoxidation or photosensitized oxidation. The fragmentation patterns observed by isobutane CI are compared with our previous decomposition studies based on GC-(EI)-MS to elucidate the pathways to volatile oxidation products of flavor and biological significance. The ammonia CI-MS was also investigated on two hydroperoxy cyclic peroxides.

EXPERIMENTAL

Hydroperoxy cyclic peroxides were prepared from methyl linoleate subjected to photosensitized oxidation (7) and from methyl linolenate subjected to either autoxidation (9) or photosensitized oxidation. The hydroperoxy bis-cyclic peroxides and dihydroperoxides were derived from the photosensitized oxidation of methyl linolenate (10). The procedure involved silicic acid column separation of monohydroperoxides from secondary oxidation products followed by high pressure liquid chromatography (4). The functional purity of each chromatographic fraction was checked by thin layer chromatography using previously characterized hydroperoxy cyclic peroxides and dihydroperoxides as reference standards. The same mass spectrometer (Finnigan 4535/TSQ) and CI-MS procedure with direct exposure probe was used as that previously described for monohydroperoxides of methyl linoleate (2).

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RESULTS AND DISCUSSION

When applied to monohydroperoxides of methyl linoleate, the direct exposure CI technique previously has been shown to produce spectra with more intense mass fragments than obtained with the standard probe (2). The mass fragments obtained provided structural information on the location of the hydroperoxide group and could be explained by well-recognized homolytic and heterolytic decomposition pathways (3,5,11). The direct exposure CI technique was applied in this study to intact hydroperoxy cyclic peroxides and dihydroperoxides using either isobutane or ammonia as reacting gases (Figs. 1-3).

The isobutane CI spectra of the monounsaturated hydroperoxy cyclic peroxides I and II from methyl linoleate showed weak fragments at m/z 341 and 325, corresponding to $(MH\text{-}H_2O)^+$ and $(MH\text{-}H_2O_2)^+$, respectively. The base peaks at m/z 187 for peroxide I correspond to the protonated C-9 aldehyde ester formed from cleavage A between the hydroperoxide group and the peroxide ring after homolytic or heterolytic loss of OH (Fig. 1A). Other fragments produced from this cleavage include m/z 173 corresponding to $(MH\text{-}186)^+$, 155 from cleavage A and 139 $(155\text{-}O)^+$. Alternatively, peak 155 may be rationalized as derived from 187 with the loss of methanol (32). This assignment is more consistent with that made below for the bis-cyclic peroxides IV and V (Figs. 2B and 2C). Ion fragment 139 may also be derived from protonated nonadienal by cleavage A after opening of the peroxide ring and loss of OH. Cleavage B across the peroxide ring produced another major fragment at m/z 113, corresponding to protonated heptenal. The isobutane CI spectrum of peroxide II indicates the same fragmentation pattern as peroxide I, but here the base peak at m/z 199 corresponding to protonated C-10:1 aldehyde ester is consistent with cleavage B across the peroxide ring (Fig. 1C). Fragments produced from cleavage A between the hydroperoxide group and the peroxide ring include m/z 101 corresponding to protonated hexanal, 225 $(241\text{-}OH)^+$ and 241 $(M\text{-}100\text{-}OH)^+$. Cleavage C between the peroxide ring and the allylic double bond accounts for peaks at m/z 187 $(186+H^+)$ and 157 $(172\text{-}O=156+H^+)$.

Previous studies of thermal decomposition of cyclic peroxides I and II (7) support the identity of fragment ions 187, 113, 199, and 101 as due to the respective protonated species of C-9 aldehyde ester, 2-heptenal, C-10:1 aldehyde ester and hexanal. The relative intensities of

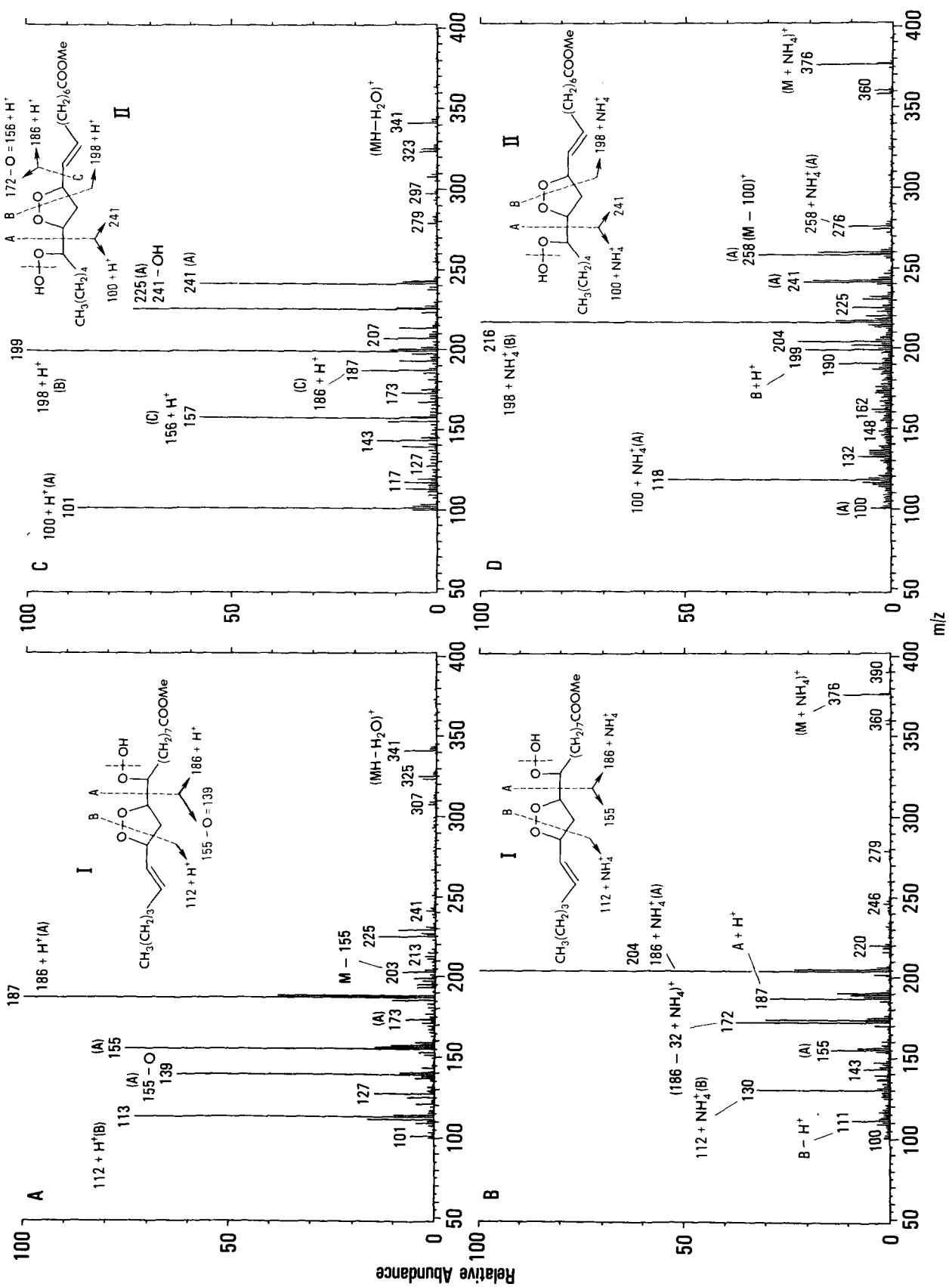

FIG. 1. (A) Isobutane CI-MS of methyl 9-hydroperoxy-10,12-epidioxy-13-octadecenoate (I); (B) Ammonia CI-MS of I; (C) Isobutane CI-MS of methyl 13-hydroperoxy-10,12-epidioxy-8-octadecenoate (II); (D) Ammonia CI-MS of II.

these fragment ions observed in the spectra of these cyclic peroxides (Figs. 1A and 1C) vary in the same direction as the relative concentrations of the corresponding fragmentation products previously found by thermal decomposition of I (39% C-9 aldehyde ester from cleavage A and 27% 2-heptenal from cleavage B) and II (29% C-10:1 aldehyde ester from cleavage B and 45% hexanal from cleavage A) (7).

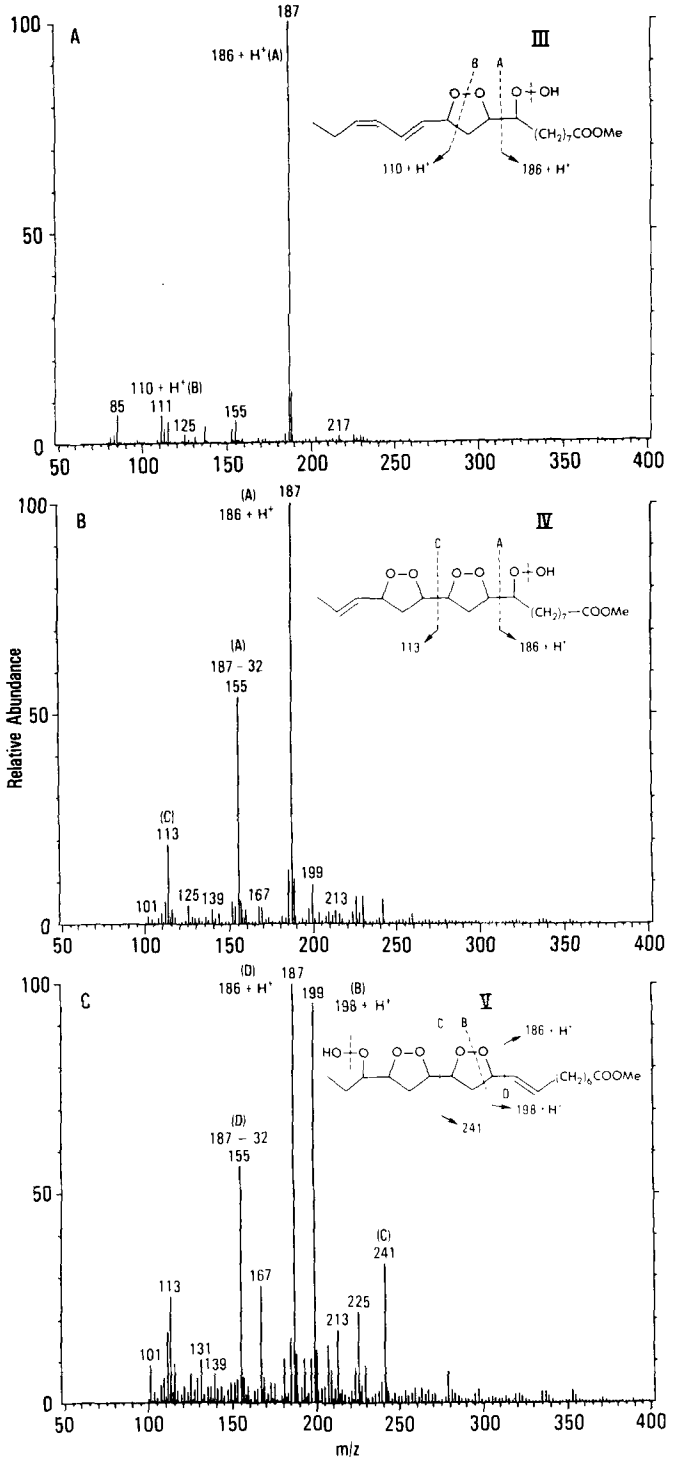

FIG. 2. Isobutane CI-MS of (A) methyl 9-hydroperoxy-10,12-epidioxy-13,15-octadecadienoate (III); (B) methyl 9-hydroperoxy-10,12,13-bisepidioxy-16-octadecenoate (IV); (C) methyl 16-hydroperoxy-10,12,13,15-bisepidioxy-8-octadecenoate (V).

The ammonia CI spectra of peroxides I and II showed weak fragments at m/z 376 and 360 corresponding to $(M+NH_4)^+$ and $(M+NH_4\text{-}O)^+$, respectively (Figs. 1B and 1D). With a standard probe, the ammonia CI spectrum of a mixture of peroxide isomers I and II was reported to give the adduct ions 376 $(M+18)^+$ and 358 $(M+18\text{-}H_2O)^+$ as intense peaks (88.7 and 100% rel int, respectively) (12). Figure 1B shows the base peak for peroxide I at m/z 204, corresponding to the ammonia adduct of C-9 aldehyde ester from cleavage A after loss of OH. The peak from the protonated C-9 aldehyde ester at m/z 187 is also important, as well as the other fragments consistent with cleavage A at m/z 172 (186-32=154+$NH_4)^+$ and 155 $(172\text{-}O)^+$. Products from cleavage B are also observed as the ammonia adduct 130 $(112+NH_4)^+$ and ion 111 $(B\text{-}H)^+$ of heptenal. In the ammonia CI spectrum of II the base peak at m/z 216 corresponding to the adduct of C-10:1 aldehyde ester may come also from cleavage B across the ring; the corresponding protonated ion is also evident at m/z 199 (Fig. 1D). Products from cleavage A are observed as ions at m/z 118 and 100, corresponding to the ammonia adduct of hexanal and protonated hexanal, respectively. Other peaks that may be due to cleavage A after loss of OH include 258 $(M\text{-}100)^+$, 241 $(M\text{-}117)^+$ and 276 $(258+NH_4)^+$.

The isobutane CI spectrum of the conjugated diene hydroperoxy cyclic peroxide III from methyl linolenate was dominated by the base peak at m/z 187 assigned to protonated C-9 aldehyde ester derived from cleavage A (Fig. 2A). Fragment ion 111 corresponding to protonated heptadienal from cleavage B became much less important than with peroxides I and II, apparently because of the presence of the conjugated diene system. No fragment related to the molecular ions could be detected. The protonated ions observed by CI-MS of cyclic peroxide III are analogous to the thermal decomposition products previously observed under conditions of GC-(EI)-MS, with C-9 aldehyde ester as a major product (55.7%) and 2,4-heptadienal as a minor one (4.6%) (8).

The isobutane CI spectra of the hydroperoxy bis-cyclic peroxides IV and V from methyl linolenate also showed no fragments related to the molecular ions (Figs. 2B and 2C). The base peak at m/z 187 for peroxide IV may be derived from cleavage A, between the hydroperoxide group and the first peroxide ring, after loss of OH producing C-9 aldehyde ester, which becomes protonated. Peak at m/z 155 can be rationalized as derived from 187 with the loss of 32 due to methanol. Fragments expected from cleavage B across the terminal peroxide ring are too small in molecular weight to be detected. Fragment ion at m/z 113 can be attributed to cleavage C between the two peroxide rings in IV (Fig. 2B). The isobutane CI spectrum of bis-cyclic peroxide V showed peak 199 as the second most important fragment ion corresponding to protonated C-10:1 aldehyde ester from cleavage B (Fig. 2C). The base peak at m/z 187 corresponding to protonated C-9 aldehyde ester may be derived from a heterolytic cleavage D between C-9 and C-10. Alternatively, the vinylic radical from the corresponding

homolytic cleavage could react with OH radical to produce the vinylic alcohol tautomerizing to the saturated C-9 aldehyde ester (3). Fragment ion at m/z 241 can be assigned to cleavage C between the two peroxide rings in V. Previous thermal decomposition studies of bis-cyclic peroxides IV and V (8) produced analogous fragmentation patterns with C-9 aldehyde ester as a dominant product of both peroxides (44.3 and 20.3%, respectively) and C-10:1 aldehyde ester as an important product of V (23.8%).

The fragmentation pattern observed with all hydroperoxy cyclic peroxides I–V is in complete agreement with that previously observed with the same compounds subjected to thermal decomposition under GC-(EI)-MS conditions (4,7,8). The general fragmentation observed under these conditions between the peroxide ring and the hydroperoxide was considered sufficiently predictable to be used as an analytical tool for the structural characterization of these compounds. On the same grounds, CI-MS can be used to investigate the structure of these cyclic compounds if a direct exposure probe is used to magnify the intensity of fragment ions.

The isobutane of CI spectra of dihydroperoxides VI, VII and VIII from methyl linolenate show characteristic fragment ions at m/z 339 $(MH\text{-}H_2O)^+$, 323 $(M\text{-}OOH)^+$, 307 $(MH\text{-}H_2O\text{-}2\ O)^+$ and 305 $(323\text{-}H_2O)^+$ (Figs. 3A-C). The spectrum of the 10,12-dihydroperoxide VI can be explained by a fragmentation pattern involving cleavage on each side of the hydroperoxide groups. According to this mechanism, base peak 199 comes from cleavage A after loss of OH producing C-10:1 aldehyde ester (Fig. 3A). Fragment ion 187 comes from cleavage B producing C-9 aldehyde ester by either heterolysis or homolysis as discussed above for cyclic peroxide V. The identity of fragment ion 187 as due to methyl 9-oxononanoate is supported by our previous studies of thermal decomposition of dihydroperoxides VI, VII and VIII (4). Cleavage C around the second hydroperoxide group of VI produces heptadienal detected as the protonated ion 111. Fragment ion 125 may come from cleavage A producing a diunsaturated C-8 methyl ketone (Fig. 3A).

The isobutane spectra of dihydroperoxides VII and VIII revealed information only on the position of the first hydroperoxide group on C-9 and C-10 (Figs. 3B and 3C). The hydroperoxide group on C-16 produced fragments that were below the lowest mass scanned in the spectra. The spectrum of 10,16-dihydroperoxide (VII) is dominated by base peak 199 due to protonated C-10:1 aldehyde ester from cleavage A. In contrast to dihydroperoxide VI, the fragment ion 187 was found to the extent of 8% in the spectrum of VII that would be expected from cleavage B on the other side of the C-10 hydroperoxide group (Fig. 3B). Fragment ion at m/z 125 can be attributed to cleavage A with loss of OOH. The isobutane spectrum of 9,16-dihydroperoxide VIII showed evidence of cleavage A producing protonated C-9 aldehyde ester as the base peak at m/z 187 and cleavage B producing methyl octanoate at m/z 159 $(158+H)^+$ (Fig. 3C). The peak at m/z 199 indicates that the sample was not pure but contaminated with dihydroperoxide VII (Fig. 3B).

The CI-MS of dihydroperoxides produced similar but simpler fragmentation patterns than those previously observed with the same compounds under conditions of GC-(EI)-MS (4). With CI-MS, however, some structural information was lost in those compounds containing a hydroperoxide group near the end of the molecule because the fragment ions produced were too small to be detected under the direct exposure technique used in this study.

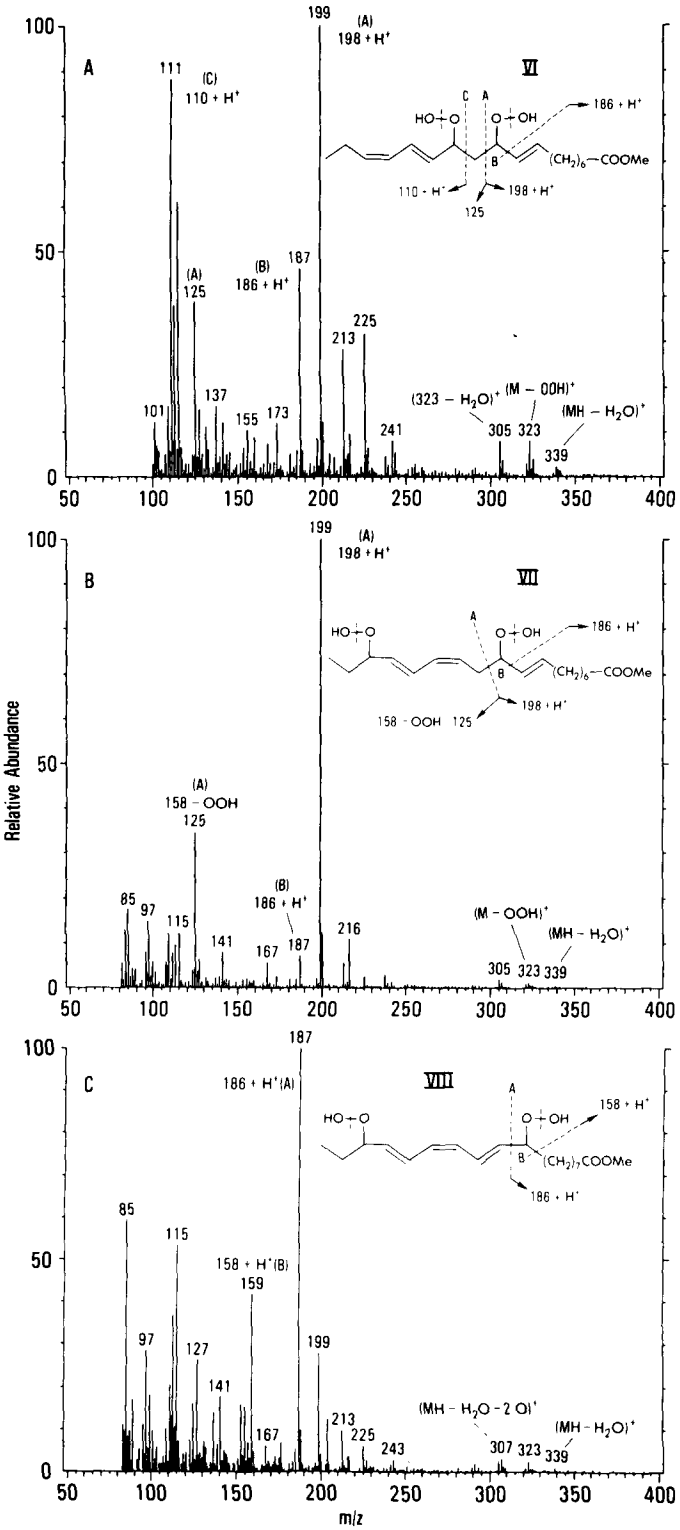

FIG. 3. Isobutane CI-MS of (A) methyl 10,12-dihydroperoxy-8,13,15-octadecatrienoate (VI); (B) methyl 10,16-dihydroperoxy-8,12,14-octadecatrienoate (VII); (C) methyl 9,16-dihydroperoxy-10,12,14-octadecatrienoate (VIII).

In general, more structural details could be gained from the fragmentation patterns observed previously under the thermal decomposition conditions used for GC and GC-(EI)-MS (3,4,8). Furthermore, the improved reproducibility achieved by capillary GC (4) makes it possible to obtain useful structural data based on retention data. MS is then needed only for confirmatory purposes. On the other hand, the CI-MS technique has the advantage of producing simpler fragmentation patterns that may be more straightforward and easier to interpret than those of the GC-(EI)-MS method.

Mechanistically, the fragmentation patterns observed under CI-MS conditions support the general decomposition pathways established under either thermal (3,4,8) or acid conditions (4-6). The findings of both homolytic and heterolytic products under conditions of CI-MS in this study with secondary oxidation products, and in the previous study with monohydroperoxides (2), indicate that the reactant ions have sufficient energy to decompose into radical ions. This decomposition process may also be increased during the rapid heating of the direct exposure probe used to introduce the sample in the CI-MS system (2). Additional work is needed to determine how fragmentation patterns obtained by CI-MS can be changed by varying the conditions of analysis. Additional studies with tandem MS-MS experiments may further elucidate the origin of key fragment ions obtained in this CI-MS study.

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Influence of Dietary Fiber on Lipids and Aortic Composition of Vervet Monkeys

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A semipurified, cholesterol-free diet containing 40% carbohydrate can produce aortic sudanophilia or aortic atherosclerosis in vervet monkeys *(Cercopithecus aethiops pygerethrus)* depending on the particular carbohydrate fed. Four groups of vervet monkeys (three males and three females per group) were fed semipurified diets containing lactose. Two of the groups were also fed 15% cellulose (C) or 15% cellulose plus 0.1% cholesterol (CC); the two other groups were fed 15% pectin (P) or 15% pectin plus 0.1% cholesterol (PC). The average serum total cholesterol and low density lipoprotein cholesterol levels over the entire feeding period (mg/dl ± SEM) were, for C, 156 ± 14 and 95 ± 5; for P, 173 ± 15 and 112 ± 8; for CC, 187 ± 27 and 122 ± 21; and for PC, 155 ± 11 and 108 ± 7. Cholesterol levels at autopsy (mg/dl ± SEM) were, for C, 103 ± 6; for P, 108 ± 16; for CC, 92 ± 9; and for PC, 106 ± 7. Aortic sudanophilia (percentage of area) was, for C, 5.9 ± 2.7; for P, 13.5 ± 9.4; for CC, 5.3 ± 2.1; and for PC, 21.6 ± 10.3. Dietary pectin led to more severe sudanophilia (increased by 129% in the absence of cholesterol and by 308% in its presence) than did cellulose. Analysis of aortic glycosaminoglycans (GAG) revealed that dermatan sulfate levels fell in both cholesterol-fed groups, and chondroitin sulfate fell in aortas of group CC. Heparan sulfate levels were unaffected by cholesterol feeding. Hexuronic acid, galactosamine and hexosamine levels were elevated in the pectin-fed monkeys, but levels were unaffected by dietary cholesterol. Pectin may contribute galactosamine and glucuronic acid towards aortic GAG.

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We have shown that a semipurified diet containing 40% carbohydrate, 25% casein, 15% cellulose, 14% coconut oil, 5% mineral mix and 1% vitamin mix can lead to hyperlipidemia, hyperlipoproteinemia and aortic sudanophilia in baboons (1) and vervet monkeys (2). The severity of the sudanophilia varies with the type of carbohydrate present in the diet. Recently we found that addition of 0.1% cholesterol to this diet led to aortic atherosclerosis in baboons fed lactose, fructose or sucrose (3). The most severe atherosclerosis and sudanophilia were observed in baboons fed the diet containing lactose. The work reported in this paper was undertaken to determine if a lactose-rich diet would also be atherogenic for vervet monkeys. Since we had previously observed that the level of aortic sudanophilia in monkeys fed the semipurified diet was also a function of the type of fiber present (4), we felt it would be interesting to compare the effects of two dietary fibers, cellulose and pectin. Thus, there were four groups of monkeys used: two fed the semipurified diet containing cellulose or pectin and two fed the different fibers plus 0.1% cholesterol.

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MATERIALS AND METHODS

Vervet monkeys were randomly assigned to four groups of six animals (three males, three females) each. The groups were maintained on commercial ration plus fruit and vegetables for a 4-wk period to establish baseline levels for lipids and lipoproteins. The monkeys were then placed in one of four diet groups. All diets contained 40% lactose, 25% casein, 14% coconut oil, 5% mineral mix and 1% vitamin mix. The compositions of the mineral and vitamin mixes have been published (1). One diet contained 15% cellulose (C) and one contained 15% pectin (P) as fiber source. Two other groups of animals were fed diets C and P in which 0.1% of the coconut oil had been replaced by cholesterol (CC and PC, respectively).

The animals were bled (under Ketamine anesthesia) at 0, 5, 10, 16 and 33 wk, and sera was analyzed for serum total cholesterol, low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol and triglycerides using the Technicon AAII Autoanalyzer (5). The HDL cholesterol was assayed after precipitation of plasma LDL and very low density lipoprotein (VLDL) with sodium phosphotungstate (6). Serum phospholipids were analyzed by the method of Sokoloff and Rothblat (7).

Monkeys were killed by exsanguination while under deep anesthesia. Serum was extracted with chloroform/methanol 2:1 (v/v) (8) and the lipids were separated by thin layer chromatography (TLC). The free and esterified cholesterol and the triglycerides were eluted from the silicic acid. The sterol was quantitated using the O-phthalaldehyde reagent of Zlatkis and Zak (9). Triglycerides were quantitated using the Van Handel modification (10) of the Van Handel and Zilversmit procedure (11). Phospholipids were analyzed by the ammonium molybdate assay of Sokoloff and Rothblat (7). Liver aliquots were homogenized in chloroform/methanol 2:1 (v/v), and the phospholipids were assayed using an aliquot of the washed chloroform extract. After separation of lipids by TLC, free and esterified cholesterol were eluted from the gel and the sterol was quantitated (9). Triglycerides were assayed as described above (10).

Aortas were fixed and stained with Sudan IV to reveal the extent of lipid deposition. The sudanophilic areas were recorded on standard diagrams and the areas were shaded. The amount of stained aortic surface was calculated from the shaded area as a percentage of total area. The aortas were then minced and homogenized in chloroform/methanol 2:1 (v/v), and the lipids were recovered as described by Kim et al. (12). The aortic lipids were quantitated by the methods described above.

Hexuronic acid, hexosamine and glycosaminoglycan (GAG) concentrations were determined according to the method of DeHoff (13). Briefly, aortas were graded for

atherosclerotic involvement (14,15), scraped to remove adventitial fat, weighed and lyophilized to determine water content. The dried aortas were minced and extracted with chloroform/methanol (2:1, v/v) twice at 4 C. Dried, defatted aortas were reweighed to determine fat content, then stored at −15 C until further analysis.

GAG were isolated from the dry, defatted aortas by serial enzymatic digestion using a modification of the procedure of Curwen and Smith (16). In this procedure, the aorta was exposed to various enzymes, including pronase B (Calbiochem, La Jolla, California), papain (Sigma Chemical Co., St. Louis, Missouri) and ribonuclease B, then deoxyribonuclease I (Worthington Biochemical Corp., Freehold, New Jersey) to remove specifically proteins and nucleic acids leaving GAG. The extract was then dialyzed exhaustively against deionized water at 4 C, transferred to storage vials, lyophilized, reconstituted in 250 μl deionized water and stored at −15 C prior to analysis for hexuronic acid, hexosamines and total GAG.

Total hexuronic acid content of the extract was assayed according to the method of Bitter and Muir (17) using glucuronic acid (Sigma) as a standard. Total hexosamine was determined on the aortic extract according to the method of Gatt and Berman (18) using glucosamine (Sigma) as a standard. Galactosamine content of the aortic GAG solutions was determined according to the methods of Blumenkrantz and Asboe-Hansen (19) and Wagner (20). Glucosamine content may be inferred by subtracting galactosamine content from the total hexosamine content in this procedure.

Hyaluronic acid, heparan sulfate, dermatan sulfate and chondrotin-4- and -6-sulfates were isolated from the aortic extracts by cellulose polyacetate strip electrophoresis (Gelman Instrument Co., Ann Arbor, Michigan) according to the method of Stefanovich and Gore (21) and quantified according to the method of Hronowski and Anastassiades (22). Reference standards for all intact GAGs for electrophoresis were purchased from Seikagako Kogyo Co. (Tokyo, Japan).

Data on hexuronic acid, hexosamines and GAG were analyzed according to Federer-Zelen's method (23) for analysis of variance (pectin and cellulose in the absence or presence of cholesterol). Means were compared by Bonferroni t statistics (24), as outlined by Gill (25).

RESULTS

Table 1 presents the average (for the entire experimental period) serum lipid data. There were no significant differences in serum cholesterol and triglyceride levels among the test groups, although cholesterol levels were 12–35% higher than starting levels and triglyceride levels were 39–58% higher. Levels of serum HDL cholesterol, unaffected in groups C and P, showed a 15% rise in group CC and a 21% fall in group PC. The starting ratio of HDL/LDL cholesterol was 0.68. This ratio was slightly elevated in group C, unchanged in group CC and reduced significantly ($p < 0.05$) in both pectin-fed groups. A low ratio of HDL/LDL cholesterol is an indicator of increased risk of coronary disease (26,27). The ratio of HDL/LDL cholesterol in both pectin-fed groups was over 20% lower than in their cellulose-fed counterparts. Addition of cholesterol to the diets led to a reduction in the HDL/LDL cholesterol ratio. The data obtained at autopsy are summarized in Table 2. Liver weight was increased in the pectin-fed monkeys, and relative liver weight was significantly higher in groups P and CC compared with groups C and PC. The serum cholesterol levels at autopsy were significantly lower than the average over the entire experiment. This discrepancy was not unexpected since the seasonal variation in serum lipids of primates is well documented (1,28–30). At autopsy, there were no differences in serum cholesterol levels. The ratios of free/esterified cholesterol in the various groups were all within the normal range (0.43–0.67, representing 60–70% ester). Serum triglyceride and phospholipid levels were lower in the two cholesterol-fed groups but the differences were not statistically significant. Liver cholesterol levels were significantly lower in monkeys fed diets CC or PC than in the other two groups. Liver esterified cholesterol was low (4–8%) in all groups. Liver triglyceride and phospholipid levels were not significantly different.

The levels of aortic sudanophilia were as follows: C, 5.9 ± 2.7%; P, 13.5 ± 9.4%; CC, 5.3 ± 2.1% and PC, 21.6 ± 10.3%. Comparison of groups C and P shows that the monkeys fed pectin exhibited more than twice the level of aortic sudanophilia. Addition of cholesterol to the cellulose-rich diet did not affect aortic sudanophilia, but it was almost doubled when 0.1% cholesterol was added

TABLE 1

Serum Lipids in Monkeys Fed Semipurified Diets Containing Cellulose or Pectin (15%) ± 0.1% Cholesterol for 36 Wk (36-wk Average)

	Regimen[a]			
	C	P	CC	PC
Number of monkeys	2 M, 3 F	2 M, 1 F	3 M, 2 F	2 M, 3 F
Serum lipids (mg/dl ± SEM)				
Total cholesterol (139 ± 6)[b]	156 ± 14	173 ± 15	187 ± 27	155 ± 11
HDL cholesterol (52 ± 2)	56 ± 9	56 ± 7	60 ± 6	41 ± 6
LDL cholesterol (83 ± 5)	95 ± 5	112 ± 8	122 ± 21	108 ± 7
HDL/LDL (0.68 ± 0.03)	0.61 ± 0.09	0.47 ± 0.04	0.53 ± 0.06	0.38 ± 0.05
Triglycerides (36 ± 2)	53 ± 11	57 ± 14	55 ± 13	50 ± 9

[a]Diets contained 40% lactose, 25% casein, 15% fiber, 14% coconut oil ± 0.1% cholesterol. C, cellulose; P, pectin; CC, cellulose-cholesterol; PC, pectin-cholesterol.

[b]Average starting value for all monkeys.

to the pectin-containing diet. The average sudanophilia (all groups) for the nine male monkeys was 3.29 ± 0.73%; for the nine females it was 19.47 ± 5.94% ($p < 0.05$). The greater susceptibility to atherosclerosis of female vervet monkeys had been observed previously (31). Aortic cholesterol levels were not significantly different among the four groups. The ratio of free to esterified cholesterol was reduced by cholesterol feeding (48% in animals fed cellulose and 63% in those fed pectin). The ratio of free to esterified cholesterol is reduced with severity of atherosclerosis in man (32) and in rabbits fed cholesterol (33) or a cholesterol-free atherogenic diet (34). Other aortic lipids were not significantly different.

The results of analysis of aortic GAG and GAG components are given in Table 3. Hyaluronic acid levels are lower in aortas of monkeys fed cholesterol, and lower in those fed cellulose than in those fed pectin. Heparan sulfate was not affected by cholesterol feeding but was slightly higher in aortas of pectin-fed monkeys. Dermatan sulfate levels were lower in the two cholesterol-fed groups. Chondroitin sulfate levels were highest in groups C and PC and lowest in group CC. Hexuronic acid galactosamine and hexosamine levels in monkeys fed cellulose were similar and were lower than those seen in aortas of monkeys fed pectin. The levels of hexuronic acid, galactosamine and hexosamine were not influenced by dietary cholesterol. Levels of galactosamine in aortas of monkeys fed cellulose were significantly ($p < 0.05$) lower than those in aortas of either pectin-fed group. Levels of aortic glucosamine were very low in all groups.

DISCUSSION

The most surprising finding was the increased aortic sudanophilia in monkeys fed pectin. Pectin-fed monkeys also exhibited considerably more GAG in their aortas than did those fed cellulose. Pectin is hypocholesterolemic in man (35) and inhibits atherogenesis in rabbits (36) and chickens (37). In the monkeys, however, dietary pectin increased aortic sudanophilia by 129% in the absence of

TABLE 2

Autopsy Data for Monkeys Fed Semipurified Diets Containing 15% Cellulose or Pectin ± 0.1% Cholesterol for 36 Wk (Data ± SEM)[a]

	Regimen[b]			
	C	P	CC	PC
Wt (kg)	3.6 ± 0.4	4.1 ± 0.4	3.5 ± 0.7	4.3 ± 0.3
Liver wt (g)	107 ± 13[a]	164 ± 4[ab]	138 ± 23	121 ± 17[b]
Relative liver wt	3.02 ± 0.25[c]	4.07 ± 0.35[cd]	4.25 ± 0.70	2.84 ± 0.05[d]
Serum lipids (mg/dl)				
Cholesterol	103 ± 6	108 ± 16	92 ± 9	106 ± 7
Triglycerides	189 ± 43	157 ± 76	106 ± 34	86 ± 51
Phospholipids	182 ± 10	182 ± 13	140 ± 34	164 ± 11
Liver lipids (mg/100 g)				
Cholesterol	215 ± 13[ef]	115 ± 15[eg]	110 ± 18[fh]	232 ± 23[gh]
Triglycerides	435 ± 86	634 ± 120	481 ± 48	553 ± 47
Phospholipids (g)	12 ± 0.9	12 ± 2.1	13 ± 0.7	14 ± 0.6

[a]Values bearing the same letter are significantly different ($p \leq 0.05$).

[b]See footnote a, Table 1.

TABLE 3

Aortic GAG and GAG Components in Monkeys Fed Cellulose or Pectin (15%) ± 0.1% Cholesterol for 36 Wk (Data ± SEM)

	Regimen[a]			
	C	P	CC	PC
GAG (μg/mg DDA[b])				
Hyaluronic acid	4.17 ± 1.41	7.66 ± 1.63	3.49 ± 1.44	4.82 ± 1.41
Heparan sulfate	3.81 ± 0.81	4.06 ± 0.87	2.66 ± 0.81	4.42 ± 0.81
Dermatan sulfate	3.32 ± 1.01	3.84 ± 1.01	2.11 ± 0.88	2.09 ± 0.88
Chondroitin sulfate	4.91 ± 1.14	2.56 ± 1.31	1.68 ± 1.14	4.37 ± 1.14
GAG components (μg/mg DDA)				
Hexuronic acid	9.37 ± 1.08	12.89 ± 1.39	8.84 ± 1.21	12.44 ± 1.08
Galactosamine	7.76 ± 1.03	11.11 ± 1.33	8.26 ± 1.15	11.30 ± 1.03
Glucosamine	0.84 ± 0.39	1.01 ± 0.51	0.41 ± 0.29	0.77 ± 0.39
Hexosamine	9.47 ± 0.69	10.15 ± 0.89	8.47 ± 0.77	10.62 ± 0.69

[a]See footnote a, Table 1.

[b]DDA: dried, defatted aorta.

dietary cholesterol and by a factor of 3 when this sterol was present in the diet. Pectin is completely degraded in the colon (38), and it is possible that its degradation products (carbohydrates, short chain fatty acids) affect lipid metabolism differently in monkeys than in other species. Another possibility is that the degradation products in the monkey differ from those produced in other species.

Lactose enhances atherosclerosis in cholesterol-fed rabbits (39) and baboons (3). Aortic sudanophilia in the cellulose-fed group (5.9%) was lower than that seen earlier in vervet monkeys fed cellulose and fructose (20.3%), but similar to levels of sudanophilia seen in monkeys fed cellulose and sucrose (2.5%) or glucose (4.5%) (2).

It has been suggested (40,41) that the susceptibility of different animal species to atherosclerosis increases as their aortic levels of GAG increase. As can be seen from Table 3, the levels of total aortic GAG in the absence of dietary cholesterol are 12% higher in the monkeys fed pectin. When cholesterol is present in the diet, aortic GAG levels in the pectin-fed group are 58% higher. These findings are consistent with the hypothesis that higher levels of GAG are associated with more atherosclerosis, but more data are required. It would be interesting to see if aortic GAG levels were consistently elevated by dietary modalities that are known to increase cholesterolemia and atherosclerosis.

In contrast to findings in other species, dietary pectin enhances aortic sudanophilia in vervet monkeys. The influence of dietary pectin is not reflected in serum, liver or aortic lipids but is consistent with levels of aortic GAG. The effect of lactose on serum lipids and aortic atherosclerosis in the vervet monkey is also different from effects seen in other species.

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Lipid Content of Swine Influenza and Other Vaccines

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An analysis of the lipids in swine influenza vaccines was performed, comparing six different lots of swine influenza, other influenza and noninfluenza vaccines. Cholesterol content and phospholipid content varied greatly, but there were no major differences between the types of vaccines. Appreciable amounts of phosphatidylethanolamine were found in only one swine influenza vaccine. The major phospholipids of influenza vaccines were phosphatidylcholine, sphingomyelin and phosphatidic acid. A detectable amount of phosphatidylserine was not found in any swine influenza vaccine, but was present in two of three nonswine influenza vaccines. Only two of six swine influenza vaccines showed trace amounts (less than 0.5 μg/ml) of ganglioside (GM_3). However, larger quantities of galactocerebroside were found (2.24–6.43 μg/ml) in all influenza vaccines examined, including swine influenza vaccines.
Lipids 21, 342–346 (1986).

An extensive immunization program conducted in the United States in 1976 to reduce the impact of an influenza epidemic caused by a swine flu strain was reported to have been associated with an increased incidence of Guillain-Barre syndrome (GBS) (1). The factor in the vaccines provoking the GBS immune disorder has not been identified. Experimental allergic neuritis (EAN) is an animal model of GBS which shares many features of the human disease and can be induced in a variety of laboratory animals by immunization with peripheral nerve emulsified in complete Freund's adjuvant (2,3).

There have been many studies on the roles of lipids, either alone or with protein, in experimental demyelinating diseases of the peripheral nervous system (PNS). When myelin is injected into animals, the majority of the organ-specific, complement-fixing activity of antimyelin, or of myelin-induced EAN serum, is directed at galactocerebroside (GalCer) (4,5). The antisera is directed at the galactose moiety of galactocerebroside (6), and immunogenicity does not depend upon the carrier protein (7). Rabbits injected repeatedly with galactocerebroside develop experimental neuritis (8,9), and antibodies to GalCer can cause demyelination or inhibit myelination of PNS cultures (10,11). Serum from animals with myelin-induced EAN show the same in vitro activity, which can be blocked by pretreating the serum with GalCer (12).

Antibodies to gangliosides are produced in mice after sciatic nerve injury (13), suggesting a role in pathologic conditions of the PNS similar to the one proposed in diseases of the central nervous system (CNS) (14). EAN-inducing activity has been reported to require gangliosides as well as P2 protein for induction (15), but this observation has been disputed (16). Recently, Zeigler et al. reported that an EAN-like disease is produced in rabbits following injection with influenza vaccines mixed with gangliosides, cholesterol and Freund's adjuvant (17).

Purified P2 protein (18) and peptides obtained from it induce EAN (19–21), although their neutritogenic potency appears weaker than that of purified myelin. Phosphatidylserine has been reported to enhance the neuritogenicity of P2 (22), perhaps by an effect upon protein conformation.

Although P2 protein is not found in swine influenza vaccines (23), an analysis of the lipids in these vaccines appeared warranted. Here we present a quantitative and qualitative study of cholesterol and other neutral lipids, phospholipids and glycosphingolipids in several vaccines, including those for swine and other strains of influenza.

MATERIALS AND METHODS

Phospholipid and neutral lipid analysis. Lyophilized samples were extracted with chloroform/methanol (C/M) and partitioned by the addition of 0.2 vol of water as described by Radin (24). The lower phases were dried under a stream of nitrogen and applied to columns of silica gel in chloroform. Ten column vol of chloroform was collected for "neutral lipids" (i.e., cholesterol, glycerides, fatty acids). Glycolipids were eluted with 40 column vol of acetone. This fraction was analyzed for GalCer or glucosylcerebroside (GlcCer) as described below. Phospholipids were eluted with 10 column vol of methanol (25).

Cholesterol in the neutral lipid fraction was quantitated spectrophotometrically as described by Veerkamp and Broekhuyse (26). Neutral lipids were separated on silica gel thin layer chromatography (TLC) plates developed in light petroleum ether/diethyl ether (96:4, v/v) and visualized by exposure to iodine vapors.

Phospholipids were separated by two-dimensional TLC using high performance TLC (HPTLC) plates. After application, samples were chromatographed in C/M/concentrated ammonia (65:35:5, v/v/v) to the top of the plate plus an additional 10 min. HPTLC plates were air-dried and held in vacuo overnight over P_2O_5 to reactivate the silica gel. Chromatography in the second direction was performed in chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5, v/v/v/v/v). After being air-dried, phospholipids were visualized by exposure to iodine vapors, matched to standards and marked. After sublimation of I_2, marked areas were carefully scraped from the glass backing, charred and assayed for liberated phosphate by the method of Ames (27). Prior to TLC, aliquots were withdrawn and assayed in the same manner for total phospholipid determination.

Glycolipid analysis. After 5 ml of vaccine samples were taken to dryness by lyophilization, lipids were extracted with 5 ml of C/M 2:1 (v/v), then 5 ml of C/M 1:2 (v/v), and finally with C/M 1:2 (v/v) containing 20% water (28). With each extraction, the samples were stirred at room temperature for 30 min, and the insoluble material was removed by centrifugation. Pooled supernatants were taken to dryness under a stream of nitrogen and saponified with 0.6 N NaOH in methanol at 37 C for 6 hr.

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Glycosphingolipid and ganglioside nomenclature is as recommended by IUPAC-IUB (*Lipids 12,* 455–468 [1977]).

Following neutralization with HCl, samples were dialyzed for several days at 4 C and dried in an evaporating centrifuge (Savant Instruments). With some samples (the influenza vaccines produced by Wyeth and the rubella vaccine from Merck, Sharp and Dohme [West Point, Pennsylvania]), it was necessary to remove excessive neutral lipids by chromatography on small columns of silicic acid (29). Final dried residues were dissolved in 100 μl of C/M, 2:1 (v/v), and 40 μl aliquots were applied to silica gel TLC plates. Development was performed in C/M/water, containing 0.25% $CaCl_2$, (60:40:9, v/v/v). Asialosyl (i.e., neutral) glycolipids were visualized with diphenylamine (30) and gangliosides were visualized with resorcinol (31). Cerebrosides were separated by TLC in C/M/50 mM borate buffer, pH 10.0 (65:17:2, v/v/v) (32). Separated compounds were quantitated by scanning densitometry with an LKB Laser Densitometer, using standard curves generated by scanning chromatograms of serial dilutions of known quantities of galactosylceramide, glucosylceramide and ganglioside GM_1.

Materials. Influenza vaccines were provided by the Center for Disease Control (Atlanta, Georgia). Other vaccines were obtained from the U.S. Justice Department or were purchased locally. The vaccines used are listed in Table 1. Fertilized eggs were supplied by the University of Connecticut (Storrs), and allantoic fluid was collected 14 days after fertilization.

Organic solvents were of the highest purity commercially available. Silica gel (BioSil-A) was purchased from BioRad Laboratories (Richmond, California). TLC and HPTLC plates were from E. Merck (Darmstadt, Federal Republic of Germany). Glycolipid standards were prepared and characterized in our laboratory. Phospholipid and neutral lipid standards were from Sigma Chemical Co. (St. Louis, Missouri), Serdary Research Laboratories (London, Ontario, Canada) or were provided by Inderjit Singh (Department of Pediatrics, The Medical University of South Carolina).

TABLE 1

Vaccines Examined in This Study

Manufacturer	Lot no.	Type
Swine flu vaccines		
Parke-Davis	913349B	Bivalent, type A
Parke-Davis	912255B	Monovalent, type A
Merrell-National Laboratories	1496FK	Monovalent, type A
Merrell-National Laboratories	1497FK	Monovalent, type A
Merrell-National Laboratories	1493FK	Bivalent, type A
Wyeth Laboratories	180801	Bivalent, type A
Other flu vaccines		
Parke-Davis	03374P	Trivalent, types A and B
Wyeth	(1982)	—
Connaught (MNL)	(1982)	—
Other vaccines		
Merck Sharp & Dohme, Heptavex B	2451H	Hepatitis, type B
Wyeth, Triple Antigen	74504	Diphtheria, tetanus and pertussis
Merck Sharp & Dohme, Rubella	0315K	Rubella, Wistar RA 27/3 strain

RESULTS

The cholesterol content of the vaccines is summarized in Table 2. The nonesterified cholesterol varied, ranging from 3.0 to 44.0 μg/ml. The influenza vaccines produced by Wyeth had the highest amounts of cholesterol. A slightly higher content was found in the allantoic fluid

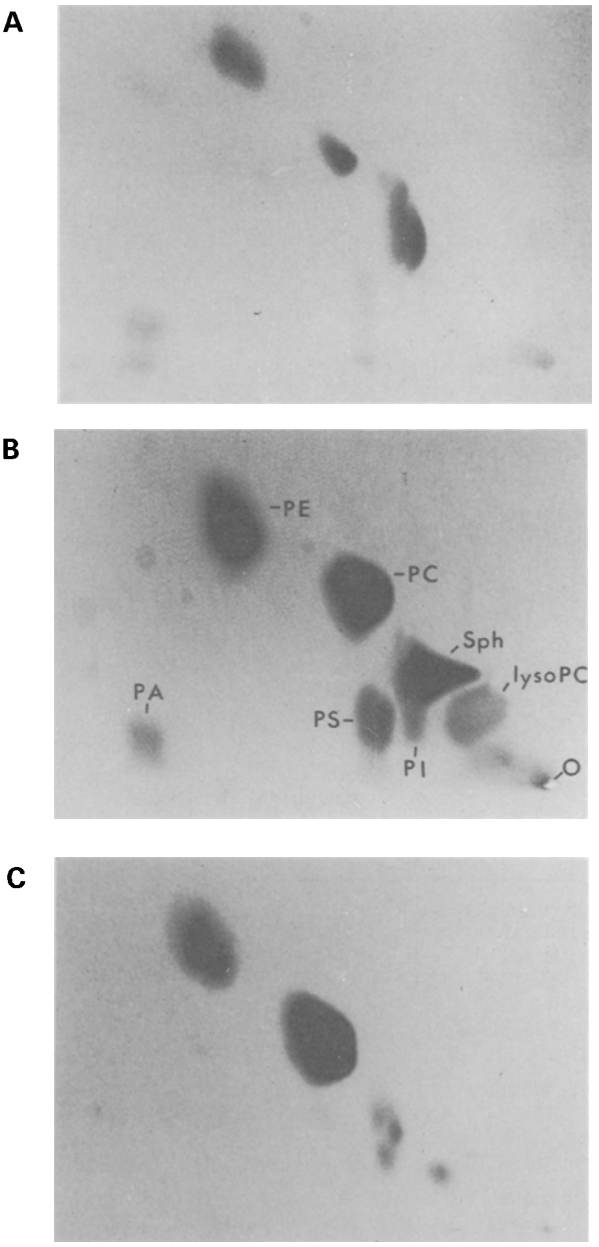

FIG. 1. Representative chromatograms of two-dimensional HPTLC of phospholipid samples. (A) Mixed phospholipid standards; (B) Parke-Davis influenza vaccine, lot 03374P; (C) chick embryo allantoic fluid. Quantitation of individual phospholipids after HPTLC separation is summarized in Table 4. O, origin; other abbreviations as denoted in Table 4.

of eggs containing 14-day chick embryos (46.7 μg/ml). Table 3 summarizes the results of semiquantitative estimation of neutral lipids, including cholesterol esters, mono-, di- and triglycerides, and free fatty acids, after separation by TLC. None of the vaccines examined contained detectable amounts of cholesterol esters, and triglycerides were only detected in the Wyeth influenza vaccines. Traces of cholesterol were observed in the non-influenza vaccines studied, yet monoglycerides could be detected.

Table 2 also summarizes the total phospholipid content of the vaccines. The values varied from 0.012 to 0.101 μmol Pi/ml vaccine, and were much less than the 13.7 μmol Pi/ml in the chick embryo allantoic fluid. Although the Wyeth influenza vaccines were high in phospholipid content, one Parke-Davis sample (nonswine influenza) had

TABLE 2

Lipid Content of Various Vaccines

Sample[a]	Cholesterol (μg/ml)	Pi[b] (μmol/ml)	Cerebroside[c] (μg/ml)	Ganglioside (μg/ml)
Swine flu vaccines				
PD 913349B	10.0	0.036	5.32	ND[d]
PD 912255B	3.0	0.019	2.94	<0.5
MNL 1496FK	5.72	0.016	3.28	ND
MNL 1497FK	3.72	0.012	2.24	ND
MNL 1493FK	6.86	0.020	4.82	ND
Wyeth 180801	44.0	0.087	4.19	<0.5
Other flu vaccines				
PD 03374P	18.8	0.101	6.43	0.78
Wyeth, 1982	30.6	0.079	4.02	<0.5
Connaught (MNL), 1982	20.0	0.039	3.56	<0.5
Other vaccines				
Heptavex B (MSD 2451H)	7.8	0.014	<0.5	ND
Triple Antigen (W 74504)	10.6	0.042	ND	ND
Rubella (MSD 0315K)	23.2	0.015	ND	ND
Chick embryo allantoic fluid	46.7	13.7	0.936	11.77

[a]Names of manufacturers have been abbreviated; see Table 1.

[b]Pi, inorganic phosphate.

[c]Cerebroside is galactosylceramide.

[d]ND, not detectable.

TABLE 3

Summary of Neutral Lipid TLC

Sample	Cholesterol esters	Triglycerides	Free FA[a]	Cholesterol	Diglycerides	Monoglycerides
Swine flu vaccines						
PD 913349B	0	trace	1	3	1	1
PD 912255B	0	trace	2	3	2	1
MNL 1496FK	0	0	trace	3	0	1
MNL 1497FK	0	0	1	3	0	1
MNL 1493FK	0	0	1	3	0	1
Wyeth 180801	0	3	3	3	1	2
Other flu vaccines						
PD 03374P	0	trace	2	3	1	1
Wyeth, 1982	0	3	3	3	1	1
Connaught, 1982	0	0	1	3	0	1
Other vaccines						
Heptavex B	0	0	0	trace	0	2
Triple Antigen	0	0	0	trace	2	2
Rubella	0	0	0	trace	2	2
Chick embryo allantoic fluid	0	3	2	3	1	2

Chromatograms were visualized by exposure to iodine vapors and photographed. Staining intensity of each band was scored on a scale of 0 (absent) to 4 (intense staining).

[a]FA, fatty acids; other abbreviations as denoted in Table 1.

the highest value. Examples of the separation of individual phospholipids by two-dimensional HPTLC are shown by representative chromatograms in Figure 1, and the quantitative data are summarized in Table 4. The major phospholipids found in the influenza vaccines were sphingomyelin, phosphatidylcholine and phosphatidic acid. There was some variation in the distribution of these compounds, and differences in the amounts of phosphatidylethanolamine, phosphatidylinositol and lysophosphatidylcholine were also noted. Appreciable amounts of phosphatidylethanolamine were found in only one vaccine (MNL 1497FK). Phosphatidylserine was not found in swine flu vaccines, but was present in two of the nonswine influenza vaccines. Triple Antigen (DPT), prepared from bacterial cultures, had a unique pattern. Only 32.1% of the total phospholipid was recovered from identifiable spots on two-dimensional HPTLC. A trace of phospholipid which co-migrated with lysophosphatidylcholine was found in the rubella vaccine.

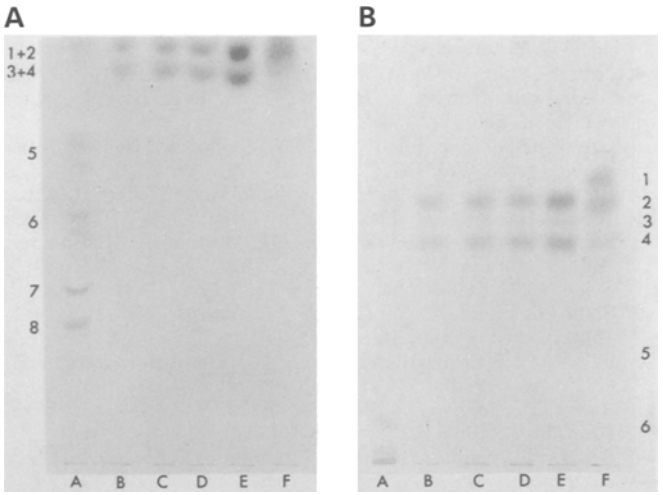

FIG. 2. TLC in two solvent systems of neutral glycolipids from representative vaccines (left panel, chloroform/methanol/water, 65:35:8, v/v/v; right panel, chloroform/methanol/50 mM borate buffer, pH 10, 65:17:2, v/v/v). Lane A, mixed glycolipid standards; lane B, MNL 1496FK; lane C, PD 03374B; lane D, Wyeth 180801; lane E, GalCer standard; lane F, mixture of GalCer and GlcCer standards. Standards: 1, GlcCer (nonhydroxyl); 2, GalCer (nonhydroxyl); 3, GlcCer (hydroxyl); 4, GalCer (hydroxyl); 5, lactosylceramide; 6, $GbOse_3Cer$; 7, $nLcOse_4Cer$; 8, $nLcOse_5Cer$. Names of manufacturers have been abbreviated; see Table 1. See text for additional details.

Glycosphingolipid determination, summarized in Table 2, revealed more than 2 μg/ml of galactosylceramide in the influenza vaccines (representative chromatograms are presented in Fig. 2). This was substantially greater than the amount of cerebroside found in chick embryo allantoic fluid or in the three noninfluenza vaccines. There were no remarkable differences between the swine flu vaccines and the other influenza vaccines. Gangliosides were detected in five vaccines, only one of which (a nonswine influenza vaccine) contained enough to measure; the others had only trace amounts. No gangliosides were detected by spectrodensitometry in four of the six swine influenza vaccines. This contrasts with chick embryo allantoic fluid, the source of these vaccines, which contained 0.936 μg cerebroside/ml in addition to substantial amounts of ganglioside, 11.77 μg/ml. In all samples, the cerebroside was galactosylceramide and not glucosylceramide, as determined by TLC separation using borate buffer (32).

DISCUSSION

Given the variety of lipids, including GalCer, gangliosides and phosphatidylserine, proposed for roles in the develop-

TABLE 4

Percentage of Total Recovered Phospholipid[a]

Sample	PC	PE	SPH	PA	PS	PI	Lyso PC
Swine flu vaccines							
PD 913349B	26.9	ND	42.3	23.1	ND	7.7	ND
PD 912255B	13.5	ND	78.8	7.7	ND	ND	ND
MNL 1496FK	9.3	ND	66.7	24.1	ND	ND	ND
MNL 1497FK	9.1	36.4	33.3	21.2	ND	ND	ND
MNL 1493FK	20.0	16.7	36.7	26.7	ND	ND	ND
Wyeth 180801	60.5	ND	17.8	7.0	ND	5.4	9.3
Other flu vaccines							
PD 03374P	14.9	13.4	34.4	9.0	16.4	11.9	ND
Wyeth, 1982	45.5	16.3	22.7	ND	7.2	8.2	ND
Connaught, 1982	6.3	16.7	38.9	38.1	ND	ND	ND
Other vaccines							
Heptavex B (MSD)	72.2	ND	27.8	ND	ND	ND	ND
Triple Antigen (W)	6.1	15.7	10.3	ND	ND	ND	ND
Rubella (MSD)	ND	ND	ND	ND	ND	ND	100
Chick embryo allantoic fluid	50.0	15.4	9.3	ND	ND	ND	25.3

[a]Values are percentage of total recovered inorganic phosphate (27); average of duplicate samples.

ND, not detectable; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPH, sphingomyelin; PA, phosphatidic acid; PS, phosphatidylserine; PI, phosphatidylinositol; lyso PC, lysophosphatidylcholine. Other abbreviations are as denoted in Table 1.

ment of experimental neuritis and the value of EAN as a model of GBS, we decided to analyze the major lipid classes in swine influenza and other vaccines.

Although the quantitative estimates of cholesterol varied (from 3.0-44.0 μg/ml vaccine), no major differences between swine influenza, other influenza and noninfluenza vaccines were found. Even at the highest value, these vaccines could not add appreciable cholesterol to the vaccinee, since human plasma contains between 1.2 and 3.1 mg cholesterol/ml (33). The qualitative finding that the noninfluenza vaccines had little cholesterol in comparison to monoglycerides, with the opposite being true for influenza vaccines, may be due either to the methods of preparation or the source of the vaccines. While influenza vaccines are prepared from the allantoic fluid of fertilized eggs, Heptavex B, Triple Antigen (DPT) and rubella vaccines are prepared from purified human plasma, bacterial cultures and human diploid cell cultures, respectively.

The total phospholipid content of the vaccines varied (from 0.12-0.101 μmol Pi/ml vaccine). Major differences between types of vaccines were not noted. The fact that allantoic fluid contained far greater phospholipid concentrations than any of the vaccines (13.7 μmol Pi/ml), which was not true of cholesterol content, indicates that purification during manufacture removes much of the phospholipid. In terms of individual phospholipids, influenza vaccines roughly parallel normal human plasma, in which phosphatidylcholine constitutes 67% of the total phospholipids and sphingomyelin constitutes 21% (34). This distribution percentage is most closely followed by the Heptavex B vaccine (noninfluenza), which is prepared from human plasma. The Triple Antigen (DPT) produced a unique pattern, in which only 32.1% of the total phospholipid was recovered from identifiable spots upon two-dimensional HPTLC. The additional unidentified phospholipids undoubtedly reflect bacterial phospholipids of a different structure.

The EAN-inducing properties of P2 protein are reported to be enhanced by phosphatidylserine (PS) (22). In this study, PS was not detected in any of the swine influenza vaccines. This, in addition to the lack of detectable P2 by radioimmunoassay in the same vaccines (23), suggests that a role for PS-protein complexes in the occurrence of GBS associated with these vaccines can be excluded.

Similarly, this study does not support the idea that gangliosides are involved. It has been reported that EAN can be induced by mixtures of gangliosides with P2 protein (15) or with influenza vaccines and adjuvants (17). Since only two of the six swine influenza vaccines examined showed traces of gangliosides (below 0.5 μg/ml), it seems unlikely that these glycosphingolipids are responsible for an increased incidence of GBS among recipients of swine influenza vaccines.

GalCer was found in the swine influenza vaccines. This glycolipid can elicit demyelination both in vivo and in vitro (5,6,8-12). While GalCer was undetected or present only in traces in the noninfluenza vaccines examined, it was not unique to the swine influenza vaccines; it was found in similar concentrations in other influenza vaccines as well. With the observation that antigalactosylceramide causes demyelination under experimental conditions (10-12), the presence of GalCer in these vaccines does warrant attention.

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Elongation of (n-9) and (n-7) *cis*- Monounsaturated and Saturated Fatty Acids in Seeds of *Sinapis alba*

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Lipids in developing seeds of *Sinapis alba* contain appreciable proportions of (n-7)octadecenoic (vaccenic) acid besides its (n-9) isomer (oleic acid), whereas the constituent very long chain (> C_{18}) monounsaturated fatty acids of these lipids are overwhelmingly composed of the (n-9) isomers. Cotyledons of developing *Sinapis alba* seed use [1-^{14}C]acetate, [1-^{14}C]malonate or [1,3-^{14}C]malonyl-CoA for de novo synthesis of palmitic, stearic and oleic acids and for elongation of preformed oleic, vaccenic and stearic acids to their higher (n-9), (n-7) and saturated homologs, respectively. Moreover, elongation of preformed (n-7)palmitoleic acid to vaccenic acid is observed. Stepwise C_2-additions to preformed oleoyl-CoA by acetyl-CoA or malonyl-CoA yielding (n-9)icosenoyl-CoA, (n-9)docosenoyl-CoA and (n-9)tetracosenoyl-CoA are by far the most predominant reactions catalyzed by the elongase system, which seems to have a preference for oleoyl-CoA over vaccenoyl-CoA as the primer. The pattern of ^{14}C-labeling of the very long chain fatty acids formed from either acetate or malonate shows a close analogy in the mode of elongation of monounsaturated and saturated fatty acids.
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The majority of *cis*-monounsaturated fatty acids in lipids of higher plants belong to the (n-9) series, which constitutes oleic acid and its homologs. It is now well established that in higher plants oleic acid is synthesized de novo via Δ^9-desaturation of stearoyl-ACP (1), and several studies suggest that in certain seeds oleoyl-CoA is elongated to very long chain (n-9) monounsaturated fatty acids (2–4).

The occurrence in higher plants of *cis*-monounsaturated fatty acids belonging to the (n-7) series, which constitutes vaccenic acid and its homologs, is unequivocally established (5,6). Vaccenic acid is known to be synthesized in animal tissues (7,8) and microorganisms (9) via Δ^9-desaturation of palmitic acid and elongation of the resulting (n-7)palmitoleic acid. A similar mechanism of formation of vaccenic acid seems to be operative in higher plants (10,11), but little is known about the elongation of vaccenic acid to its higher (n-7) homologs.

This paper records the formation in vivo of oleic and vaccenic acids in developing seeds of *Sinapis alba* and the elongation of these isomeric *cis*-octadecenoic acids to their (n-9) and (n-7) homologs, respectively. Evidence also is presented on the analogy in the mode of elongation of *cis*-monounsaturated and saturated fatty acids.

MATERIALS AND METHODS

Materials. Seeds of field-grown white mustard, *Sinapis alba,* cv. Albatros (Saaten-Union GmbH, Hannover, Federal Republic of Germany), were collected at specific intervals after flowering.

Sodium [1-^{14}C]acetate (58 Ci/mol) and [1-^{14}C]oleic acid (56 Ci/mol) were purchased from Amersham Buchler (Braunschweig, Federal Republic of Germany), and [1-^{14}C]malonic acid (6 Ci/mol) and [1,3-^{14}C]malonyl-CoA (22 Ci/mol) from NEN Chemicals (Dreieichenhain, Federal Republic of Germany). Unlabeled oleic and vaccenic acids as well as methyl esters used as reference standards for gas chromatography (GC) were obtained from Nu-Chek-Prep (Elysian, Minnesota). All chemicals were of reagent grade and the solvents were distilled before use.

Incubations. Fresh developing seeds of *Sinapis alba* collected at the sixth week after flowering were used. The seeds were sliced with a scalpel, the seed coat was removed and the cotyledon tissue (150 mg) from about 30 seeds was used in each incubation. The cotyledons were incubated for six hr with [1-^{14}C]acetate or [1-^{14}C]malonate, 5 μCi each, or with 5 μCi [1-^{14}C]oleic acid for 24 hr in a manner described elsewhere (12). Alternatively, unlabeled oleic or vaccenic acid, 2 mg each, was solubilized in a mixture consisting of 20 μl aqueous ethanol (20%, v/v) and 20 μl aqueous ammonium hydroxide (33%, w/v) and preincubated with the cotyledons for two hr in the presence of 0.5 ml sodium phosphate buffer (0.1 M, pH 6.0) under similar conditions to those mentioned above. Subsequently, [1,3-^{14}C]malonyl-CoA, 2 μCi, was added to each incubation mixture and the incubations were continued for another 20 hr.

Lipid extraction. The incubations were terminated by heating the mixtures with isopropanol and the lipids were extracted essentially as described elsewhere (12). Similarly, the lipids were extracted from the developing seeds of *Sinapis alba* at various stages of maturation.

Radioactivity measurements. Radioactivity in lipid samples was measured in a Packard Tri-carb C2425 liquid scintillation spectrometer using a toluene scintillator (Packard Instruments Co., Downers Grove, Illinois).

Thin layer chromatograms were assayed for radioactivity with a Berthold Scanner LB 2760 (BF-Vertriebsgesellschaft, Wildbad, Federal Republic of Germany).

Radio GC was carried out in a Perkin-Elmer F-22 instrument equipped with thermal conductivity detectors (Perkin-Elmer & Co. GmbH, Überlingen, Federal Republic of Germany) using helium (40 ml/min) as carrier gas. Glass columns (1.8 m × 4 mm diameter) were used. Radioactivity in the column effluent was monitored in a Packard Gas Proportional Counter, Model 894 (Packard Instruments Co.) and the peaks were integrated by an Autolab Minigrator (Spectra Physics GmbH, Darmstadt, Federal Republic of Germany).

Fractionation of methyl esters. Total lipids were transmethylated (13) and the methyl esters fractionated according to degree of unsaturation by thin layer chromatography on Silica Gel G (E. Merck, Darmstadt, Federal Republic of Germany) containing 20% (w/w) silver nitrate. The plates were developed twice with hexane/diethyl ether (9:1, v/v) and the fractions were

detected by spraying the edges of the chromatogram with 0.1% (w/v) ethanolic 2′,7′-dichlorofluorescein and viewing under UV light and/or by the thin layer radiochromatogram scanner. The fractions of saturated and *cis*-monounsaturated methyl esters were eluted from the adsorbent with water-saturated diethyl ether.

Saturated methyl esters were fractionated into individual homologs by preparative GC on 3% (w/w) OV-101/Gas-Chrom Q, 100–120 mesh (Applied Science Laboratories, State College, Pennsylvania) at 240 C using helium (15 ml/min) as carrier gas. The fractions were monitored by the thermal conductivity detector and collected in glass tubes (25 cm × 6 mm diameter) that were loosely packed with glass wool. The fractions were subsequently eluted with hexane.

Monounsaturated methyl esters were fractionated into individual homologs by reversed phase partition chromatography on precoated silica gel plates (E. Merck) using heptane as the stationary phase and a mixture of acetic acid/acetonitrile (4:6, v/v) saturated with heptane as the mobile phase (14). The fractions were detected by spraying the chromatograms with 0.1% (w/v) ethanolic 2′,7′-dichlorofluorescein and viewing under UV light. The individual fractions were then eluted with chloroform/methanol/water (5:5:1, v/v/v), dried and dissolved in hexane, and the residual dye was removed by washing with 0.1 M tris/HCl, pH 7.4, followed by water.

Purity of each labeled methyl ester fraction was checked by radio GC on 10% (w/w) Silar 5CP/Gas-Chrom Q, 80–100 mesh (Applied Science Laboratories) at 180 C. The purity of unlabeled methyl esters was ascertained by GC in a similar manner, but using flame ionization detectors for mass detection and nitrogen (40 ml/min^{-1}) as the carrier gas. Similarly the methyl esters from total lipids as well as the fractions of monounsaturated and saturated methyl esters were analyzed.

Degradative analysis of methyl esters. Aliquots of each monounsaturated methyl ester fraction were subjected to reductive ozonolysis according to the procedures commonly followed in our laboratory (15). The products of ozonolysis, i.e., aldehydes and aldesters, were analyzed by Radio GC on 3% (w/w) OV-101/Gas-Chrom Q, 100–120 mesh (Applied Science Laboratories). After sample injection, the column temperature was held at 70 C for two min, then raised to 270 C (4 C/min), and kept at this final temperature for four min before cooling. With unlabeled methyl esters the mass detection was carried out with flame ionization detectors. The ozonolysis fragments were identified by comparison of retention times with those of the aldehydes and aldesters that were derived by reductive ozonolysis of the authentic (n-9) and (n-7) monounsaturated methyl esters of various chain lengths. Distribution of radioactivity in the (n-9) and (n-7) isomers of the monounsaturated methyl esters of a particular chain length was determined by adding up the percentage radioactivity in the aldehydes and the corresponding aldester fragments. For example, the distribution of radioactivity between methyl oleate and methyl vaccenate in a methyl octadecenoate fraction was determined from the percentage of radioactivity in the C_9-aldehyde plus C_9-aldester and that in the C_7-aldehyde plus C_{11}-aldester.

A part of each monounsaturated methyl ester fraction was hydrogenated using Adam's catalyst (16) and the completeness of hydrogenation was ascertained by radio GC as described above.

Aliquots of saturated methyl ester fractions obtained by preparative GC and those obtained via hydrogenation of the monounsaturated methyl ester fractions were hydrolyzed (17) to yield the corresponding fatty acids. Each of the labeled saturated fatty acids was subjected to chemical α-oxidation using potassium permanganate in acetone essentially as described elsewhere (18). The resulting mixture, consisting of the unoxidized parent fatty acid and the chain-shortened acids derived therefrom, was converted to methyl esters with diazomethane and analyzed by radio GC. The separations were carried out on 3% (w/w) OV-101/Gas Chrom Q, 100–120 mesh (Applied Science Laboratories); the column temperature was programmed from 120 C to 250 C (4 C/min) and held at the final temperature for 16 min. The relative specific activity of each component was determined as the ratio of percentage area of the radioactive peak to that of the mass peak and expressed with reference to the specific activity of the parent fatty acid as unity.

RESULTS AND DISCUSSION

The lipids of *Sinapis alba* seed contain varying proportions of (n-9) and (n-7) isomers of octadecenoic, icosenoic, docosenoic and tetracosenoic acids at various stages of seed development, as shown by the data given in Table 1. The proportion of oleic acid increases rapidly till the fifth week after flowering and then levels off, while from this period onward the (n-9) isomers of icosenoic, docosenoic and tetracosenoic acids are accumulated. These findings indicate elongation of de novo synthesized oleic acid to very long chain (n-9) monounsaturated fatty acids, as observed in several other lipid-rich seeds (2–4).

The data given in Table 1 also show that vaccenic acid constitutes more than one-half of the octadecenoic acids at the third week after flowering, but with progressing seed maturation the proportion of vaccenic acid decreases. It appears that vaccenic acid is synthesized mainly in the early stages of seed development, which coincides with the phase where extensive synthesis of palmitic acid also occurs, as evident from the fatty acid composition of the developing *Sinapis alba* seed (19). These results suggest that vaccenic acid might be derived from palmitic acid via Δ^9-desaturation and elongation in a similar manner as in animal tissues (7,8) and microorganisms (9). This is supported by the occurrence of (n-7)hexadecenoic acid in the seed lipids (Table 1). Some decrease in the proportion of vaccenic acid during the course of seed development and the concomitant appearance of (n-7)icosenoic and docosenoic acids (Table 1) indicate that preformed vaccenic acid is elongated to very long chain (n-7) monounsaturated fatty acids in a similar manner to the elongation of preformed oleic acid to its higher (n-9) homologs. It is noteworthy that the very long chain monounsaturated fatty acids are predominantly composed of the (n-9) isomers (Table 1).

To study the formation of (n-9) and (n-7) monounsaturated fatty acids in vivo, the cotyledons of developing *Sinapis alba* seed at six wk after flowering

TABLE 1

Changes in the Content of (n-9) and (n-7) Monounsaturated Fatty Acids in Lipids of *Sinapis alba* Seed at Various Stages of Development

Wk after flowering	Lipid content (%)	Content of (n-9) and (n-7) isomers in individual acids (% of total fatty acids)[a]								
		16:1	18:1		20:1		22:1		24:1	
		(n-7)	(n-9)	(n-7)	(n-9)	(n-7)	(n-9)	(n-7)	(n-9)	(n-7)
3	0.5		4.2 (0.21)	5.0 (0.25)	0.5 (0.003)		0.5 (0.003)		0.4 (0.002)	
4	2.0	1.5 (0.03)	17.6 (3.52)	7.9 (1.58)	3.7 (0.74)	1.1 (0.22)	6.0 (1.20)	0.3 (0.06)	0.9 (0.018)	
5	4.4	1.0 (0.04)	21.7 (9.55)	6.1 (2.68)	7.7 (3.39)	0.9 (0.39)	13.2 (5.81)	0.2 (0.09)	1.1 (0.05)	tr[b]
6	8.3		23.2 (19.26)	2.5 (2.08)	9.3 (7.72)	1.2 (0.99)	28.1 (23.32)	0.5 (0.42)	1.6 (0.13)	tr
Mature seed	24.9		20.0 (49.80)	1.3 (3.24)	8.9 (22.16)	0.8 (1.99)	37.5 (93.38)	0.6 (1.49)	2.1 (0.52)	tr

[a]Figures in parentheses are mg/g seed tissue.
[b]tr = Traces.

TABLE 2

Radioactive Labeling of (n-9) and (n-7) Monounsaturated Fatty Acids after Incubation of *Sinapis alba* Cotyledons with [1-^{14}C]Acetate or [1-^{14}C]Malonate

Substrate	Fatty acid	Radioactivity (% of total acids)	Specific radioactivity	(n-9):(n-7)	Distribution of radioactivity in ozonolysis fragments		Specific radioactivity of chain-shortened acids obtained by α-oxidation of the parent (C_n) acid						
					C_9-Aldehyde:C_{n-9}-aldester	C_7-Aldehyde:C_{n-7}-aldester	C_n	C_{n-1}	C_{n-2}	C_{n-3}	C_{n-4}	C_{n-5}	C_{n-6}
Acetate[a]	18:1	32	1.0	97:3	48:52	28:72	1.00	0.65	0.71	0.77	0.74		
	20:1	13	1.1	95:5	13:87	tr:∼100[b]	1.00	0.26	0.32	0.22	0.25	0.35	
	22:1	34	1.1	100:0	12:88		1.00	0.84	0.81	0.40	0.25	0.34	0.28
	24:1	2	0.9	100:0	5:95		1.00	0.76	0.61	0.51	0.73	0.68	0.48
Malonate[c]	18:1	11	1.2	∼100:tr	40:60		1.00	1.14	1.48	1.57	1.52	1.58	1.56
	20:1	14	4.1	94:6	7:93	tr:∼100	1.00	0.11	0.16	0.14	0.19	0.23	0.18
	22:1	54	6.4	99:1	4:96	tr:∼100	1.00	0.78	0.75	0.25	0.30	0.21	0.18
	24:1	7	9.6	100:0	1:99		1.00	0.77	0.61	0.49	0.52	0.33	0.25

[a]Incorporation of radioactivity in total lipids = 48%.
[b]tr = Traces.
[c]Incorporation of radioactivity in total lipids = 7%.

were incubated with various radioactively labeled substrates, and the incorporation of label into individual monounsaturated fatty acids of the total lipids was determined.

Table 2 shows the pattern of labeling of monounsaturated fatty acids after incubation of the cotyledons with sodium [1-^{14}C]acetate or [1-^{14}C]malonic acid. It can be seen that labeled octadecenoic acids and very long chain monounsaturated fatty acids are formed from both acetate and malonate; however, the pattern of labeling from the two substrates is somewhat different. Thus, more very long chain monounsaturated fatty acids and less octadecenoic acids are formed from malonate than from acetate. Furthermore, the relative specific radioactivity of all the monounsaturated fatty acids is quite similar when acetate is the substrate, whereas with malonate as substrate the relative specific radioactivity of the monounsaturated fatty acids is substantially increased with increasing chain length of these acids. Apparently, both acetyl-CoA and malonyl-CoA are used for the biosynthesis of very long chain monounsaturated fatty acids. It is conceivable that

acetyl-CoA formed from acetate is converted to malonyl-CoA, which is finally used for the elongation reactions.

The results given in Table 2 also show that from both acetate and malonate predominantly the (n-9) monounsaturated fatty acids are formed. From both substrates, most of the labeled oleic acid formed is a product of de novo synthesis, as evidenced by a roughly equal distribution of label in its ozonolysis fragments, i.e., C_9-aldehyde and C_9-aldester. This is further supported by analysis of the products of α-oxidation, which shows that with respect to the parent octadecenoic acid all the chain-shortened acids are extensively labeled (Table 2).

In contrast to oleic acid, the labeled (n-9)icosenoic, docosenoic and tetracosenoic acids are formed mostly by elongation of unlabeled endogenous oleic acid, as evidenced by the preponderance of label in the aldester part of their ozonolysis fragments (Table 2). This is further supported by α-oxidation, which shows that in (n-9) icosenoic acid the carboxyl carbon atom is most extensively labeled, as seen from the specific radioactivity of the chain-shortened acids relative to that of the parent fatty acid. Furthermore, in the products of α-oxidation of (n-9)docosenoic and tetracosenoic acids the chain-shortened acids down to C_{19} have a distinctly higher specific radioactivity than the chain-shortened acids from C_{18} and below (Table 2). Analysis of the ozonolysis products also shows a distinctly higher labeling of the aldester fragments from the very long chain (n-9) monounsaturated fatty acids when malonate is the substrate rather than acetate (Table 2). This again shows that malonyl-CoA is the primer for elongation of preformed oleic acid.

When the cotyledons of developing *Sinapis alba* seed are incubated with exogenous [1-^{14}C]oleic acid, formation of labeled icosenoic and docosenoic acids is observed (12). Analysis of these very long chain fatty acids by reductive ozonolysis and α-oxidation reveals that exogenous [1-^{14}C]oleic acid is elongated to (n-9)[3-^{14}C]icosenoic acid and (n-9)[5-^{14}C]docosenoic acid (data not shown). Obviously, oleoyl-CoA formed from exogenous oleic acid is elongated by endogenous malonyl-CoA to the very long chain (n-9) monounsaturated acyl-CoA derivatives. Similar observations have been reported for developing seeds of *Crambé abyssinica* (2).

The pattern of labeling of the (n-7) monounsaturated fatty acids from acetate and malonate (Table 2) shows the following: With acetate as substrate, preponderance of label in the aldester part of the ozonolysis fragments from vaccenic acid suggests that a considerable portion of labeled vaccenic acid is formed by elongation of unlabeled endogenous (n-7)hexadecenoic acid rather than by de novo synthesis. In the ozonolysis products from (n-7)icosenoic acid, derived either from acetate or malonate, and those from (n-7)docosenoic acid, derived from malonate, the label is located alomost exclusively in the aldester fragments (Table 2). These findings strongly suggest that endogenous unlabeled vaccenic acid and/or (n-7)hexadecenoic acid are elongated to very long chain (n-7) monounsaturated fatty acids in a similar manner as the elongation of preformed oleic acid to very long chain (n-9) monounsaturated fatty acids.

In order to study the elongation of exogenous oleic vs vaccenic acid, the *Sinapis alba* cotyledons were incubated for two hr with either of these unlabeled fatty acids to enable the formation of their acyl-CoA derivatives. Subsequently, [1,3-^{14}C]malonyl-CoA was added for chain elongation and the incubations were carried out for another 20 hr. The pattern of labeling of the monounsaturated fatty acids formed in both incubations is recorded in Table 3. These results show that substantial proportions of labeled oleic and vaccenic acids are formed in both experiments in addition to very long chain monounsaturated fatty acids. In the presence of either of the exogenous octadecenoic acids, [1,3-^{14}C]malonyl-CoA is partially used for de novo synthesis of oleic acid and elongation of endogenous (n-7)hexadecenoic acid. This is evident from a roughly equal labeling of the ozonolysis fragments from oleic acid and preponderance of label in the aldester fragment from vaccenic acid (Table 3).

The pattern of labeling of the very long chain monounsaturated fatty acids derived by incubation with unlabeled exogenous octadecenoic acids in conjunction

TABLE 3

Radioactive Labeling of (n-9) and (n-7) Monounsaturated Fatty Acids after Incubation of *Sinapis alba* Cotyledons with Unlabeled Oleic or Vaccenic Acids in Conjunction with [1,3-^{14}C]Malonyl-CoA

Substrate	Fatty acid	Radioactivity (% of total fatty acids)	(n-9):(n-7)	Distribution of radioactivity in ozonolysis fragments		Specific radioactivity of chain-shortened acids obtained by α-oxidation of the parent (C_n) acid					
				C_9-Aldehyde: C_{n-9}-aldester	C_7-Aldehyde: C_{n-7}-aldester	C_n	C_{n-1}	C_{n-2}	C_{n-3}	C_{n-4}	C_{n-5}
Oleic acid +[1,3-^{14}C] malonyl-CoA[a]	18:1	25	83:17	43:57	20:80	1.00	0.91	0.69	1.57	1.12	1.18
	20:1	18	100:0	0:100		1.00	0.34	0.36	0.19	0.44	
	22:1	6	100:0	0:100		1.00	0.63	0.82	0.78	0.27	0.33
Vaccenic acid + [1,3-^{14}C]malonyl-CoA[b]	18:1	19	93:7	41:59	19:81	1.00	1.05	0.63	0.78	1.09	0.73
	20:1	15	43:57	0:100	0:100	1.00	0.27	0.24	0.12	0.21	0.17
	22:1	19	97:3	0:100	0:100	1.00	0.62	0.36	0.34	0.12	0.18

[a]Incorporation of radioactivity into total lipids = 7%.
[b]Incorporation of radioactivity into total lipids = 9%.

with [1,3-^{14}C]malonyl-CoA reveals striking differences in the elongation of oleic and vaccenic acids.

With exogenous oleic acid and radioactive malonyl-CoA as substrates, the labeled icosenoic and docosenoic acids formed are exclusively composed of the (n-9) isomers that are derived by elongation of oleic acid only. This is evident from the almost exclusive location of the label in the elongated part of (n-9)icosenoic and docosenoic acids, since in their ozonolysis products only the aldester fragments are labeled, and in their α-oxidation products essentially the chain-shortened acids down to C_{19} are found to be mainly radioactive (Table 3). Apparently, in the presence of exogenous oleic acid in the incubation mixture only this acid is extensively elongated to very long chain (n-9) monounsaturated fatty acids, whereas the elongation of endogenous vaccenic acid is completely suppressed.

With exogenous vaccenic acid and radioactive malonyl-CoA as substrates, more than one-half of the labeled icosenoic acids formed are composed of the (n-7) isomer (Table 3). The labeled (n-9) and (n-7)icosenoic are formed by elongation of unlabeled oleic and vaccenic acids, respectively, as evident from exclusive location of the label in the elongated part of the icosenoic acids. Thus, in the ozonolysis products from the icosenoic acids only the aldester fragments are found to be labeled, and in their α-oxidation products essentially the chain-shortened acids down to C_{19} are seen to be mainly radioactive (Table 3). It is thus obvious that in the presence of exogenous vaccenic acid in the incubation mixture, substantial proportions of this acid are also elongated by two carbon atoms, yet large proportions of endogenous oleic acid are elongated as well. The distribution of label in the ozonolysis fragments from docosenoic acids and in their products of α-oxidation reveals that labeled (n-9) and (n-7)docosenoic acids are also formed by elongation of unlabeled oleic and vaccenic acids, respectively, yet the labeled docosenoic acids are overwhelmingly composed (97%) of the (n-9) isomer (Table 3). Apparently, even in the presence of exogenous vaccenic acid in the incubation mixture, the endogenous oleic acid is selectively elongated by four carbon atoms. This is very likely due to preference of the elongase system for oleoyl-CoA; however, nonavailability of vaccenoyl-CoA at the site of elongation could also be a possible reason for preferential elongation of oleic acid.

The above findings show conclusively that the elongase system in seeds of *Sinapis alba*, and probably of some other plants, selectively convert oleic acid rather than vaccenic acid to very long chain monounsaturated fatty acids. Preponderance of the (n-9) isomers in icosenoic, docosenoic and tetracosenoic acids in the seeds of *Sinapis alba* (Table 1) and other Crucifereae (5,6) corroborate the strong preference of the elongase system for oleic acid. This is possibly the case with mammalian tissues too, in which the very long chain monounsaturated fatty acids found belong predominantly to the (n-9) series (20). It has been observed in rat liver microsomes that although both oleic acid (60%) and vaccenic acid (40%) are formed from malonyl-CoA via elongation/desaturation of endogenous palmitic acid and elongation of endogenous (n-7)hexadecenoic acid, respectively, only the oleic acid is further elongated to (n-9)icosenoic acid (21). A high substrate specificity with regard to the position of the double bond of octadecenoic acids has been observed earlier in their esterification to phospholipids of plant cell cultures (22).

Evidences presented in Tables 2 and 3 support the view that in seeds of *Sinapis alba* the elongation of oleic and vaccenic acids occurs by condensation of their CoA derivatives with malonyl-CoA. In developing seeds of *Simmondsia chinensis* (3) and *Limnanthes alba* (4), both acetate and malonate have been found to be extensively used for the elongation of preformed oleic acid. In animal tissues, such as rat liver, both acetyl-CoA and malonyl-coA are used for the elongation of C_{16} and C_{18} fatty acids; however, separate mechanisms of elongation seem to exist in different subcellular fractions (23). Thus, the microsomal elongation reaction requires fatty acyl-CoA plus malonyl-CoA (24), whereas the mitochondial elongation reaction requires fatty acyl-CoA plus acetyl-CoA (25). It is conceivable that a

TABLE 4

Radioactive Labeling of Saturated Fatty Acids after Incubation of *Sinapis alba* Cotyledons with [1-^{14}C]Acetate or [1-^{14}C]Malonate

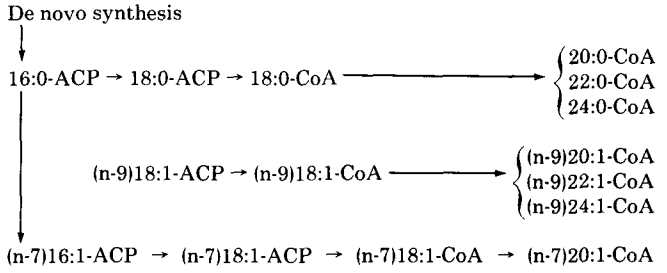
Substrate	Fatty acid	Radioactivity (% of total fatty acids)	Specific radioactivity	Specific radioactivity of chain-shortened acids obtained by α-oxidation of the parent (C_n) acid									
				C_n	C_{n-1}	C_{n-2}	C_{n-3}	C_{n-4}	C_{n-5}	C_{n-6}	C_{n-7}	C_{n-8}	C_{n-9}
Acetate[a]	16:0	6.7	1.0	1.00	0.75	0.87	0.77	0.89	0.54				
	18:0	1.7	0.8	1.00	0.77	0.76	0.87	0.65	0.60	0.47	0.86		
	20:0	1.4	1.7	1.00	0.20	0.25	0.14	0.16	0.24	0.35	0.20	0.21	
	22:0	0.9	1.9	1.00	1.55	1.33	0.34	0.29	0.21	0.15	0.29		
	24:0	1.4	3.0	1.00	0.48	0.65	0.61	0.79	0.46	0.29	0.16	0.06	0.09
Malonate[b]	16:0	1.9	1.0	1.00	0.56	0.81	0.53	0.78	0.61				
	18:0	1.3	2.2	1.00	0.82	0.79	0.65	0.72	0.60	0.62	0.54		
	20:0	1.0	4.3	1.00	0.34	0.19	0.42	0.26	0.49				
	22:0	1.8	13.6	1.00	0.89	0.44	0.13	0.13	0.08	0.05	0.09	0.08	
	24:0	1.5	11.4	1.00	0.63	0.52	0.42	0.26	0.05	0.14	0.07	0.09	0.10

[a]Incorporation of radioactivity into total lipids = 48%.
[b]Incorporation of radioactivity into total lipids = 7%.

similar situation exists in developing seeds in which very long chain monounsaturated fatty acids are synthesized.

Several studies in the past have indicated that very long chain saturated fatty acids are formed in higher plants by elongation of preformed palmitoyl-CoA and/or stearoyl-CoA by malonyl-CoA (4,26,27), although acetate also serves as a primer for these elongation reactions (28,29). It appears that essentially similar pathways are involved in the biosynthesis of very long chain saturated and monounsaturated fatty acids. To verify the existence of such a common pathway, the very long chain saturated fatty acids were examined that were formed when the cotyledons of developing *Sinapis alba* seed were incubated with [1-^{14}C]acetate or [1-^{14}C]malonate.

The results given in Table 4 show that both radioactive acetate and malonate produce labeled very long chain saturated fatty acids in addition to palmitic and stearic acids. Although the relative proportions of labeled very long chain saturated fatty acids formed from either acetate or malonate are quite similar, their relative specific activities are distinctly different. Thus, with acetate as substrate, the relative specific radioactivity of the saturated fatty acids is slightly increased with increasing chain length of these acids (Table 4). Analogously, the relative specific radioactivity of the monounsaturated fatty acids formed from acetate is barely altered with increasing chain length of these acids (Table 2). On the other hand, with malonate as substrate, the relative specific radioactivity of the saturated fatty acids is greatly increased with increasing chain length (Table 4); the relative specific radioactivity of the monounsaturated fatty acids derived from malonate is also considerably increased with increasing chain length (Table 2). These findings strongly suggest that the very long chain saturated and monounsaturated fatty acids are formed by the same mechanism, and malonyl-CoA appears to be the primer in these elongation reactions, as outlined below (acyl moieties are designated by the number of carbon atoms:number of double bonds):



The validity of the above pathways in the formation of very long chain saturated fatty acids is further evidenced by analysis of the products of α-oxidation of the saturated fatty acids derived from acetate or malonate (Table 4). The data show that the parent palmitic and stearic acids as well as the chain-shortened acids derived from each of these fatty acids by α-oxidation are all extensively labeled. Obviously, labeled palmitic and stearic acids are formed predominantly by de novo synthesis from radioactive acetate or malonate. On the other hand, in each of the very long chain saturated fatty acids, the label is located overwhelmingly between the terminal carboxyl carbon atom and the C_{19} carbon atom, counting from the methyl end of the fatty acid chain. This is evident from analysis of the products of α-oxidation of the very long chain saturated fatty acids, which shows that only the parent fatty acids and the chain-shortened acids down to C_{19} are extensively labeled (Table 4) similar to the very long chain monounsaturated fatty acids (Table 2). Obviously, preformed stearic acid is elongated by radioactive acetate or malonate in a similar manner to the elongation of preformed oleic acid to very long chain (n-9) monounsaturated fatty acids.

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Quantitation of Dolichyl Phosphate and Dolichol in Major Organs of the Rat as a Function of Age

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Previous studies in mice and humans have shown age-related increases in the levels of dolichol in all organs investigated. In the present study, the levels of dolichyl phosphate, the physiologically active form of dolichol, as well as dolichol and cholesterol were determined in five major organs of the rat from 4 to 14 wk of age. As observed for mice and humans, the levels of dolichol increased in all tissues examined, especially testis where an eightfold increase was found. Cholesterol levels remained relatively constant in all tissues examined except brain, where a threefold increase was observed. Hepatic dolichyl phosphate levels decreased slightly during growth while nonhepatic tissues showed moderate (1.2–1.7-fold) increases. It is proposed that steady-state levels of hepatic dolichyl phosphate are maintained in the face of constant de novo synthesis by a combination of two pathways: export, either via the circulation or the previously demonstrated fecal route (Connelly and Keller [1984] *Bioscience Reports 4*, 771-776) and conversion to dolichol with subsequent accumulation.
Lipids 21, 353–355 (1986).

Dolichol is a long chain polyisoprenoid which occurs ubiquitously in eucaryotes. It exists in the cell in several forms including the free alcohol, fatty acid esters and the various phosphorylated derivatives which participate in N-linked glycoprotein synthesis (1,2). Recently, we have begun to address the subject of the mechanism of turnover of dolichol compounds. Interest in this aspect of dolichol metabolism has been stimulated by findings (3–6) showing that the levels of dolichol increase substantially in human brain and murine tissues as a function of age, and the discovery that brain levels of dolichol (7) and dolichyl phosphate (Dol-P) (8) are elevated in the disease neuronal ceroid lipofuscinoses. To date, in vivo studies examining the fate of radiolabeled dolichol have failed to demonstrate significant metabolism of the polyisoprenoid backbone (9,10). We have recently shown that, although present in similar levels in rat liver (11), Dol-P is excreted at a rate 2–3 times that of dolichol from rats maintained on a dolichol-free diet (12). This finding raised the possibility that the levels of Dol-P may be maintained at a constant level in some or all tissues by a combination of conversion to dolichol (with subsequent accumulation) and export. In the present study we have examined this possibility directly by determining the levels of Dol-P and dolichol as a function of age in five major organs of the rat.

MATERIALS AND METHODS

Pig liver dolichol and dolichol-11 were obtained from Sigma Chemical Co. (St. Louis, Missouri). The procurement and preparation of all other chemicals has been described in previous publications (11,13).

The extraction of Dol-P as well as dolichol and cholesterol has been described previously (ref. 11, method 1). Briefly, the tissue is first saponified in the presence of internal standards and extracted three times with diethyl ether. All three lipids extract quantitatively. The ether extract is backwashed with 5% acetic acid, taken to dryness and dissolved in chloroform/methanol (2:1, v/v). The sample is chromatographed on DEAE-cellulose (13) to obtain a run-through fraction (cholesterol and dolichol) and an ammonium acetate eluate (Dol-P). Recovery from DEAE-cellulose is nearly quantitative for cholesterol and dolichol and 80–90% for Dol-P. The cholesterol-dolichol fraction is taken to dryness, dissolved in reagent alcohol (Fisher Scientific, Fairlawn, New Jersey; composition is methanol/ethanol/isopropanol, 5:95:5, v/v/v) and applied to a 1 × 30 cm column of Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, New Jersey). The column is equilibrated and eluted with reagent alcohol. The dolichol fraction, which elutes at about 22 ml, is taken to dryness and quantitated by silica high performance liquid chromatography (HPLC) (13). The cholesterol fraction, which elutes at about 40 ml, is analyzed by silica HPLC using 0.7% reagent alcohol in hexane as mobile phase. The Dol-P fraction is treated to remove salt (13), taken to dryness and quantitated by silica HPLC (11). Overall yield for all three lipids ranged from 60–90%. Internal standards, added to the tissue prior to saponification, were Dol-11-P, unsaturated polyprenol-19 and [4-^{14}C]-cholesterol. The former two compounds elute about 1 min apart from Dol-P and dolichol, respectively, from HPLC (11,14) and thus serve as convenient recovery standards.

Three-week-old male Sprague-Dawley rats were obtained from Harlan Industries (Madison, Wisconsin). They were housed five animals per cage and fed Purina Lab Chow and water ad libitum. Animals were killed every two wk by decapitation and organs were quickly frozen for subsequent analysis.

RESULTS AND DISCUSSION

We have recently described a new procedure for the isolation of total tissue Dol-P which involves saponification, ether extraction, purification by ion exchange chromatography and analysis by HPLC on nonbonded silica (11). We showed that the values for tissue levels of Dol-P were substantially greater than had theretofore been determined. In fact, in some tissues the levels were higher than those of dolichol. In the present work, we have used this new procedure to determine the levels of Dol-P as a function of age in major organs of the rat. In addition, we have also determined the levels of total nonphosphorylated dolichol and total cholesterol.

The most striking finding was that the levels of dolichol in all tissues examined increased two- to

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TABLE 1

Effect of Age on Cholesterol, Dolichol and Dol-P Levels in Five Major Organs of the Rat*

Tissue	Age (wk)	Body wt (g)	Wet wt (g)	Chol† (μg/g)	Dol-OH (μg/g)	Dol-P (μg/g)
Brain	4	79±8	1.26 ±0.09	3.82^a ± 0.26	9.36^a ± 0.52	15.3^a ± 1.9
	6	161±9	1.48 ±0.06	11.1^b ± 0.3	14.2^b ± 1.1	13.2^a ± 4.4
	8	234±17	1.61 ±0.06	12.4^c ± 1.1	17.8 ± 1.1	15.8^a ± 1.3
	10	288±25	1.71 ±0.08	9.85^d ± 0.49	23.2^d ± 3.0	13.8^a ± 1.6
	14	328±16	1.80 ±0.06	9.83^d ± 0.41	27.9^c ± 0.8	19.8^b ± 1.8
	fold increase			2.6	3.0	1.3
Liver	4	79±8	3.58 ±0.35	2.21^a ± 0.13	21.1^a ± 1.4	22.0^a ± 1.5
	6	161±9	6.78 ±0.39	2.80^b ± 0.11	32.1^b ± 2.2	22.0^a ± 2.8
	8	234±17	9.34 ±0.59	3.29^c ± 0.18	41.6^c ± 4.1	21.2^a ± 1.6
	10	288±25	10.49 ±1.26	2.79^b ± 0.10	44.5^c ± 5.9	17.8^b ± 0.9
	14	328±16	10.62 ±0.45	2.27^a ± 0.16	52.2^d ± 4.5	16.7^b ± 1.6
	fold increase			—	2.5	.76
Spleen	4	79±8	0.36 ±0.03	2.85^a ± 0.12	58.9^a ±12.4	11.6^a ± 1.8
	6	161±9	0.72 ±0.08	2.29^b ± 0.07	58.1^a ±11.3	15.9^b ± 2.1
	8	234±17	0.69 ±0.05	2.49^a ± 0.21	57.9^a ± 7.4	18.6^c ± 0.7
	10	288±25	0.59 ±0.04	3.19^c ± 0.09	97.9^b ± 1.4	19.1^c ± 1.7
	14	328±16	0.65 ±0.08	2.72^a ± 0.17	157^c ±26	24.8^c ± 1.5
	fold increase			—	2.7	1.7
Kidney	4	79±8	0.87 ±0.08	3.81^a ± 0.11	12.3^a ± 1.2	22.0^a ± 1.0
	6	161±9	1.37 ±0.07	3.70^a ± 0.05	10.2^a ± 1.1	16.0^b ± 4.9
	8	234±17	1.94 ±0.15	3.49^a ± 0.29	13.2^b ± 1.4	16.8^b ± 1.7
	10	288±25	2.11 ±0.12	3.86^a ± 0.16	15.6^c ± 1.9	23.9^a ± 1.9
	14	328±16	2.22 ±0.11	3.96^a ± 0.14	24.1^d ± 1.2	20.0^a ± 2.3
	fold increase			—	2.0	1.25
Testis	4	79±8	0.74 ±0.11	3.73^a ± 0.49	3.34^a ± 0.22	11.8^a ± 0.9
	6	161±9	2.12 ±0.13	5.91^b ± 0.17	5.50^b ± 0.28	18.2^b ± 1.1
	8	234±17	3.00 ±0.18	1.31^c ± 0.04	9.79^c ± 0.82	13.5^c ± 0.7
	10	288±25	3.28 ±0.20	1.59^c ± 0.06	11.3^d ± 1.1	16.7^d ± 1.2
	14	328±16	3.38 ±0.11	1.28^c ± 0.09	24.3^c ± 0.6	19.1^b ± 0.9
	fold increase			—	7.4	1.6

*Values are mean ±SD of five samples for each. Levels with different letter superscripts are significantly different at 95% confidence level. Analysis of variance was carried out using Duncan's multiple range test (15).

†Chol, cholesterol.

eightfold during the 10-wk period of investigation, while the levels of Dol-P rose only slightly or not at all (Table 1). For example, dolichol levels in the testis increased from 3.3 μg/g to 24.3 μg/g, while Dol-P levels in the same organ increased less than twofold. Interestingly, the levels of liver Dol-P showed a slight decrease, declining 24% over the time course investigated. The spleen was found to exhibit the highest level of dolichol at all ages and the level increased approximately threefold during the period studied. As noted previously (11), for a given age the ratio of dolichol/Dol-P was not constant in the various tissues assayed. There also appears to be no correlation of either dolichol or Dol-P levels when the same tissues from rats and human are compared. Thus, human testis has relatively high levels of dolichol (16,17) and Dol-P (17) whereas the levels in rat testis are less than average for both lipids. Eggens et al. (17) have noted that human pituitary exhibits the highest level of dolichol of any tissue examined. With the improved assay for Dol-P developed in this laboratory, it should now be possible to assay for Dol-P levels in this organ in both humans and rodents.

The values we report here for tissue levels of dolichol and Dol-P are not in complete agreement with those of a previous study (11). Although the source of this discrepancy is not known with certainty, it is most likely due to biological variation. The animals used in the present study were purchased as a single shipment at post-weaning and therefore were raised in our facilities. The rats used in the previous study (11) were purchased as 200 g animals raised by the vendor. This fact, along with possible seasonal variation and different vendors, may have contributed to the differences in values observed.

It should also be noted that there is a wide range of values reported for rat liver dolichol. We previously reported values of 17.1 μg/g in 200–300 g rats (11). Tavares et al. (18) reported 22.6 μg/g and 26.1 μg/g in Wistar rats (starting weight 150 g) fed for two wk a control and cholesterol-enriched diet, respectively. Wenstrom and Hamilton reported 17.4 μg/g in 450–500 g male rats from Charles River Labs (19). Eggens et al. (17) reported 43 μg/g in starved Sprague-Dawley rats (180–200 g). Chaudhary et al. (20) reported 60.5 μg/g in 200 g male Wistar rats. Wong et al. (21) reported 0.1–0.2 μg/mg protein in 150 g Sprague-Dawley rats, equivalent to 22–44 μg/g wet weight of liver. Yamada et al. (22) recently reported 17.2 μg/g in male Sprague-Dawley rats (200–250 g). From the present work as well as others (7), it is clear that in the future, the exact age and weight of the rats should be noted, since dolichol levels rise rapidly during growth.

As it appears that most, if not all, tissues exhibit a measurable de novo dolichol synthesis pathway (see, e.g., 19,23,24) the question arises as to the fate of the newly synthesized dolichol compounds. Studies by Keenan et al. (9) and Rip and Carroll (10) have indicated little or no metabolism of the polyisoprenoid backbone of exogenously administered radiolabeled dolichol. If the same holds true for endogenously synthesized dolichol compounds, then the only fates of de novo Dol-P synthesis may be conversion to dolichol and excretion (12). This assumes conversion of Dol-P to dolichol takes place in vivo. In our opinion this pathway has never been conclusively demonstrated, although enzymes which can interconvert dolichol and Dol-P are well known. In fact, studies by Ekström et al. (25) have raised the possibility that dolichol and Dol-P are synthesized via separate, unconnected pathways. Clearly, more in vivo-type studies will be necessary to elucidate the mechanisms which control the tissue levels of Dol-P and dolichol.

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METHODS

Quantitative Analysis of Polyenoic Phospholipid Molecular Species by High Performance Liquid Chromatography

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The quantitative analysis of phospholipid molecular species containing polyenoic fatty acids is described. Dinitrobenzoyl derivatives of diacylglycerols prepared from phospholipids were separated into individual molecular species by reversed-phase high performance liquid chromatography (HPLC) using a combination of two solvent systems and were quantified at 254 nm. Thirty-six molecular species were resolved from the phosphatidylcholines of rat hearts, human platelets and Chinese hamster V79-R cells. The derivatives of alkenylacyl molecular species from platelet phosphatidylethanolamine were resolved concomitantly with diacyl molecular species.
Lipids 21, 356-361 (1986).

Biological membranes consist of various phospholipid classes which control membrane fluidity and function. In animal cells, phospholipids have an abundance of polyunsaturated fatty acids. The analysis of phospholipid molecular species by argentation thin layer chromatography (TLC) of acetyldiacylglycerols has been reported from this laboratory (1). However, the method requires a large amount of sample and it remains difficult to clearly separate polyunsaturated molecular species. Recently, HPLC of intact phospholipids (2,3) or of acetyldiacylglycerol derivatives (4) was developed for this purpose, and the separated molecular species were detected at 205 nm. In this case, phosphorus determination or gas liquid chromatography (GLC) of fatty acid methyl esters is required for quantitative determinations due to differences in the specific absorption of individual molecular species.

Benzoyl (5), *p*-nitrobenzoyl (6) or dinitrobenzoyl (7) derivatives of diacylglycerol prepared from phospholipids, which have a chromophore with strong UV absorption, can be separated by HPLC and directly determined with a UV detector at 230 or 254 nm. However, it is still difficult to separate all of the molecular species using a single solvent system. The method by Snyder and co-workers required argentation TLC combined with HPLC for complete resolution (5). In this paper, we describe the complete separation of phospholipid molecular species of biological origin by HPLC using a combination of two solvent systems.

MATERIALS AND METHODS

Materials. Phospholipase C (grade I, from *Bacillus cereus*) was from Boehringer Mannheim (Mannheim, Federal Republic of Germany), 3,5-dinitrobenzoylchloride (DNBC) from Dojindo Laboratories (Kumamoto, Japan), pyridine (silylation grade) from Pierce Chemical Co. (Rockford, Illinois) and dilauroyl phosphatidylcholine from Sigma Chemical Co. (St. Louis, Missouri). Acetonitrile, methanol and 2-propanol were of HPLC grade; all other chemicals were of reagent grade. Phospholipids were extracted and separated by TLC according to previously described methods (8). Rat heart phosphatidylcholine was isolated from 1-g hearts of rats (8) that were fed sardine oil (containing 20% of 20:5) or corn oil for 10 days (9). Chinese hamster V79-R phosphatidylcholine was obtained from 10^8 V79-R cells cultured in Eagle's minimum essential medium containing 6% delipidated fetal calf serum (10), the medium supplemented with 20:4, and the medium supplemented with 20:5. Platelet phosphatidylcholine and phosphatidylethanolamine were obtained from 10^9 platelets (11) prepared from normal human blood (12).

Preparation of the derivatives. The method described previously (7) was used with a few changes. Approximately 0.5 mg of the phospholipid (containing 10 μg of butylated hydroxytoluene [BHT]) in 1.25 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 30 mM H_3BO_3 was dispersed by sonication. Then 200 units of phospholipase C and 2 ml of water-saturated diethyl ether were added to the suspension. After shaking at room temperature for 30 min, the ether layer containing diacylglycerol was removed and the remaining diacylglycerol was extracted twice with water-saturated diethyl ether. Phosphatidylcholine was completely hydrolyzed under this condition. For complete hydrolysis of phosphatidylethanolamine, incubation was carried out at 30 C for 12 hr. The combined ether extracts were dried under a flow of N_2. The diacylglycerol and 25 mg of DNBC were then dried for 30 min in vacuo prior to use. The mixture was dissolved in 0.5 ml of dry pyridine and heated in a sealed vial at 60 C for 10 min. After being cooled in an ice bath, 2.0 ml of 0.1 N HCl was added and the product was extracted three times with *n*-hexane. The combined extract was dried under a flow of N_2. The residue was dissolved in *n*-hexane and washed with 0.1 N HCl (three times), 0.1 N $NaHCO_3$, 1 N NaCl and water. For storage, 10 μg of BHT was added to the sample.

HPLC. HPLC was carried out on a Hitachi model 655-15 liquid chromatograph. The separated molecular species were determined at 254 nm with a Hitachi model 638-41 variable wavelength UV monitor. Data were processed with a Hitachi model 655-60 processor. An Ultrasphere ODS column (5 μm, 4.6 mm i.d. × 250 mm, supplied by Altex Scientific, Berkeley, California) was used for separation with the following solvent system: acetonitrile/2-propanol (80:20, v/v) or methanol/2-propanol (95:5, v/v). The flow rate was 1.0 ml/min and the column temperature was 25 C. Samples were dried under a flow of N_2 to remove the solvent and the residue was dissolved in acetonitrile or methanol. A 20-100 μl aliquot of the sample (1-100 μg) was injected into the liquid chromatograph using a 200-μl loop.

Identification of the HPLC peaks. The fraction of each peak collected from HPLC was methylated with sodium methoxide or boron trifluoride in methanol, and the fatty acid methyl esters or dimethylacetals were analyzed by

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GLC and GLC-mass spectrometry as described previously (9).

RESULTS AND DISCUSSION

By using acetonitrile/2-propanol (80:20, v/v) as solvent, HPLC was done on the dinitrobenzoyldiacylglycerol (DNB-DG) derivatives from phosphatidylcholines of human platelets, V79-R cells and the hearts of rats fed sardine or corn oil diets (Fig. 1). Twenty-nine peaks with 36 molecular species were identified in these samples (Table 1). Rat heart phosphatidylcholine consisted mainly of 16:0-20:4, 18:0-20:4, 16:0-22:6, and 18:0-22:6 species. The molecular species containing 20:4 decreased in the hearts of rats fed sardine oil as compared to those fed corn oil, whereas the molecular species containing 22:6 increased. The molecular species containing 20:5 were newly synthesized in the hearts of rats which were fed sardine oil. Among the phosphatidylcholine molecular species of human platelets, the 16:0-20:4 and 18:0-20:4 species

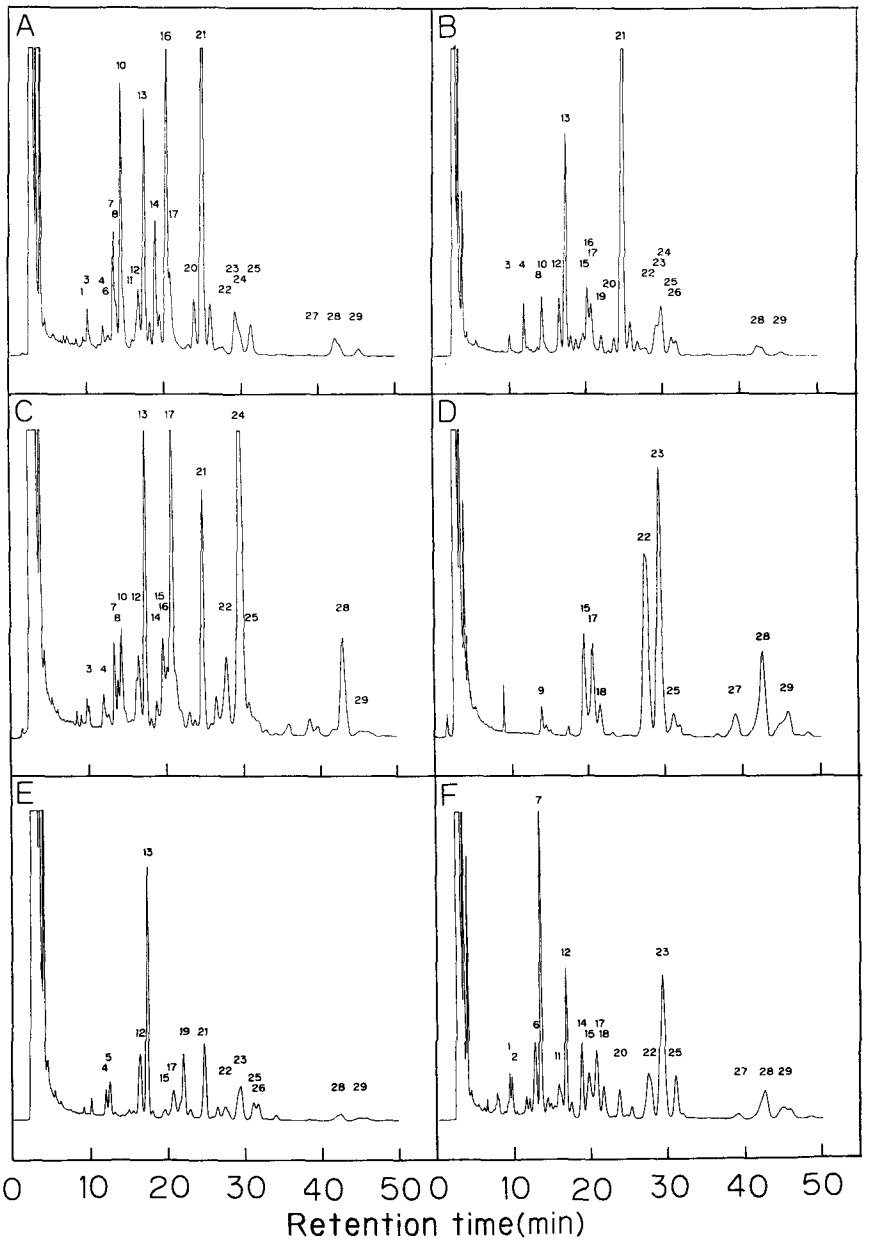

FIG. 1. HPLC separations with acetonitrile/2-propanol (80:20, v/v) of the DNB-DG derivatives prepared from phosphatidylcholines of the following origins: A, hearts of rats fed sardine oil diet; B, hearts of rats fed corn oil diet; C, human platelets; D, V79-R cells cultured in the lipid-free medium; E, V79-R cells cultured in the medium supplemented with 20:4; F, V79-R cells cultured in the medium supplemented with 20:5. Peak numbers correspond to those listed in Table 1. Approximately 5–20 μg of sample was injected. Detection was by absorption at 254 nm. The full-scale absorbance was 0.16.

TABLE 1

Identification and Quantification of Molecular Species Separated by HPLC with Acetonitrile/2-Propanol (80:20, v/v)

Peak no.[a]	RRT[b]	Molecular species	Phosphatidylcholine					
			Rat heart		Human platelet	V79-R		
			SO[c]	CO[d]		Lipid-free	20:4	20:5
1	1.15	16:1-20:5n3	T[e]	—	—	—	—	1.6 ± 0.2[f]
		18:2-20:5n3		—	—	—	—	—
2	1.19	14:0-20:5n3	—	—	—	—	—	1.5 ± 0.0
3	1.23	16:1-22:6n3	1.5 ± 0.0	—	—	—	—	—
		18:2-22:6n3		T	T	—	—	—
4	1.47	16:1-20:4n6	T	2.4 ± 0.0	—	—	2.1 ± 0.0	—
		18:2-20:4n6			1.1 ± 0.0	—	—	—
5	1.52	14:0-20:4n6	—	—	—	—	4.0 ± 0.0	—
6	1.55	18:1-20:5n3	T	—	—	—	—	5.0 ± 0.0
7	1.63	16:0-20:5n3	5.7 ± 0.0	—	2.0 ± 0.0	—	—	14.8 ± 0.0
8	1.68	18:1-22:6n3		T	1.2 ± 0.0	—	—	—
9	1.73	16:1-16:1	—	—	—	1.3 ± 0.0	—	—
10	1.75	16:0-22:6n3	10.9 ± 0.0	4.1 ± 0.0	3.3 ± 0.2	—	—	—
11	1.93	18:1-22:5n3	T	—	—	—	—	3.2 ± 0.0
12	2.04	18:1-20:4n6	3.8 ± 0.0	4.1 ± 0.0	2.4 ± 0.0	—	9.7 ± 0.0	—
		16:0-22:5n3			—	—	—	7.7 ± 0.0
13	2.13	16:0-20:4n6	10.4 ± 0.0	13.6 ± 0.0	9.1 ± 0.1	—	23.5 ± 0.3	—
14	2.30	18:0-20:5n3	6.1 ± 0.0	—	1.0 ± 0.0	—	—	4.7 ± 0.0
15	2.41	18:1-16:1	—	—	—	7.5 ± 0.1	1.5 ± 0.0	5.0 ± 0.0
		18:1-18:2	—	2.1 ± 0.0	3.9 ± 0.0	—	—	—
16	2.48	18:0-22:6n3	15.6 ± 0.0	5.2 ± 0.0	1.8 ± 0.0	—	—	—
17	2.54	16:0-16:1	—	—	—	7.7 ± 0.1	5.4 ± 0.0	6.0 ± 0.0
		16:0-18:2	3.2 ± 0.1	4.4 ± 0.0	15.4 ± 0.1	—		—
		18:1-22:4n6	—		—	—		—
18	2.66	16:0-14:0	—	—	—	2.6 ± 0.0	9.8 ± 0.1	2.7 ± 0.0
19	2.70	16:0-22:4n6	—	1.6 ± 0.0	—	—		—
20	2.91	18:0-22:5n3	3.0 ± 0.0	1.4 ± 0.0	—	—	—	2.2 ± 0.1
21	3.04	18:0-20:4n6	22.0 ± 0.1	35.6 ± 0.1	9.2 ± 0.1	—	10.9 ± 0.1	—
22	3.38	18:1-18:1	T	1.1 ± 0.0	4.9 ± 0.1	23.8 ± 0.2	3.1 ± 0.1	6.3 ± 0.0
23	3.59	16:0-18:1	4.7 ± 0.0	3.0 ± 0.1	24.2 ± 0.2	27.2 ± 0.1	8.5 ± 0.1	17.4 ± 0.1
24	3.67	18:0-18:2		5.9 ± 0.1		—	—	-
25	3.83	16:0-16:0	2.2 ± 0.0	2.1 ± 0.0	2.6 ± 0.2	2.8 ± 0.1	3.0 ± 0.0	4.4 ± 0.3
26	3.90	18:0-22:4n6	—	1.5 ± 0.0	—	—	2.9 ± 0.1	—
27	4.85	20:1-18:1	T	—	—	3.6 ± 0.0	—	T
28	5.24	18:0-18:1	2.4 ± 0.0	2.4 ± 0.0	6.5 ± 0.0	12.8 ± 0.1	2.7 ± 0.1	5.2 ± 0.0
29	5.59	18:0-16:0	T	T	T	6.2 ± 0.0	1.1 ± 0.1	2.4 ± 0.5
Total of T			4.4	1.8	1.4	0.0	1.8	0.9
Unidentified			4.1	7.7	10.0	4.5	10.0	9.0

[a]Peak numbers correspond to those shown in Figure 1.

[b]RRT, relative retention time: that of 12:0-12:0 is regarded as 1.00.

[c]SO, rats fed sardine oil diet.

[d]CO, rats fed corn oil diet.

[e]T, trace amounts (less than 1%).

[f]Values are average mol % ± S.D. based on triplicate analyses of the identical samples.

comprised 9.1 and 9.2%, respectively. V79-R phosphatidylcholine cultured in the lipid-free medium was mainly composed of the 18:1-18:1 (23.8%), 16:0-18:1 (27.2%) and 18:0-18:1 (12.8%) species. The species containing 20:4 and 20:5 were newly produced with a consequent decrease of the species containing 18:1 in the cells grown in media supplemented with 20:4 and 20:5. The DNB-DG derivative of 12:0-12:0 species was used as the base peak for calculation of relative retention time (RRT) values. However, the RRT values changed slightly due to the difference in the lot numbers of the commercial column. We found that the peak area of each fraction was proportional to the amount of the molecular species and was not affected by differences in the structures of the molecular species (7). Therefore, the 12:0-12:0 or 18:0-18:0 species can be added as the internal standard to determine the absolute amount of each fraction.

When the DNB-DG derivatives from the heart phosphatidylcholines of rats which were fed sardine or corn oil diets were analyzed by HPLC using methanol/2-propanol (95:5, v/v) as the solvent, 17 peaks were identified. However, many peaks contained more than one species (Fig. 2 and Table 2). This solvent system could not separate the 18:0-20:4 species from the 16:0-16:0, 16:0-18:1 and 18:1-18:1 species.

Graphical representation of the RRT values for the molecular species indicates the difference in the elution profile by the two solvent systems (Fig. 3). This shows a possibility that the peaks that cannot be resolved with acetonitrile/2-propanol are separated with methanol/2-propanol. When the fraction of the unseparated peak (peak 12 in Fig. 1A) on HPLC with acetonitrile/2-propanol (80:20) was collected and rechromatogrammed with methanol/2-propanol (95:5), the overlapping molecular species were successfully separated (peaks 12a and 12b in Fig. 4A). Similarly, peaks 23 and 24 in Fig. 1A were well resolved (Fig. 4B). Two molecular species containing the common fatty acid at one side chain and 16:1 or 18:2 at the other side chain could not be resolved with acetonitrile/2-propanol (Table 1). However, they were separated with methanol/2-propanol (Table 2). Snyder and co-workers used argentation TLC prior to HPLC to resolve overlapping peaks in HPLC (5). However, by this procedure, precision of analysis may suffer.

The DNB-DG derivatives of the molecular species prepared from human platelet phosphatidylethanolamine

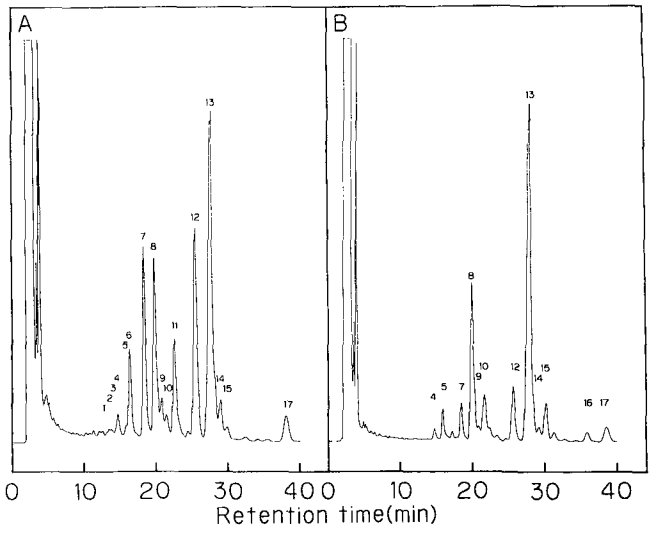

FIG. 2. HPLC separations with methanol/2-propanol (95:5, v/v) of the DNB-DG derivatives prepared from phosphatidylcholines of the following origins: A, hearts of rats fed sardine oil diet; B, hearts of rats fed corn oil diet. Peak numbers correspond to those listed in Table 2. Approximately 5-20 μg of sample was injected. Detection was by absorption at 254 nm. The full-scale absorbance was 0.16.

TABLE 2

Identification and Quantification of Molecular Species Separated by HPLC with Methanol/2-Propanol (95:5, v/v)

Peak no.[a]	RRT[b]	Molecular species	Phosphatidylcholine, Rat heart, SO[c]	Phosphatidylcholine, Rat heart, CO[d]
1	1.55	16:1-20:5n3	T[e]	—
2	1.67	18:2-20:5n3	T	—
3	1.70	16:1-22:6n3	T	—
4	1.82	18:2-22:6n3 16:1-20:4n6	1.7 ± 0.0[f]	T
5	1.96	18:2-20:4n6		2.9 ± 0.0
6	2.02	18:1-20:5n3 16:0-20:5n3	6.1 ± 0.0	— —
7	2.27	18:1-22:6n3 16:0-22:6n3	11.6 ± 0.1	3.7 ± 0.0
8	2.45	18:1-20:4n6 16:0-20:4n6	11.9 ± 0.0	17.2 ± 0.0
9	2.57	18:1-22:5n3 16:0-22:5n3	2.6 ± 0.0	1.2 ± 0.0
10	2.66	16:0-18:2 18:1-18:2	1.8 ± 0.0 —	6.1 ± 0.0
11	2.80	18:0-20:5n3	8.0 ± 0.0	—
12	3.16	18:0-22:6n3 16:0-22:4n6	15.8 ± 0.1 —	6.5 ± 0.0
13	3.42	18:0-20:4n6 18:1-18:1 16:0-18:1 16:0-16:0	28.8 ± 0.2	44.9 ± 0.1
14	3.60	18:0-22:5n3	3.2 ± 0.0	1.8 ± 0.0
15	3.71	18:0-18:2	1.1 ± 0.0	4.9 ± 0.0
16	4.43	18:0-22:4n6	—	1.4 ± 0.1
17	4.75	18:0-18:1 18:0-16:0	3.1 ± 0.1	2.9 ± 0.1
Total of T			1.2	0.8
Unidentified			3.1	5.7

[a]Peak numbers correspond to those shown in Figure 2.
[b]RRT, relative retention time: that of 12:0-12:0 is regarded as 1.00.
[c]SO, rats fed sardine oil diet.
[d]CO, rats fed corn oil diet.
[e]T, trace amounts (less than 1%).
[f]Values are average mol % ± S.D. based on triplicate analyses of the identical samples.

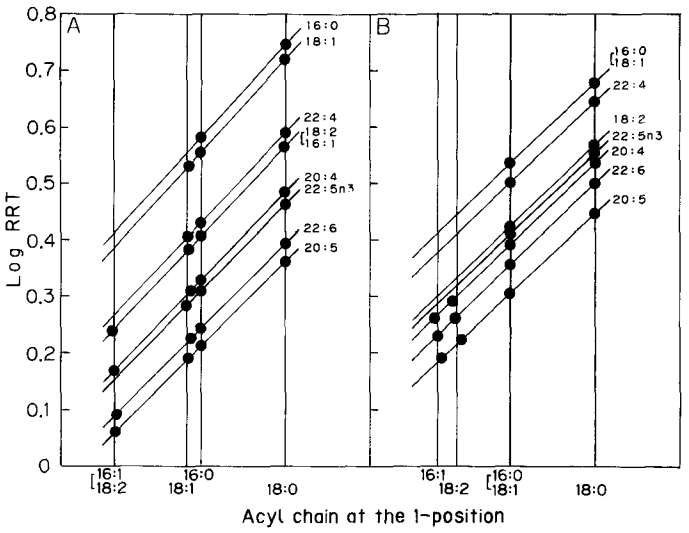

FIG. 3. Relationship between RRT values and fatty acyl moieties of molecular species. Plotting and drawing were carried out according to Patton et al. (3) and Nakagawa and Horrocks (4). A, in acetonitrile/2-propanol (80:20, v/v); B, in methanol/2-propanol (95:5, v/v). A fatty acid of which the number of double bonds is smaller than that of the other fatty acid was regarded as the fatty acid at the 1-position.

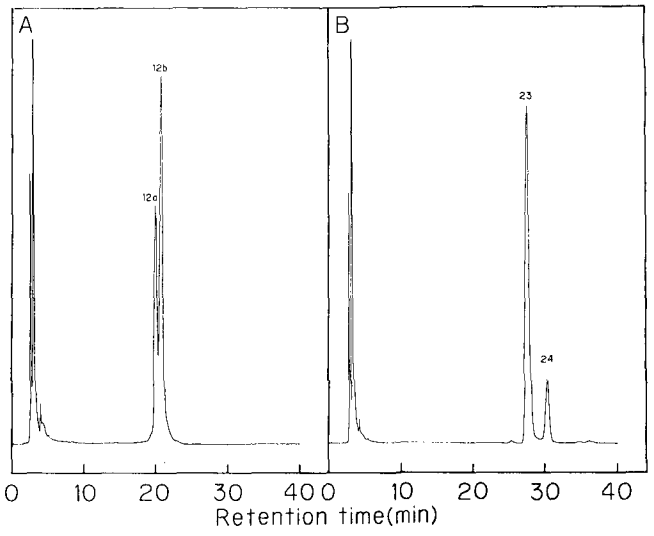

FIG. 4. Rechromatography with methanol/2-propanol (95:5, v/v) of the overlapping peaks shown in Fig. 1A. A, peak 12; B, peaks 23 and 24. Peak 12a, 18:1-20:4n6; 12b, 16:0-22:5n3; 23 and 24 (see Table 1).

after hydrolysis by phospholipase C (see Methods) were separated by HPLC similarly to those from phosphatidylcholine (Fig. 5). Polyenoic alkenylacyl and diacyl molecular species were well resolved. However, separation of alkylacyl species is now under examination.

GLC coupled with a flame ionization detector (13) and HPLC combined with mass spectrometry (14,15) have the disadvantage of loss of sample during analysis. However, the method described here allows the quantitative preparation of molecular species. Hence, radiolabeled fatty acyl chains or glycerol moieties of phospholipids are available. In addition, the wavelength of 254 nm used in this method is superior to detection at 230 nm for

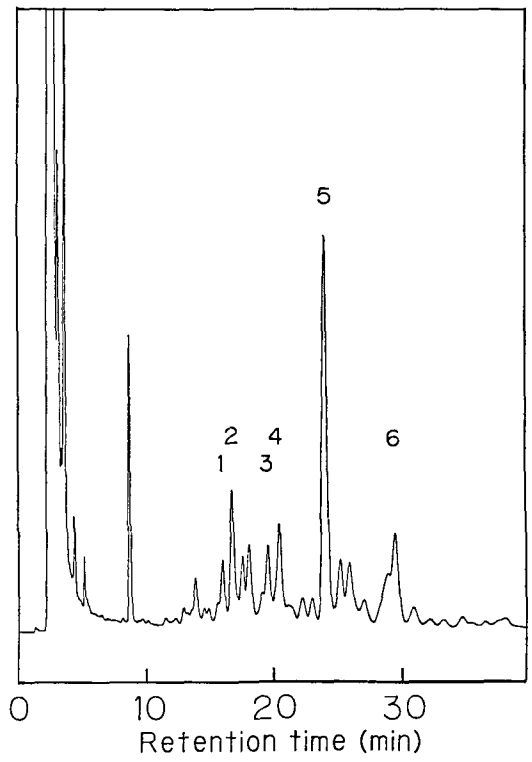

FIG. 5. HPLC separation with acetonitrile/2-propanol (80:20, v/v) of the DNB-DG derivatives of alkenylacyl and diacyl molecular species from human platelet phosphatidylethanolamine. 1, 18:1(acyl)-20:4; 2, 16:0(acyl)-20:4; 3, 18:1(alkenyl)-20:4; 4, 16:0(alkenyl)-20:4; 5, 18:0 (acyl)-20:4; 6, 18:0(alkenyl)-20:4.

benzoyl diacylglycerols (5), because interference from absorption of solvents and impurities is less likely.

Recently, Krüger et al. (16) reported the HPLC analysis of naphthylurethane derivatives. However, high temperature (85 C) and long incubation time (2 hr) were required for the preparation of the derivatives. In addition, the separation was not sharp in spite of the long gradient elution time (16). Using our method, incubation at 60 C for 10 min and short isocratic elution time permitted the preparation and the resolution of the DNB-DG derivatives. Therefore, this appears to be suitable for routine analyses of the polyenoic molecular species.

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Fast Atom Bombardment and Tandem Mass Spectrometry for Determining Iso- and Anteiso- Fatty Acids

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Branched fatty acids can be distinguished from isomeric straight chain fatty acids by collisionally activating the $(M-H)^-$ ions desorbed by using fast atom bombardment (FAB) mass spectrometry (MS). In particular, an acid with iso- fatty branching can be readily distinguished from one containing anteiso- branching; the latter undergoes loss of the elements of CH_4 and C_2H_6 but not C_3H_8. These decompositions are another example of remote charge site fragmentation. Mixtures of homologs and isomers can be investigated by using the combination of FAB and tandem mass spectrometry (MS-MS).
Lipids 21, 362-365 (1986).

Many lower life forms such as bacteria and marine organisms possess the ability of producing a variety of structurally modified fatty acids. One such modification is branching near the alkyl terminus of the acid in either the iso- or anteiso- position. These modified acids are of interest not only because they are unusual but also because they are useful for characterizing organisms.

Structural elucidation of branched acids is a challenging problem, and mass spectrometry (MS) has been shown to be useful for many cases. The study of methyl esters of branched chain acids was included in the pioneering studies of electron ionization (EI) MS of fatty acids by Stenhagen and coworkers (1-3). When branching occurs on the carbon chain, there is a preferential cleavage next to the tertiary carbon atom, with the charge being retained by the fragment of highest relative stability. This method is quite satisfactory for single branches relatively near the carboxylate. Unfortunately, iso- and anteiso- branching, which are the most common in nature, are quite difficult to locate from EI spectra because the diagnostic fragment ions are of high mass and low abundance (4,5).

Several workers have suggested using ratios of specific ion abundances for distinguishing normal from iso- and anteiso-branched acids (6,7). This is difficult at best and only useful for determining the position of a branch in an acid which has been tentatively characterized by other means. In practice, EI mass spectra are usually considered in combination with infrared spectra (8,9) or gas chromatography data (5,10) for establishing the structure of terminally branched acids. Authentic standards are usually required to complete the assignment.

Derivatization followed by MS analysis is another approach for structural determination of branched acids. Derivatization methods include conversion of methyl esters of fatty acids to their corresponding fatty alcohols (4) or methyl ethers (11), or formation of dicyanomethylene (12), pyrrolidide (13-17) or picolinyl (18) derivatives. Of these derivatives, pyrrolidides are probably most frequently used. However, they are somewhat limited by low abundance, high mass diagnostic ions for determining terminal branching (ca. 1% rel. abundance). Picolinyl derivatives have been introduced recently and appear to be a satisfactory derivatization approach in terms of both ease of preparation and abundance of diagnostic ions. Nevertheless, this requires the additional step of derivatization prior to analysis.

In recent years, we have shown that FAB (19) combined with tandem mass spectrometry (often called mass spectrometry-mass spectrometry [MS-MS]) can be a powerful tool for identification and structural analysis of many biomolecules either in pure form or as components of mixtures (20-24). More specifically, we have found that collisional activation (25,26) of FAB-desorbed carboxylate anions of nonderivatized fatty acids yields information which allows the substance to be identified as a fatty acid and modifications such as double bonds to be located (27,28). In this paper, we show that FAB/MS-MS methods are highly suited to the problem of structural analysis of iso- and anteiso-branched acids, in terms of simplicity of analysis and clarity and quality of information obtained.

MATERIALS AND METHODS

Materials. Fatty acids were obtained from Foxboro/Analabs (North Haven, Connecticut) and included eicosanoic, 18-methyl nonadecanoic acid, 16-methyl heptadecanoic acid, 16-methyl octadecanoic acid and 14-methyl hexadecanoic acid. These acids were analyzed directly without prior treatment or derivatization.

Mass spectrometry. Mass spectra were obtained with a Kratos MS-50 triple analyzer tandem mass spectrometer which has been described previously (29). This instrument consists of a high-resolution MS-I of Nier-Johnson geometry followed by an electrostatic analyzer used as MS-II. It is equipped with a standard Kratos FAB source and and Ion Tech saddle field ion gun (Teddington, United Kingdom).

Each sample was dissolved in triethanolamine matrix, and a drop of the resulting solution was placed on the copper target of the FAB direct insertion probe for analysis. The samples were bombarded with 8 KeV Xe atoms, and the ions produced were accelerated through 8 KeV. All spectra were acquired in the negative ion mode. Full mass spectra were acquired at a resolution of ca. 2000 by scanning MS-I and leaving MS-II fixed to pass all ions. Collisional activation spectra were obtained by selecting the desired ion with MS-I, colliding the selected ion beam with sufficient He in the collision cell located between MS-I and MS-II to cause a 50% reduction in the selected beam, and scanning MS-II to obtain a spectrum of the daughter ions formed as a result of collisional activation of the parent ion. Spectra were acquired, signal-averaged and processed with a standard DS-55 data system using software written at this laboratory (30).

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RESULTS AND DISCUSSION

Fatty acids are ideally suited to negative ion FAB/MS analysis because carboxylate anions are readily desorbed from the basic triethanolamine matrix into the gas phase. Mass spectra of the negative ions produced by FAB contain relatively little information except for a strong $(M-H)^-$ ion and associated cluster ions. No detectable fragment ions are found (see Fig. 1a).

If the abundant $(M-H)^-$ ions (presumably $RCOO^-$) are selected by using MS-I and are collisionally activated, distinctive and very reproducible fragmentation occurs. A unique feature of this fragmentation is a series of losses of the elements C_nH_{2n+2} from the alkyl terminus of the $(M-H)^-$ ion (27,28). The collisionally activated decomposition (CAD) spectrum of the $(M-H)^-$ ion of eicosanoic acid (Fig. 1) is a representative example of a straight chain saturated carboxylate anion containing 10 or more carbons. Elimination of the elements of CH_4, C_2H_6, C_3H_8 and C_4H_{10} gives rise to the ions of m/z 295, 281, 267, and 253, respectively. Although the elements of alkanes are lost, the losses are probably of H_2 and neutral alkenes (28). The only other ions formed from carboxylate anions are those resulting from H_2O loss from the $(M-H)^-$ ion and ions of m/z 58, 71 and 86. The latter three ions are produced in all cases and are of relatively low abundance for acids having longer chains.

This unique fragmentation behavior, which we have termed remote charge site fragmentation, gives rise to a pattern in CAD spectra that is expected to be perturbed if the carbon chain of the acid is modified. This is true for monounsaturated acids that show enhanced abundances for fragments that arise from cleavages of the allyl bond and sparse fragments for cleavages of the vinyl or double bonds (27). Because nonderivatized acids containing one or more double bonds (27,31) can be determined, it is likely that the method would be informative for distinguishing iso- and anteiso-branched acids.

Full mass spectra of ions desorbed from samples of 16-methyl heptadecanoic acid, 18-methyl nonadecanoic acid, 14-methyl hexadecanoic acid and 16-methyl octadecanoic acid were obtained. As is typical for carboxylic acids, only an abundant $(M-H)^-$ ion, a less abundant $[M-H-H_2]$ and associated C-13 and other isotope-containing ions are desorbed into the gas phase.

Collisional activation of the $(M-H)^-$ ions of each of these acids yielded the spectra of fragment ions shown in Figure 2. Clearly branching is distinguished because certain remote site fragmentations are suppressed owing to the presence of a branch point. For 16-methyl heptadecanoate and 18-methyl nonadecanoate iso-branched acids, the loss of the elements of CH_4 from the $(M-H)^-$ is enhanced presumably because there are two terminal methyl groups. The loss of the elements of

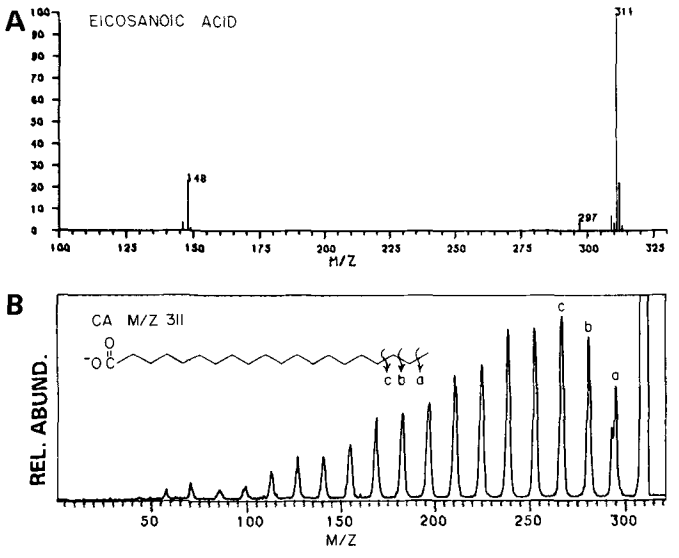

FIG. 1. (A) Mass spectrum of negative ions produced by FAB desorption of eicosanoic acid. Ion of m/z 148 is from the triethanolamine matrix. (B) Spectrum of the daughter ions produced by collisionally activating the $(M-H)^-$, m/z 311, of eicosanoic acid (CAD spectrum).

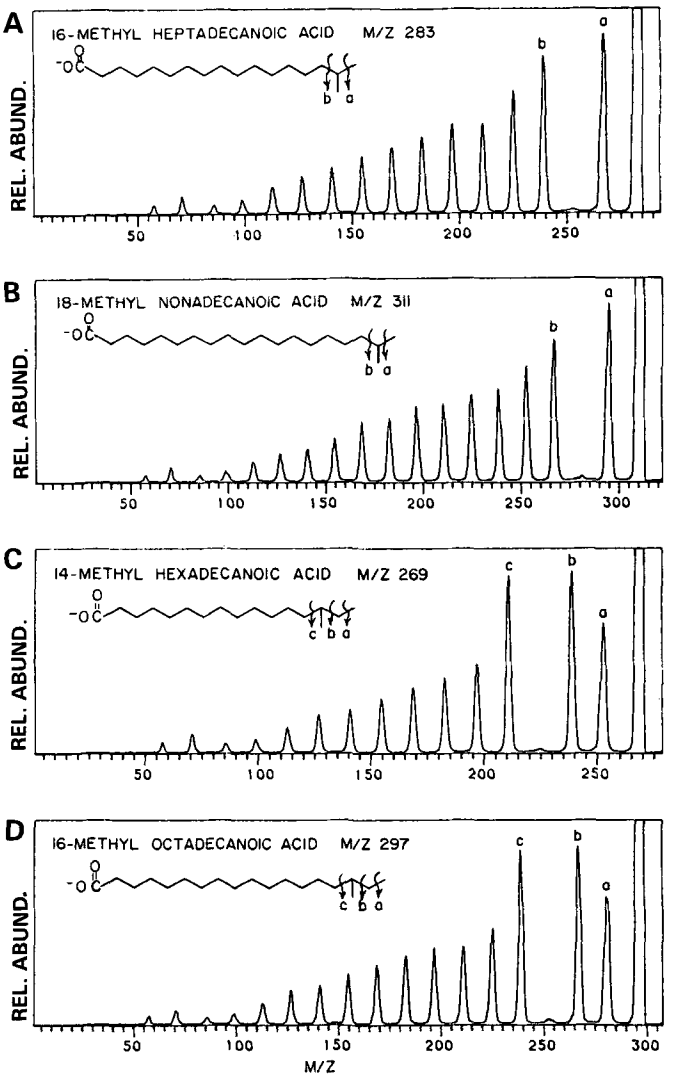

FIG. 2. Spectra of the daughter ions produced by collisionally activating the $(M-H)^-$ ions of iso- and anteiso-branched acids. (A) 16-Methyl heptadecanoic, m/z 283. (B) 18-Methyl nonadecanoic, m/z 311. (C) 14-Methyl hexadecanoic, m/z 269. (D) 16-Methyl octadecanoic, m/z 297.

C_2H_6 is dramatically suppressed as the second carbon from the alkyl terminus has a methyl substituent, and elimination of C_2H_6 is not readily accomplished. Once the branch point is passed, normal remote charge site fragmentation resumes (compare Figs. 2a and 2b with Fig. 1). Similarly, the ion corresponding to the loss of C_3H_8 is suppressed for 14-methyl hexadecanoate and 16-methyl octadecanoate, which are the anteiso-branched acids (Figs. 2c and 2d).

This method is quite applicable to mixture analysis. The lack of fragmentation in the FAB desorption process facilitates selection of each component of a mixture of fatty acid anions for collisional activation. This is demonstrated in the FAB mass spectrum (Fig. 3a) obtained of a 1:1:1:1 (v/v/v/v) mixture of the four acids discussed above. Each anion may be readily selected and collisionally activated to yield CAD spectra identical to those of the pure acids.

A mixture of isomeric fatty acids presents a potential problem. However, the general distribution of the abundances of ions in remote site fragmentation is so reproducible that the presence of both eicosanoic and 18-methyl nonadecanoic (both of m/z 311) can be recognized from the CAD spectrum of the $(M-H)^-$ (m/z 311) of a 1:1 (v/v) mixture of the two (Fig. 3b). Distinction of the two is also possible for a 10:1 (v/v) mixture of eicosanoic to 18-methyl nonadecanoic (see inset of Fig. 3b). The diagnostic feature is the ion corresponding to the loss of the elements C_2H_6. The ion is of reduced abundance compared to the corresponding ion in the spectrum of pure eicosanoic acid (Fig. 1a) and of enhanced abundance compared to that of the pure branched acid (Fig. 2b). While the number of ions desorbed from the matrix by FAB may not represent the number in solution, the FAB mass spectrum is nevertheless very reproducible. The use of appropriate standards would make quantification possible for mixtures containing comparable amounts of these acids.

FAB and MS-MS with collisional activation of carboxylate anions provide an effective method for identifying branched fatty acids. The location of branch points is easily discerned without separating the acid components and derivatizing them. This method is well suited for iso- and anteiso-branched acids since, in contrast to other MS methods, the most abundant fragment ions are the informative high mass ones.

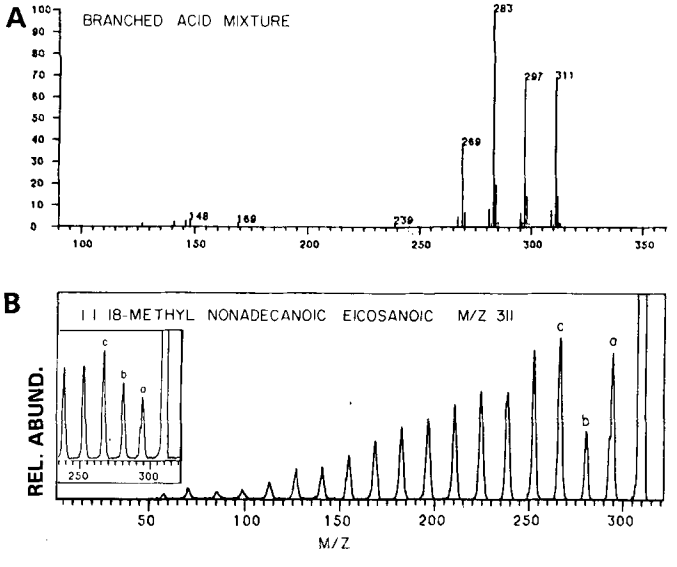

FIG. 3. (A) Mass spectrum of the negative ions produced by FAB desorption of a 1:1:1:1 (v/v/v/v) mixture of 16-methyl heptadecanoic, 18-methyl nonadecanoic, 14-methyl hexadecanoic, 16-methyl octadecanoic. (B) CAD spectrum of m/z 311 from a 1:1 (v/v) mixture of eicosanoic to 18-methyl nonadecanoic. Inset shows diagnostic peaks for a 10:1 (v/v) mixture of eicosanoic and 18-methyl nonadecanoic acids.

ACKNOWLEDGMENT

This research was supported by the National Science Foundation (Grant CHE 8302388) and by the Midwest Center for Mass Spectrometry, an NSF Instrumentation Facility (Grant CHE 8211164).

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[Received November 6, 1985]

COMMUNICATIONS

Stimulation of Fatty Acid Synthesis by 4β-Phorbol-12-Myristate-13-Acetate in Isolated Rat Hepatocytes

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The tumor-promoting agent 4β-phorbol-12-myristate-13-acetate (TPA) is shown to be a potent stimulator of fatty acid synthesis in isolated rat hepatocytes. The maximal effect of TPA is seen at 10^{-6} M, and the concentration for half-maximal effect is ca. 10^{-8} M. Stimulation of fatty acid synthesis by TPA is shown not to require the presence of extracellular Ca^{++}. TPA produces a significant increase in lactate and pyruvate accumulation. The possible involvement of protein kinase C in short-term regulation of fatty acid synthesis in the liver is discussed.
Lipids 21, 366-367 (1986).

Recent studies have demonstrated that hormones can transmit their message across the plasma membrane by a variety of mechanisms, including the generation of cyclic AMP and the stimulation of phosphoinositide metabolism (for reviews, see refs. 1 and 2). In isolated hepatocytes, it is well documented that the rate of fatty acid synthesis is inhibited by the addition of glucagon (3,4) or dibutyryl cyclic AMP (5). On the other hand, at present, very little is known about the regulation of fatty acid synthesis by a Ca^{++}-linked, cyclic AMP-independent mechanism. The potent tumor-promoter TPA can stimulate Ca^{++}-activated, phospholipid-dependent protein kinase (C-kinase) directly by substituting for diacylglycerol (6). Therefore, TPA appears to be a good probe for studying the possible role of C-kinase in the regulation of hepatic lipogenesis.

In the present study, we investigated the effect of TPA on fatty acid synthesis in isolated rat hepatocytes. We found that TPA produces a marked stimulation of this process.

MATERIALS AND METHODS

Hepatocytes were isolated from ad libitum-fed male Wistar rats (300-400 g) by the method of Berry and Friend (7) with modifications described by Harris (5). The cells were suspended (35-45 mg wet wt) in 2 ml of Krebs-Henseleit buffer (pH 7.4) supplemented with 2.5% (w/v) bovine serum albumin (essentially fatty acid-free and dialyzed, Fraction V, Sigma Chemical Co., St. Louis, Missouri) under an atmosphere of 95% O_2/5% CO_2 (v/v) in stoppered 25-ml Erlenmeyer flasks. Phorbol esters were dissolved in dimethyl sulfoxide. An equal volume of dimethyl sulfoxide was added to control incubations. Incubations were conducted in a shaking water bath at 37 C and terminated with $HClO_4$. Lactate and pyruvate were measured in KOH-neutralized $HClO_4$ extracts of cell suspensions spectrophotometrically by the enzymatic method described by Hohorst et al. (8). The rate of fatty acid synthesis was estimated by ^3H_2O (0.5 mCi/ml) incorporation as described by Harris (5). The incorporation of ^3H_2O into fatty acids is thought to be the most reliable measure of total rate of fatty acid synthesis (9,10).

Collagenase (Type II) was obtained from Worthington Biochemical Corp. (Freehold, New Jersey). TPA and 4α-phorbol-12,13-didecanoate were purchased from Sigma. Tritiated water and scintillation solution were obtained from New England Nuclear (Boston, Massachusetts).

The results are usually expressed as means ± SEM. Statistical evaluation of the data was made by means of Student's t-test for paired data.

RESULTS AND DISCUSSION

Figure 1 shows the time course of the incorporation of ^3H_2O into fatty acids in the presence and absence of TPA. It is clearly indicated that TPA stimulates fatty acid synthesis during the 60 min of incubation. Figure 2 illustrates that TPA produces dose-dependent stimulation of fatty acid synthesis in 30 min incubation. It was found that maximal effect (83 ± 20% increase, $P < 0.05$) was achieved at 1 μM TPA, while 10 nM TPA gave half-maximal stimulation. In contrast with TPA, the inactive phorbol ester 4α-phorbol-12,13-didecanoate (11) (10 μM) was found to produce no effect (Fig. 2). While this paper was in preparation, Vaartjes and de Haas (12) reported a similar stimulation of fatty acid synthesis by TPA in isolated rat hepatocytes.

Experiments on the effect of Ca^{++} depletion were performed to investigate whether the effect of TPA in

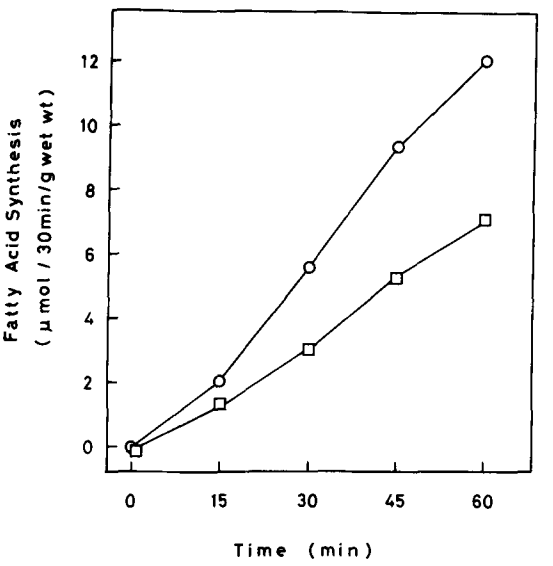

FIG. 1. Time course of ^3H_2O incorporation into fatty acids. □, control; ○, TPA (10^{-6} M). All points are the means of duplicate determinations.

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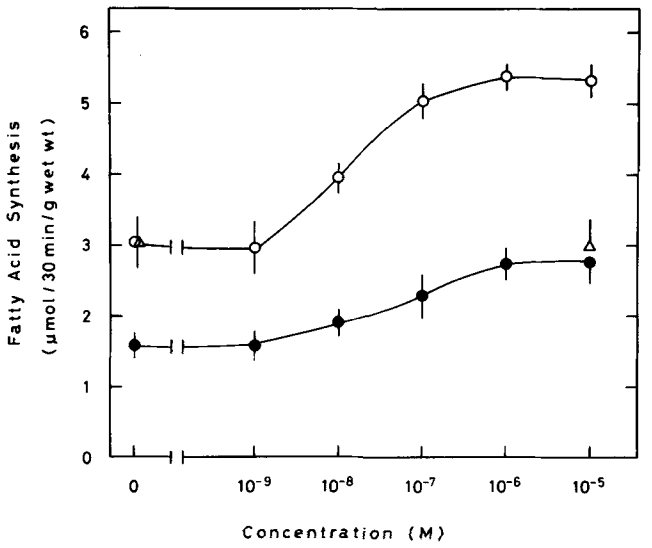

FIG. 2. Effects of phorbol esters on fatty acid synthesis. Incubations were conducted for 30 min. ○, TPA in the presence of Ca^{++}; △, 4α-phorbol-12,13-didecanoate in the presence of Ca^{++}; ●, TPA in the absence of Ca^{++}. Results are shown as means ± SEM for three hepatocyte preparations.

stimulating fatty acid synthesis was dependent on the presence of Ca^{++} in the medium. Hepatocytes were incubated for 30 min in Krebs-Henseleit buffer containing 1 mM EGTA but no calcium. It was found that the rate of fatty acid synthesis was decreased by calcium depletion per se by 48 ± 4%. However, it is clear in Figure 2 that TPA caused a significant stimulation of fatty acid synthesis in the absence of Ca^{++}, too. The maximal effect (71 ± 11% increase, $P < 0.05$) was observed at 1 μM TPA. Thus, results in the present study indicate that the stimulation of fatty acid synthesis by TPA does not require the presence of extracellular calcium.

In isolated hepatocytes, rapid rates of fatty acid synthesis have been found to correlate with rapid rates of glycolysis, i.e., lactate and pyruvate accumulation (for review, see ref. 10). In the present study, it was found that 1 μM TPA produced a statistically significant increase ($P < 0.05$) in lactate and pyruvate accumulation (control [N = 4], 21.1 ± 1.3 μmol/30 min/g wet wt; TPA [N = 4], 25.5 ± 1.9 μmol/30 min/g wet wt). It has been documented that both glycolysis and fatty acid synthesis are acutely stimulated by insulin (13) or by vasopressin (14). Whether or not the response to these hormones requires the participation of C-kinase needs to be investigated further.

Our results suggest that C-kinase may play an important role in short-term regulation of fatty acid synthesis in isolated rat hepatocytes, although the exact mechanism for the stimulation remains to be elucidated.

ACKNOWLEDGMENT

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ERRATA

The table of contents for the April 1986 issue of *Lipids* (21:4) lists incorrect page numbers for two articles. The correct listings:

301–304	**Ether Lipid Derivatives: Antineoplastic Activity In Vitro and the Structure-Activity Relationship** W.E. Berdel, D.D. Von Hoff, C. Unger, H.D. Schick, U. Fink, A. Reichert, H. Eibl and J. Rastetter
305–307	**The Metabolism of Malondialdehyde** H.H. Draper, L.G. McGirr and M. Hadley

An Essential Fungal Growth Factor Derived from Ergosterol: A New End Product of Sterol Biosynthesis in Fungi?

Leo W. Parks, Russell J. Rodriguez and Christopher Low (*Lipids 21,* 89–91, 1986)

Page 89: In the Abstract (line 1), the ergosterol concentration given should be "1.2 nM." The first sentence of the Introduction should read: "Yeast sterol auxotrophs are unable to grow on cholestanol (saturated cholesterol) (12 μM) unless a minute quantity (25 nM) of a C-5,6 unsaturated sterol such as ergosterol is available (1,2)."

Fatty Acid Composition of Individual Plasma Steryl Esters in Phytosterolemia and Xanthomatosis

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[a]Banting and Best Department of Medical Research and [b]Department of Medicine, University of Toronto, Toronto, Ontario M5G 1L6 Canada; and [c]Department of Pediatrics and [d]Medicine, and the [e]Julia McFarlane Diabetes Research Unit, Faculty of Medicine, The University of Calgary, Calgary, Alberta T2N 4N1 Canada

The bulk of the plasma plant sterol in phytosterolemia occurs in the esterified form and is carried mostly in the low and high density lipoproteins. We have determined the fatty acid composition of the individual plasma steryl esters from a newly discovered subject with phytosterolemia and xanthomatosis. For this purpose the intact steryl esters were subject to high temperature gas liquid chromatography (GLC) on a polar capillary column, which separated the major esters on the basis of molecular weight and degree of unsaturation of the fatty acids. The saturated and unsaturated sterols esterified to saturated, monoenoic, dienoic and tetraenoic fatty acids were identified by GLC analysis of the sterol moieties of the corresponding $AgNO_3$-TLC fractions of the steryl esters. The GLC results were confirmed by reversed phase high performance liquid chromatography combined with mass spectrometry via direct liquid inlet interface. It was found that, in general, each fatty acid was esterified to the same complement of sterols, and that the esterified sterols possessed a composition comparable to that of the free plasma sterols, which was comprised of about 75% cholesterol, 6% campesterol, 4% 22,23-dihydrobrassicasterol and 15% β-sitosterol. The fatty acid composition of the steryl esters differed from that of the 2-position of the plasma phosphatidylcholines, which contained significantly less palmitic and oleic and more linoleic acid. On the basis of these results and a review of the literature it is suggested that the plasma cholesteryl and plant steryl esters in phytosterolemia originate from both synthesis in plasma via the lecithin-cholesterol acyltransferase and synthesis in tissues via the acylCoA-cholesterol acyltransferase.
***Lipids* 21, 371-377 (1986).**

Phytosterolemia is a rare lipid storage disease characterized by the accumulation of plant sterols in the blood and tissues (1). Only some 20 cases of this disease have been described so far (2). We have recently reported the discovery of another subject with this disease (3) and have demonstrated the usefulness of plasma total lipid profiling in its diagnosis (4). In the present study we performed a detailed investigation of the fatty acid composition of the individual steryl esters, which had not been examined previously in this disease. Bhattacharya and Connor (5) originally reported that as much as 60% of the total plant sterol in plasma might occur in the esterified form in phytosterolemia (5,6), while Salen et al. (6) later showed that most of the plasma plant sterol was carried in the free form in this disease. The present study demonstrates that the plant steryl esters, including their α-stanol derivatives, possess fatty acid compositions comparable to those of the plasma cholesteryl esters, although the stanols and the plant sterols are somewhat less extensively esterified (60%) than cholesterol (75%).

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MATERIALS AND METHODS

Reference standards. The plant steryl esters were available in the laboratory from previous studies or were newly synthesized by previously described methods (7). Cholesteryl esters of the common fatty acids were purchased from Supelco Inc. (Bellefonte, Pennsylvania). Reference sterols (8) and quantitative standard mixtures (9) were as previously reported.

Subjects and plasma samples. Complete clinical and biochemical description of the patient, including the criteria for phytosterolemia and xanthomatosis, has been presented elsewhere along with that of the family members included as controls (3,4). Fasting blood was drawn from the patient on three different occasions about three months apart and from the family members on one occasion. All subjects ate regular food, except for the patient prior to the second and third blood sampling, when a low plant sterol diet had been advised. Blood was collected into tubes containing solid EDTA after an overnight fast (16 hr).

Isolation of steryl esters and phosphatidylcholines. Total steryl esters were isolated from the plasma and from selected lipoprotein fractions (patient only) of the subjects by extraction with chloroform/methanol 2:1 (v/v) followed by thin layer chromatography (TLC) using a neutral lipid system, as previously described (10). The total steryl ester fraction, which was well resolved from the triacylglycerols, was recovered by extracting the silica gel with chloroform. The phosphatidylcholines were recovered from the origin of the plate with chloroform/methanol/water/acetic acid (50:39:10:1, v/v/v/v) and were rechromatographed in a phospholipid solvent system (11). Very low (VLDL) and combined low (LDL) and high (HDL) density lipoproteins of the plasma from the initial visit were prepared as the top and bottom fractions, respectively, by ultracentrifugation at d = 1.006, as previously described (12).

A small aliquot of the total steryl ester fraction was saponified and the fatty acids were determined by gas chromatography/mass spectrometry (GC/MS) (8). Other aliquots of the total steryl ester fraction were subjected to direct GLC on polar capillary columns and to liquid chromatography (LC)/MS as described below. The rest of the ester fraction was resolved by argentation TLC into subfractions of uniform degree of unsaturation of the fatty acids, using chloroform/methanol 99:1 (v/v) as the developing solvent as described for triacylglycerols (14). The resolved fractions were recovered by extracting the gel with chloroform/methanol 2:1 (v/v) and were saved for subsequent examination by GLC and LC/MS.

The phosphatidylcholines were subjected to hydrol-

ysis with phospholipase A_2 and the positional distribution of fatty acids was determined as described (15).

GLC of steryl esters. Direct GLC of steryl esters was performed on glass capillary columns (10 m × 0.25 mm) coated with SP-2330 liquid phase (Supelco) as previously described for the TMS ethers of diacylglycerols (13). The columns were installed in a Hewlett-Packard model 5880A gas chromatograph equipped with a hydrogen flame ionization detector (Hewlett-Packard, Palo Alto, California). The carrier gas was H_2 at a head pressure of 8 psi. The column temperature was 250 C (isothermal); injector and detector temperatures were held at 270 C. The injections were made using a split ratio of ca. 10:1. The steryl ester peaks were identified by reference to standard esters of cholesterol, campesterol and β-sitosterol. Other GLC runs were made with the steryl ester fractions recovered from $AgNO_3$-TLC prefractionation.

LC/MS of steryl esters. HPLC analyses were performed with a Hewlett-Packard Model 1084B liquid chromatograph equipped with a Supelcosil LC-18 column (Supelco) using a gradient of 30–90% propionitrile in acetonitrile as the eluting solvent, as previously described (16–18). The columns were operated at a flow rate of 1.5 ml/min and 30 C oven temperature. About 1% of the column effluent was admitted to a Hewlett-Packard 5985B quadrupole mass spectrometer via a Hewlett-Packard direct liquid inlet interface. The data were recorded and analyzed by means of a Hewlett-Packard data system (Model HP 1000E) and graphics terminal (Model HP 2648A) as previously reported (17).

The mass spectra were limited to masses above 200 and were taken every seven sec over the entire elution profile. The contributions of the solvent peaks (e.g., m/z 252, 391) were removed by subtracting a scan made with propionitrile alone, where no steryl esters were being eluted.

GLC and GC/MS of sterols. Saturated and unsaturated sterol derivatives were resolved on a 15 m capillary column (Supelcowax 10, Supelco), while the 24α-methyl and 24β-methyl cholesterols were separated on a 25 m RSL-300 capillary column (supplied by Dr. P. Sandra) as described elsewhere (Myher, J.J., and Kuksis, A., unpublished results). In one instance the sterol fraction was subjected to oxidative destruction of the unsaturated sterols prior to GLC and GC/MS analysis (19). The cholesterol and plant sterol moieties of the steryl esters were resolved by GC/MS using a packed GLC column (30 m × 0.3 cm I.D.) containing 1% OV-liquid phase (8).

GLC of fatty acids. The fatty acids from the 2-position of phosphatidylcholine, from the total steryl esters and from the $AgNO_3$-TLC fractions were determined by polar capillary GLC (13) following methylation with methanolic 1N sodium methoxide or methanolic 6% sulfuric acid.

RESULTS

Identification of plant sterols. Prior to the resolution and determination of the mixed steryl esters, a detailed investigation was made of the composition of the sterol moieties. Figure 1 shows the sterol profile as obtained by chromatography on a Supelcowax 10 capillary column. When run as the acetates, the sterols are resolved according to molecular weight and degree of unsaturation. A mixture of the common dietary plant sterols (upper tracing) shows major peaks for campesterol (Peak 5), stigmasterol (Peak 6) and β-sitosterol (Peak 10), with smaller amounts of the corresponding β-hydroxy α-stanols. The sterols from the plasma of the patient obtained at the initial visit (lower tracing) show a large peak for cholesterol (Peak 2), smaller peaks for campesterol (Peak 5), β-sitosterol (Peak 10) and avenasterol (Peak 15). The campesterol peak was comprised of 60% campesterol (24α-methyl) and 40% 22,23-dihydrobrassicasterol (24β-methyl) (20). This mixture of sterols also contains readily detectable amounts of cholestanol (Peak 1), campestanol (Peak 4) and stigmastanol (Peak 9). The identity of these sterols was confirmed by GC/MS and LC/MS. In addition, there are small amounts of other sterols, with elution times corresponding to brassicasterol (Peak 3) and 24-methylenecholesterol (Peak 8).

Table 1 gives the quantitative estimates of the proportions of the sterols in the free sterol, the total

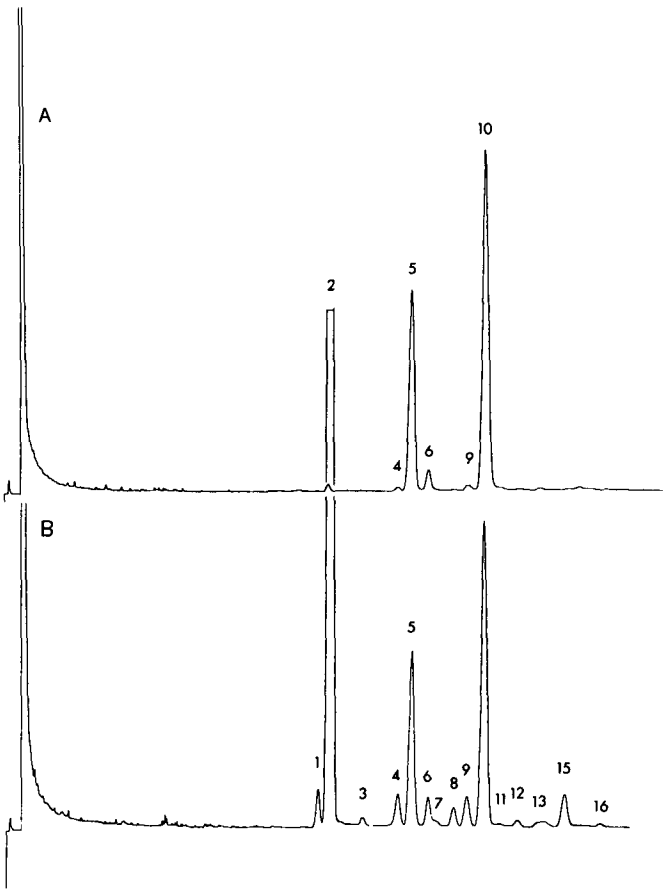

FIG. 1. GLC elution profile of sterol moieties of total steryl esters. Upper tracing, common dietary plant sterols; lower tracing, plasma steryl esters from a patient with phytosterolemia. Peak identification: 1, cholestanol; 2, cholesterol; 3, brassicasterol; 4, campestanol; 5, campesterol; 6, stigmasterol; 7, unknown; 8, 24-methylenecholesterol; 9, stigmastanol; 10, β-sitosterol; 11–14, unknown; 15, avenasterol; 16, unknown. Column: Supelcowax 10 on flexible quartz capillary column (15 m × 0.3 mm I.D.). Temperature: 250 C, isothermal. Carrier gas: H_2, 5 psi inlet pressure. Sample: steryl acetates. Instrument and other operating conditions as given elsewhere (Myher, J.J., and Kuksis, A., unpublished results).

steryl ester fraction and in the saturate + monoene, diene and tetraene subfractions obtained by argentation TLC. It is interesting to note that the stanols make up a much greater proportion of the total plant sterol than of the total cholesterol fraction. The proportion of plant sterols is higher in the free sterol (27.5%) than in the esterified sterol (19.6%) fraction. With the exception of stigmasterol, which is much lower or absent in the esterified sterol fraction, the overall composition of the plant sterols is very similar in both fractions. After three months on a diet low in plant sterols and cholesterol, the plasma sterol levels had decreased by about 50% without significant alteration in the relative proportions of the different sterol types. Following an additional three months, however, the plasma cholesterol and plant sterol levels had returned to the levels found for the initial visit sample: the patient had failed to adhere to the prescribed diet. The general composition of the total sterols is similar to that reported by Salen et al. (2,6) and by Miettinen (21), who also noted the presence of Δ-5 avenasterol in the plant sterols recovered from plasma of a patient with sitosterolemia.

Direct GLC of steryl esters. For the GLC examination of the intact steryl ester composition, the plasma lipoproteins of the initial visit sample were resolved into VLDL and LDL+HDL fractions. Figure 2 gives the steryl ester composition of the VLDL and the combined LDL+HDL fractions. There is a complete separation of the corresponding saturated and monounsaturated as well as of the di- and polyunsaturated fatty acid esters. From the polar liquid phase the polyunsaturated fatty acid esters are eluted last and suffer some losses. As a result, the contribution of the arachidonoyl species is probably underestimated. In addition to the peaks for the cholesteryl palmitate, oleate, linoleate and arachidonate, there are readily detectable peaks corresponding to campesteryl plus dihydrobrassicasteryl, and β-sitosteryl palmitate, oleate and linoleate. In addition, there are discernable elevations in the base line with retention times expected for the plant steryl arachidonates. On the SP-2330 liquid phase no resolution was obtained between the stanyl and stenyl esters or between saturated and unsaturated sterols either in the free form or in the form of their acetates or TMS ethers. Also, the fatty acid esters of campesterol and of dihydrobrassicasterol were not resolved. The identity of the plant steryl ester peaks was confirmed by GLC of the steryl ester bands recovered from argentation TLC (data not shown). The relative amounts of cholesterol, campesterol plus dihydrobrassicasterol and β-sitosterol esterified to palmitic, oleic and linoleic acids were about the same when assessed for the total steryl ester fraction of the initial visit sample. This agrees with the results of the analyses of the sterol moieties of the esters resolved by $AgNO_3$, as given in Table 1. Apparently the cholesteryl and plant steryl esters in phytosterolemia are derived from the same fatty acid pool. Table 2 gives the quantitative estimates for the various steryl esters in the initial sample of the patient's plasma. There are minor differences in the composition of the steryl esters between the two lipoprotein fractions. The VLDL fraction contains relatively more of esterified cholesterol than plant sterol when compared to the combined LDL+HDL fraction.

A comparison of the fatty acid composition of the 2-position of plasma total phosphatidylcholine with that of the cholesteryl and plant steryl esters shows that they are markedly different (data not shown). While the 2-position of the phosphatidylcholine contains palmitic, oleic and linoleic acids in the ratio of 1:4:11, respectively,

TABLE 1

Relative Quantitative Composition of Sterols in the Steryl Ester Fraction from Control (Total) and Phytosterolemia Plasma (LDL+HDL)

Peak no.	Sterols	Phytosterolemia[a] (mol %)					Control—Total (mol %)
		Free	Total	Saturated + monoenes	Dienes	Tetraenes	
1	Cholestanol	1.1	1.3	1.0	1.0	1.1	0.5
2	Cholesterol	71.4	79.1	81.4	80.2	78.0	98.6
3	Brassicasterol	0.3	0.2	0.1	0.2	0.2	
4	Campestanol	1.3	0.8	1.4	1.2	2.0	
5	Campesterol[b]	7.0	5.9	5.3	5.6	5.9	0.4
6	Stigmasterol	1.2	0.1	0.1	0.1	0.2	
7	Unknown	0.2	0.1	0.1	0.2	0.2	
8	24-Methylene cholesterol	0.8	0.5	0.4	0.2	0.3	
9	Stigmastanol	1.4	0.7	0.7	0.8	0.8	
10	β-sitosterol	13.1	9.5	8.0	9.1	10.2	0.5
11	Unknown						
12	Unknown	0.3	0.3	0.2	0.2	0.3	
13	Unknown	0.2	0.2	0.1	0.1	0.2	
14	Unknown	0.2	0.2	0.2	0.1	0.2	
15	Avenasterol	1.5	1.0	1.0	1.0	1.1	
16	Unknown	0.2	0.1	0.1	0.1	0.1	

Sterols were resolved and identified as acetates using a polar capillary GLC column as described elsewhere (Myher, J.J., and Kuksis, A., unpublished results).

[a]Plasma sample from initial visit.

[b]60% Campesterol (24α-methyl) and 40% 22,23-dihydrobrassicasterol (24β-methyl).

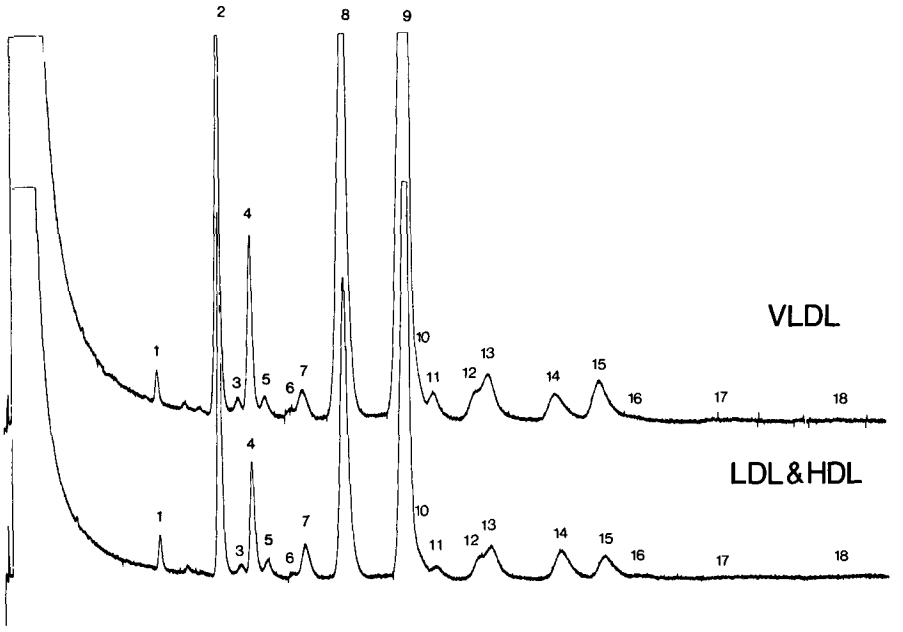

FIG. 2. GLC elution profile of steryl esters of VLDL (upper tracing) and LDL+HDL (lower tracing) fractions of plasma from a patient with phytosterolemia. Peak identification: 1, cholesteryl myristate; 2, cholesteryl palmitate; 3, unknown; 4, cholesteryl palmitoleate; 5, campesterol palmitate; 6, unknown; 7, β-sitosterol palmitate + cholesteryl stearate; 8, cholesteryl oleate; 9, cholesteryl linoleate; 10, campesteryl oleate; 11, unknown; 12, β-sitosteryl oleate; 13, campesteryl linoleate; 14, β-sitosteryl oleate; 15, cholesteryl arachidonate; 16, unknown; 17, campesteryl arachidonate; 18, β-sitosteryl arachidonate. Column SP-2330 on glass capillary column (10 m × 0.25 mm I.D.). Temperature 250 C, isothermal. Carrier gas: H_2, 8 psi inlet pressure. Sample: intact steryl esters. Instrument and other operating conditions as given elsewhere (13).

TABLE 2

Quantitative Composition of Steryl Esters of the VLDL and LDL+HDL Fractions of Plasma from a Patient with Phytosterolemia as Estimated by GLC on Polar Capillary Columns

Peak no.	Steryl esters	Total	Lipoproteins (mol %)	
			VLDL	LDL+HDL
1	Chol.14:0	0.8	0.6	0.9
2	Chol. 16:0	12.9	11.8	13.6
3	Chol. 16:1(n-9)		0.5	0.6
4	Chol. 16:1(n-7)	4.6	5.0	4.9
5	Camp. 16:0	0.7	0.6	1.1
6	Unknown		0.3	0.4
7	Sito. 16:0 + Chol. 18:0	1.9	1.0	2.2
8	Chol. 18:1	23.3	25.3	19.3
9	Chol. 18:2	41.6		
10	Camp. 18:1		44.0	36.5
11	Unknown			
12	Sito. 18:1	1.3	1.3	3.1
13	Camp. 18:2	2.0	1.6	3.8
14	Sito. 18:2	4.2	1.8	5.7
15	Chol. 20:4	3.2	2.9	4.9
16	Chol. 20:3?		0.3	1.0
17	Camp. 20:4	tr	0.3	0.5
18	Sito. 20:4	tr	ND	0.4
Other			2.7	1.0

tr, Trace; ND, not determined. Steryl esters were resolved intact on a polar capillary GLC column as previously described for TMS ethers of natural diacylglycerols (13). Plasma sample from initial visit.

the corresponding ratios of the acids in the esters of cholesterol and plant sterols are 1:2:5. Clearly, the steryl esters contain much more of the palmitate and oleate and less of the linoleate than is available in the 2-position of the corresponding plasma phosphatidylcholine. Comparable differences were seen between the fatty acids of the 2-position of the phosphatidylcholine and the steryl esters of the LDL+HDL fraction of the plasma from this patient. However, similar differences were found between the fatty acid composition of the 2-position of plasma phosphatidylcholine and the cholesteryl esters of control subjects (data not shown). The steryl ester composition also differed from that of the plasma free fatty acids, which was characterized by a high proportion of stearic acid.

LC/MS of steryl esters. Figure 3 shows the HPLC elution pattern obtained for the steryl esters of the LDL+HDL fraction of the plasma from the initial visit of the patient along with the single ion plots for the steroid nuclei of the stenyl esters. From the reversed phase HPLC column the polyunsaturated steryl esters are eluted first and the less polar saturated esters last. As a result the polyenoic species of both cholesterol and plant sterols are recovered from the HPLC column in

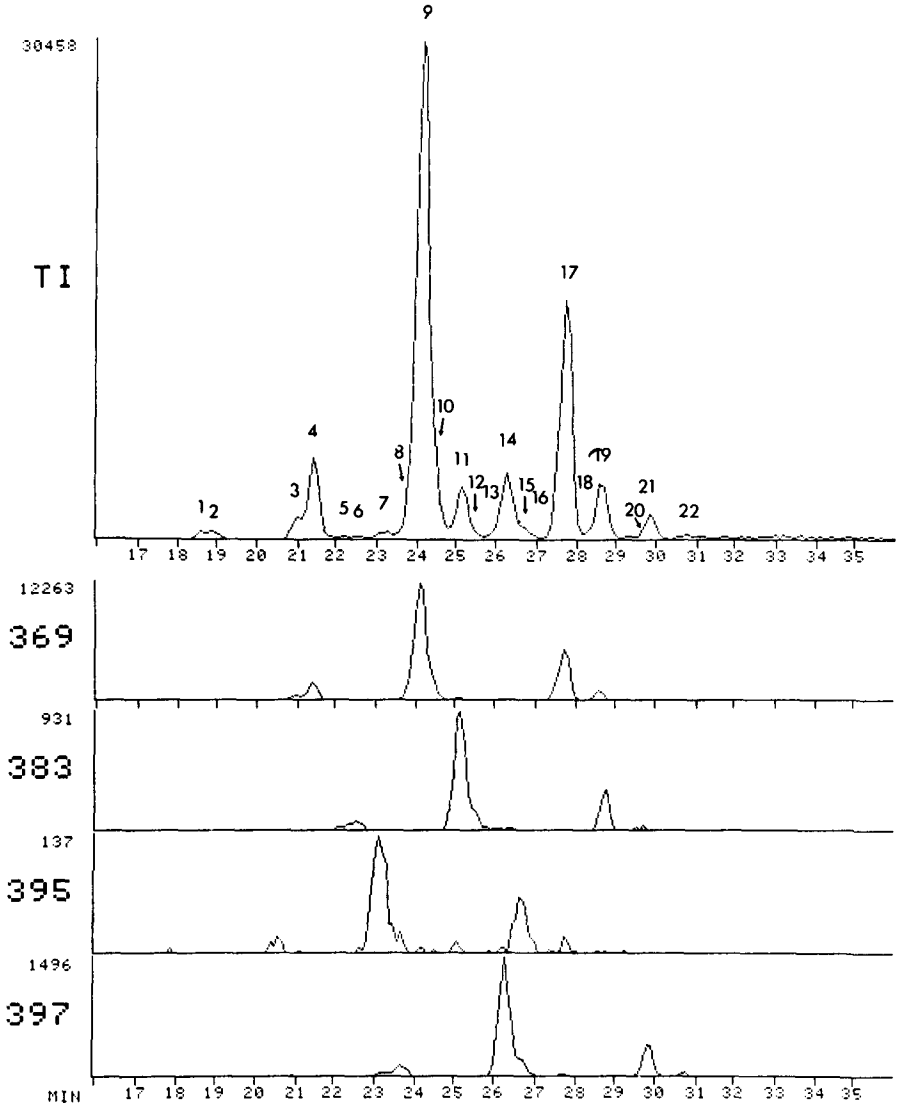

FIG. 3. LC/MS elution profile of steryl esters of the LDL+HDL fraction of plasma from a patient with phytosterolemia. TI, total ion current; 369, 383, 395 and 397, fragment ions representing the steroid nuclei of cholesterol, campesterol, stigmasterol and avenasterol, and β-sitosterol. Peak identification: 1, cholesteryl docosahexaenoate; 2, cholesteryl eicosapentaenoate; 3, cholesteryl linoleate; 4, cholesteryl arachidonate; 5, unknown; 7, avenasteryl linoleate; 8, β-sitosteryl arachidonate; 9, cholesteryl linoleate; 10, cholesteryl palmitoleate; 11, campesteryl linoleate; 12 and 13, unknown; 14, β-sitosteryl oleate; 15, avenasteryl oleate; 16, unknown; 17, cholesteryl oleate; 18, unknown; 19, cholesteryl palmitate; 20, unknown; 21, β-sitosteryl oleate; 22, unknown. Column: reversed phase Supelcosil C_{18} (25 cm × 0.25 I.D.). Eluent: a linear gradient of 30–90% propionitrile in acetonitrile. Instrument and other operating conditions as given in text.

somewhat higher proportions than from the polar capillary GLC columns. The chromatogram contains peaks for cholesteryl arachidonate (Peak 4), linoleate (Peak 9), oleate (Peak 17) and palmitate (Peak 19), which are seen in the control plasma, as well as readily detectable peaks for the linoleates, oleates and palmitates of campesterol and β-sitosterol, and smaller peaks for the minor esters of cholesterol and plant sterols. These peaks were identified from the relative retention times of standards and the identities were confirmed by MS. The parent ions were present in low yields, but in many instances could also be used to establish the identity of the components in a mixed steryl ester fraction. The distribution of the steryl esters is indicated by the ions characteristic of the steroid nuclei, e.g., m/z 369 (cholesterol), 383 (campesterol + dihydrobrassicasterol), 395 (stigmasterol + avenasterol) and 397 (β-sitosterol).

The LC/MS system also allowed an examination of each steryl ester peak for the presence of the corresponding 5α-stanyl esters. The presence of cholestanol (m/z 371) and stigmastanol (m/z 399) could be discerned for each of the major ester peaks at its characteristic retention time (results not shown). The fragment ions of the stanols possess the same mass as the Parent+2 ions of their unsaturated homologues and must be clearly differentiated from them on the basis of the retention times of the steryl ester peaks. The LC/MS results confirmed the presence of both saturated and unsaturated plant sterols in the various fatty acid classes as observed from GLC analyses of the sterols in the $AgNO_3$-TLC fractions of the intact steryl esters (see above). In control plasma only small amounts of the saturated steryl esters were found (0.4% of total). In contrast, substantial amounts of the 5α-saturated stanols were present in the phytosterolemia plasma. The LC/MS approach did not allow differentiation between the fatty acid esters of campesterol and its isomer 22,23-dihydrobrassicasterol, which were eluted with similar retention times and possessed very similar chemical ionization mass spectra. The relative contributions of the saturated and unsaturated species of each sterol type to the total amount of the major fatty acid esters are given in Table 1. The proportions obtained for the esters of the unsaturated sterols by LC/MS and polar capillary GLC were similar, except for the palmitates of the plant sterols, which apparently were underestimated by LC/MS. The proportions of the esterified stanols were obtained by LC/MS only. The estimated ratios of the oleates and linoleates of cholestanol, campestanol and its 24β-methyl isomer, and stigmastanol were similar to those obtained for the esters of the corresponding unsaturated sterols by GLC and LC/MS.

DISCUSSION

Resolution of intact steryl esters. The separation of intact steryl esters by GLC and HPLC avoids the need for saponification of the sample and the analysis of fatty acids, which results in losses of the polyunsaturates and may lead to contamination with fatty acids from other sources. The GLC separations on the polar capillary columns are similar to those obtained for the fatty acid methyl esters. Previously Smith (22) resolved intact cholesteryl esters on a 10 m SP 2330 fused-silica column using helium as a carrier gas. The use of hydrogen as the carrier resulted in a partial hydrogenation of the sample on the fused silica capillary column (23). We did not observe any reduction of the double bonds in either the sterol or the fatty acid part of the ester employing glass capillary columns coated with SP 2330 and hydrogen as the carrier gas. Although the method requires less material than conventional analyses, the advantages are minimal for the determination of the fatty acid esters of a single species of sterol. The resolution of intact molecules is essential for the identification of the fatty acid esters of mixed sterols.

HPLC provides an alternative method for the resolution of intact steryl esters. Again the separations of the esters of a single sterol moiety offer marginal advantages over analyses of the released fatty acids, but resolution of intact esters is indispensible for the determination of fatty acid esters of mixed sterols. We have previously used the LC/MS system for the separation of the fatty esters of cholesterol in normal plasma (16) and of campesterol and β-sitosterol found together in vegetable oils (17–18). The method allows an effective recovery and identification of the saturated and unsaturated fatty acid esters of all sterols, but certain critical pairs of steryl esters tend to overlap (e.g., cholesteryl linoleate and cholesteryl palmitoleate). Likewise, certain critical combinations of cholesteryl and β-sitosteryl esters tend to overlap (e.g., cholesteryl palmitate and campesteryl oleate). These esters were identified by an MS examination of the appropriate steryl ester peaks. Some of the overlaps could be avoided if the steryl esters were subjected to argentation TLC prior to the LC/MS run. The present study provides the first direct evidence of the fatty acid composition of the stanol and of the plant sterol fraction of the plasma steryl esters in phytosterolemia.

Origin of steryl esters in phytosterolemia. The results of this study confirm and extend our knowledge regarding the biochemical defects in phytosterolemia and xanthomatosis. About 60% of the plasma plant sterols was found to be esterified, which is slightly less than the 75% level of esterification commonly found for plasma cholesterol (24). The campesterol (24α-methyl) and the 22,23-dihydrobrassicasterol (24β-methyl) homologues of cholesterol were esterified in the proportion in which they occurred in the free sterol fraction of the plasma, but stigmasterol remained largely in the free form. The present study demonstrates that both cholesterol and plant sterols are esterified to fatty acids of about the same composition. This fatty acid composition does not correspond exactly to that of the 2-position of plasma phosphatidylcholine, although certain similarities are obvious (predominantly 18:2). The fatty acid composition of the steryl esters of the patient plasma is typical of that of normal subjects (12) as is the fatty acid composition of the 2-position of phosphatidylcholine (25). In the past these superficial similarities in the fatty acid patterns have been accepted as evidence of formation of plasma steryl esters via the lecithin-cholesterol acyltransferase (24). Therefore, the action of lecithin-cholesterol acyltransferase could have accounted for the composition of the steryl esters. Bhattacharya and Connor (1) have suggested this enzyme as the cause of the plant steryl esters in plasma. A possible involvement of the lecithin-cholesterol

acyltransferase would have been anticipated from the demonstration that lecithin-cholesterol acyltransferase is capable of esterifying cholestanol (26) and plant sterols (27) in vitro, although at a significantly slower rate than cholesterol. Since campesterol and β-sitosterol are known to appear in lymph largely in the free form (28), the possibility of absorption of intact steryl esters as well as their intestinal biosynthesis would appear to be excluded as a potential source of discrepancy. In keeping with a minimal contribution of steryl esters from the intestine is the finding of a significantly higher proportion of the free sterols in the VLDL fraction in comparison to the LDL+HDL fraction. However, there are good reasons for considering the acylCoA-cholesterol acyltransferase of tissues as a likely additional source of plasma steryl esters (29). This enzyme is present in the liver, which has been shown to take up plant sterols and return them to plasma as part of lipoproteins (30). The acylCoA-cholesterol acyltransferase of liver is known to yield largely the palmitoyl and oleoyl species of cholesterol esters (31) and therefore could account for the observed excess of these esters in the plasma of the patient with phytosterolemia as well as in the plasma of normal subjects.

Cholestanol is present in normal plasma at very low levels (3,6) as are campestanol and stigmastanol in the common dietary fats and oils (18). Salen et al. (32) have reported that cholestanol is formed from cholesterol via the ketonic intermediate, 4-cholesten-3-one, and that the liver microsomes from subjects with cerebrotendinous xanthomatosis reduce it much more readily than do livers from control subjects. In such a case, the plant sterols could have been taken up by the liver and both reduced and esterified there along with the cholesterol. This would have been expected to result in fatty acid composition enriched in palmitic and oleic acids. The present study, however, shows that the stanols as well as the plant sterols were esterified to linoleic and oleic acids in about the same proportion as the other sterols. Hence the lecithin-cholesterol acyltransferase and possibly the acylCoA-cholesterol acyltransferases must have contributed in about the same relative proportion to the formation of both stanyl and stenyl esters of plasma in phytosterolemia.

ACKNOWLEDGMENTS

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Serum Lipid Abnormalities in a Chemical/Viral Mouse Model for Reye's Syndrome

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Neonatal mice given nontoxic dermal applications of an industrial surfactant, Toximul MP8 (Tox), and subsequently infected with sublethal doses of mouse-adapted human Influenza B (Lee) virus (FluB) develop many of the biochemical features of Reye's Syndrome (RS). To determine whether these also include abnormal circulating lipid, we examined serum lipid profiles in the mouse model throughout the treatment course using Iatroscan-TH10. Following 10 days of exposure to surfactant, serum phospholipid and cholesterol levels were significantly reduced relative to control animals. These reductions were transient; however, four days following virus administration, significant differences in serum lipid were again evident. These abnormalities coincided and correlated with increased animal mortality. Animals that received combined Tox + virus treatment had significant decreases in serum total lipids relative to control animals, a reflection of a reduction in all lipid classes, including phospholipid, cholesterol, neutral glycerides (triglycerides plus diglycerides) and free fatty acids. Phospholipid (specifically phosphatidylcholine and lysophosphatidylcholine) and free fatty acid levels in the Tox + virus group were also significantly lower than those in animals that received virus alone. This study has demonstrated that suckling mice given chemical/viral treatment have the serum hypopanlipidemia but not the freefattyacidemia that are characteristic of RS.
***Lipids 21*, 378-382 (1986).**

Reye's Syndrome (RS), a rare but frequently fatal disease of childhood, is generally associated with several distinct clinical features, including serum freefattyacidemia (1-4) in the presence of hypopanlipidemia (1), fatty infiltration of the visera, particularly the liver (5), and severe, nonspecific cerebral encephalopathy (6). It is believed that the initial insult in RS is a loss of hormonal regulation of adipose tissue lipolysis (7). Free fatty acids are released into the circulation and can be taken up by the liver in a concentration-dependent manner (8), with the result that normal routes of metabolism (e.g., complex lipid synthesis, β-oxidation) may become overburdened. Aberrations of hepatic lipid metabolism, which are evident from increased levels of nonesterified short chain fatty acids (2) and/or synthesis of dicarboxylic acids (9), may be related to the development of cerebral edema, the major cause of death in RS patients.

Several animal models have been developed in an attempt to elucidate the etiology of RS; however, only a few investigations have concerned the associated abnormalities in lipid metabolism (10-14). Much of the information from these studies has been difficult to interpret, in part because lipid changes were examined at single time points, although we know from clinical data that in RS both serum (2) and hepatic (15) lipid changes are transient in nature.

We have developed a chemical/viral mouse model for RS that has many of the biochemical (16), morphological (16) and immunological (17) features of the human disease, and have now examined the time course of changes in serum lipids in our mouse model. We report here that mice exposed to nontoxic doses of industrial surfactant (Toximul MP8 [Tox]) and subsequently infected with sublethal doses of mouse-adapted Influenza B (Lee) virus (FluB) have serum lipid profiles that are consistent in some but not all respects with those seen in RS.

EXPERIMENTAL PROCEDURES

Chemical/viral mouse model. Newborn Swiss White CFW mice (bred from animals obtained from Canadian Biobreeding Farms, Quebec City, Quebec, Canada) were pooled at 24 hr of age and randomly assigned to one of four groups (ca. 84 animals/group) and given to nursing females (seven pups/mother). Each mother and her pups were housed in a separate cage. Twenty-four hours later, corn oil was applied dermally to abdomens of half of the mice (controls); the other half received Tox (Charles Tennant & Co., Toronto, Ontario, Canada, lot #9-30162), 1% dissolved in corn oil. This procedure was continued daily for 10 days. For the first five days, 8.6 $\pm$ 2.6 mg of Tox (in solution) per mouse was applied by painting the animals with a small brush; during the last five days the dose was increased to 25.8 $\pm$ 9.0 mg per animal. Details regarding this procedure have been reported elsewhere (16). On day 13 of the experiment, mouse-adapted human FluB was administered intranasally under light ether anesthetic to half of each of the control and Tox groups; the other half of each group received only anesthetic. The experiment continued with four treatment groups: controls, Tox alone, virus alone and Tox + virus. Infected animals (and their mothers) were housed in a level B containment facility; those that were not infected were kept in the regular animal care quarters. Deaths were recorded each morning throughout the experiment. On days 1, 12, 15 and 17, six live animals were removed from each group, the mice were sacrificed by decapitation and blood was collected for lipid analysis. Each sample was coded, and the samples were analyzed at random before being decoded. Due to the sensitivity of the Iatroscan method (18), it was possible to quantitatively analyze the lipid-class profiles from individual serum samples.

Lipid extraction and analysis. The procedure for lipid separation and Iatroscan analysis have been described in detail elsewhere (18). Briefly, lipids were extracted from individual serum samples (ca. 50 μl serum from each animal) with chloroform/methanol (C/M; 2:1, v/v), essentially as described by Folch et al. (19). Following

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extraction, lipids were suspended in chloroform, spotted on silica-coated quartz rods (Chromarod-S: Newman-Howell Assoc. Ltd., Winchester, United Kingdom; Canadian suppliers, Technical Marketing Assoc., Halifax, Nova Scotia, Canada), and after being equilibrated over saturated NaCl at 30.5% humidity for 10 min, were separated using the following sequence of solvent systems: (i) C/M/H_2O (65:35:4, v/v/v; 15 min); (ii) C/M/H_2O (65:35:4, v/v/v; 15 min); and (iii) hexane/diethyl ether/formic acid (98:2:1, v/v/v; 58 min). Rods were oven-dried (105 C) for 3 min and rehumidified over an NaCl solution for 10 min between changes in solvent systems. After the final development, the rods were passed through the Iatroscan-TH10 (Newman-Howells Assoc. Ltd.) with an air flow rate of 2000 ml/min and H_2 pressure of 0.8 kg/cm^2. Each serum sample was run in quintuplicate; the mean variability between analyses for each sample was 12.2 ± 1.7%. (For a detailed discussion of the reproducibility of the Iatroscan analysis, see ref. 20.) Individual lipids were identified and quantitated by comparison with authentic standards run simultaneously. A Spectra Physics 4200 computing Integrator (Technical Marketing Assoc.) was used for quantitation.

Statistical analysis. Data represent the means of values obtained from a minimum of three animals. Statistical analysis was carried out using one-way analysis of variance, and differences were accepted as significant when $p < 0.05$.

RESULTS

As demonstrated previously (16), dermal application of nontoxic doses of Tox to neonatal mice followed by infection with sublethal doses of mouse-adapted human FluB results in a significantly higher rate of mortality than that observed with virus infection alone (Fig. 1). Increases in mortality in the groups treated with either virus alone or with Tox + virus were first evident at day 17, four days following administration of virus. Two days later, the mortality rates had climbed to 60% and 90% in the groups treated with virus alone and Tox + virus, respectively. There were no deaths among the mice that received only Tox (and anesthetic). The relatively high incidence of mortality (4/33 animals) in the control group was very unusual; in most experiments there are no deaths in either control animals or in those that receive Tox alone.

The effects of the four treatment courses on total serum lipids are shown in Figure 2. Examination of the lipid profiles on day 12 demonstrated that the animals that had received Tox topically for 10 days had significantly reduced serum lipid content; this was due specifically to decreases in the phospholipid and cholesterol fractions. Reductions in these fractions (relative to control) were 48.5% and 36.5%, respectively (Figs. 3A and 3B). Levels of free fatty acid (FFA) and neutral glyceride did not appear to be affected by exposure to Tox.

On day 15, two days following viral infection, there were no differences in either total serum lipids (Fig. 2) or in any of the individual lipid classes (Fig. 3). Interestingly, FFA levels increased at this time in the controls and in all treatment groups (Fig. 3D).

Significant differences in lipid profiles again became apparent on day 17, the day when the mortality rates in the

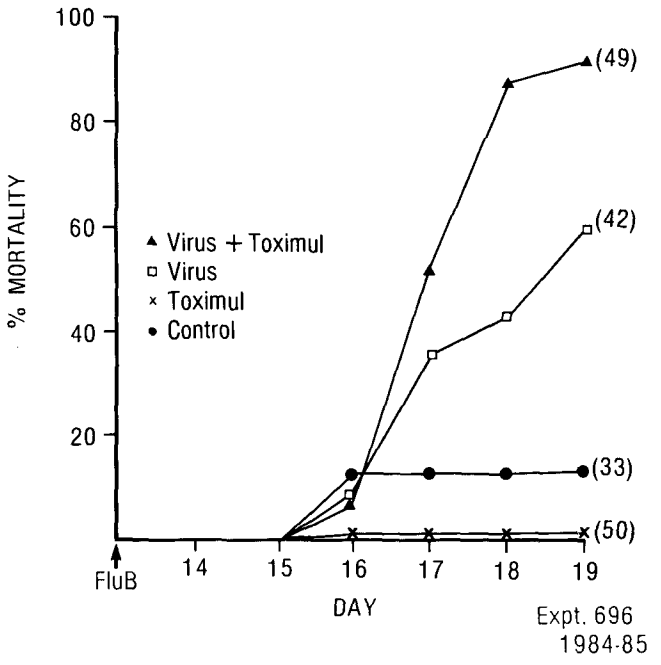

FIG. 1. Cumulative mortality rate in the mouse model for RS. Forty-eight-hr-old mice received daily abdominal applications of corn oil or Toximul (in corn oil) for 10 days. On day 13, half of the mice from each group received intranasal inoculations of Influenza B (Lee) under light ether anesthesia. The other half received anesthetic alone. Deaths were recorded daily; data are expressed as the percentage of mice that had died relative to the number alive in each of the four treatment groups 24 hr following administration of anesthetic (day 14). Numbers in parentheses refer to the total number of animals in each group at day 14.

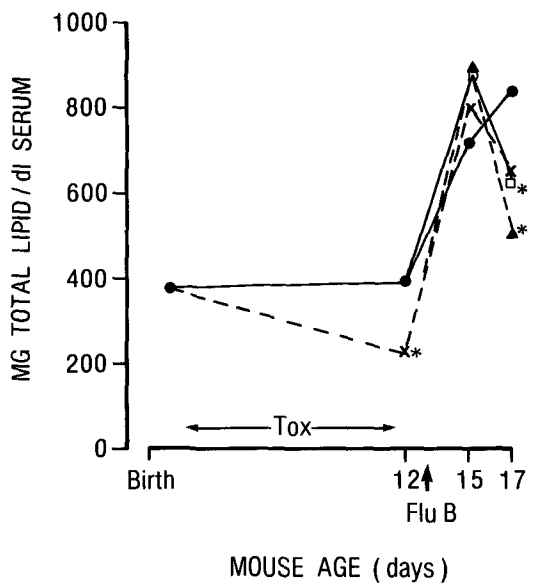

FIG. 2. Changes in serum total lipid content. Live mice were removed from each treatment group and decapitated, and the serum was extracted and analyzed for lipid content as described in the text. Data represent the sum (mg/dl serum) of phospholipid, cholesterol (cholesterol esters plus free cholesterol), neutral glycerides and free fatty acids from at least three animals. ▲, Virus + Toximul; □, virus; ×, Toximul; •, control. *$p < 0.05$ relative to control values.

groups treated with virus and Tox + virus began to increase. In the animals that received Tox + virus, total lipid content was significantly lower relative to that of control animals (59.0 ± 7.0% of control values) (Fig. 2). This reflected significant decreases in all lipid classes relative to control animals (Fig. 3). The ratios of cholesterol esters to free cholesterol (ca. 4:1) did not differ significantly between treatment groups (not shown). Of particular interest was the observation that levels of phospholipid and free fatty acid differed significantly between the animals treated with Tox + virus and those that had received only virus.

Relatively few investigators have examined the effects of viral infection on mouse serum lipids. It is clear from this study that infection of young mice with mouse-adapted human FluB by itself leads to significant reductions in circulating FFA and neutral glycerides (Figs. 3C and 3D).

During the Iatroscan analysis, phospholipids were separated into phosphatidylethanolamine (PE) + phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SPH) and lysophosphatidylcholine (lyso PC). We compared phospholipid class profiles of serum from groups receiving Tox + virus and virus alone to determine whether the significant difference in phospholipid levels between the two was due to a change in one or more specific phospholipid classes. Figure 4 demonstrates that the reduction in phospholipid in the animals that received the combined Tox + virus treatment was due specifically to significant decreases in the PC and lyso PC fractions.

DISCUSSION

The development of abnormalities in serum lipids generally associated with RS cannot be studied ideally in RS patients, since the hypopanlipidemia (reduction in total lipid) with hyperfreefattyacidemia occurs either before and/or during the antecedent viral illness, that is, before the earliest symptoms of the disease are apparent. Several animal models have been developed to study the metabolic defects underlying RS. However, we believe

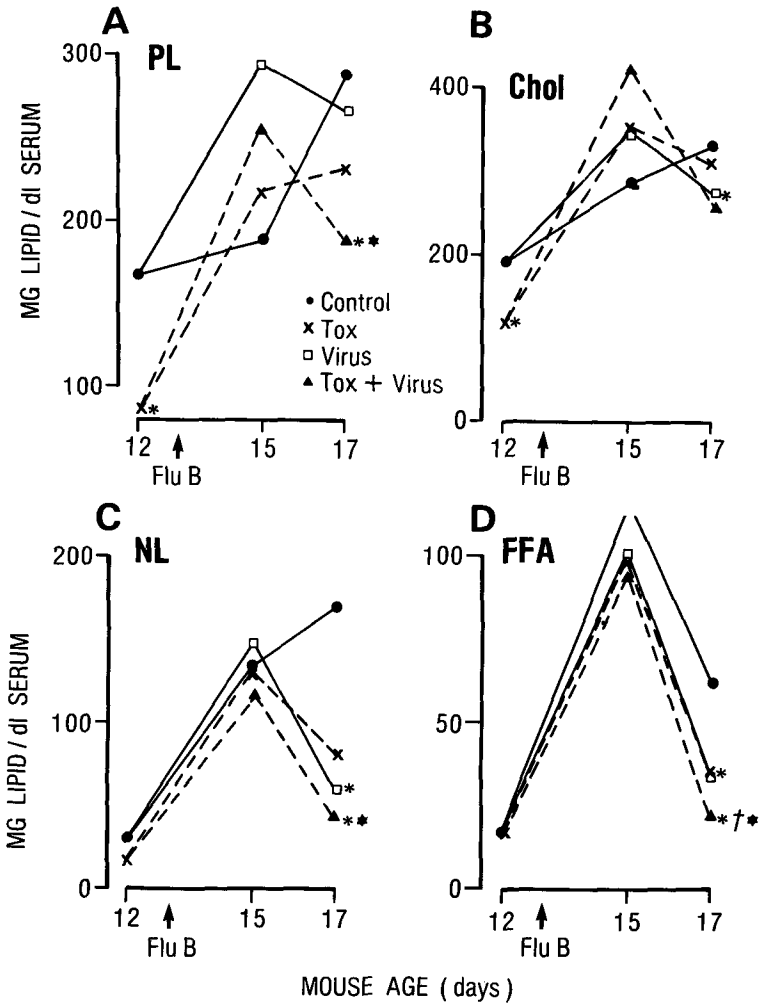

FIG. 3. Serum profiles for four lipid classes in the mouse model for RS. Data represent means of values obtained from at least three mice, and demonstrate changes in serum content of (A) total phospholipid (PL); (B) total cholesterol (Chol); (C) neutral glyceride (triglyceride + diglyceride) (NL); (D) free fatty acid (FFA). *$p < 0.05$, relative to control; †$p < 0.05$, relative to values in Tox-treated animals; ★$p < 0.05$, relative to virus-treated animals.

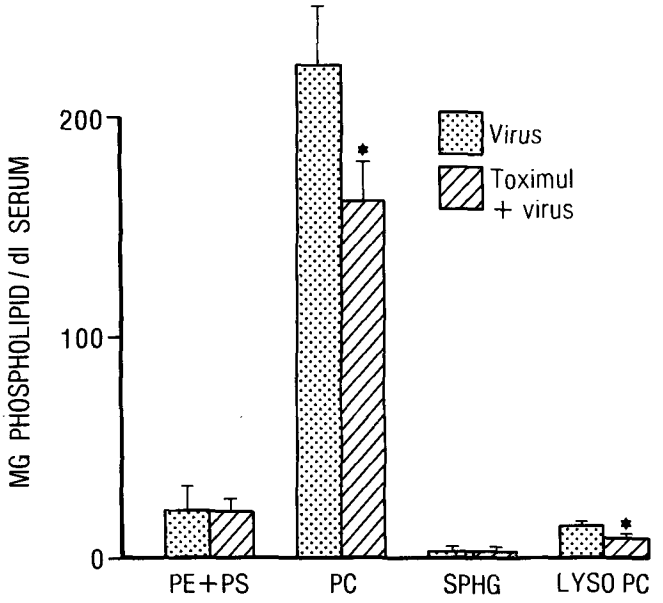

FIG. 4. Phospholipid class profiles in mice treated with FluB compared to those treated with Tox + FluB. The data represent the means (±SE) of the amount (mg/dl serum) of each phospholipid in the sera at day 17. ★$p < 0.05$ relative to values obtained in virus-treated animals.

that most are inappropriate for studies of the lipid abnormalities associated with this disorder. Several models are based upon injection of either short or long chain fatty acids (21,22) and, as such, are studies of the effects of hyperfreefattyacidemia and not of lipid pathogenesis. Other investigators have demonstrated elevated serum FFA and liver lipids in ferrets fed arginine-deficient diets or injected with jackbean urease (13), in monkeys fed aflatoxin B_1 (10) and in rats injected with *E. coli* endotoxin (14). However, none of these models involved a viral infection, and we know that prodromal infection is a consistent feature of almost all (>85%) cases of RS (23). Studies with models that have included viral infection, either with (24,25) or without (26,27) an associated toxin, have limited their observations to hepatic lipid changes based on either histological examination or total lipid determination. Ours is the first study to examine extensively the time course of serum lipid changes in an animal model.

The data in Figure 3 clearly demonstrate that serum lipid profiles in the experimental mice were abnormal at at least two distinct points during the treatment course; the first followed 10 days of exposure to Tox and before viral infection (day 12), and the second was when the mortality rate first began to increase (day 17). Ten days of topical application of Tox resulted in significant reduction in total serum lipid content, a reflection of specific reductions in the cholesterol (Fig. 3B) and phospholipid (Fig. 3A) components. Preinfection changes were of particular interest to us, since the results of several studies have demonstrated that alterations in cellular lipids, which could be accompanied by concomitant reductions in circulating lipid, can alter susceptibility to viral infection, as well as viral maturation and virulence (31,32). Interestingly, hepatic cholesterol is elevated in the Tox-treated mice on day 12 (unpublished observations). The Tox-associated decreases in serum phospholipid content observed in the mice have also been seen in macaque monkeys fed aflatoxin B_1 that had been isolated from a sample of rice, some of which had been eaten by a Thai child who had died of RS (10). Bourgeois et al. (10) suggested that this reduction may have been due to failure of hepatic lipid transport. Whether or not this is the case in our Tox-treated mice is not clear at present. At least one alternative explanation for losses in circulating phospholipid is a toxin-stimulated increase in lipid peroxidation (33). This deleterious process, which has been implicated in the etiology of RS (34), could account for much of the cellular damage seen in the disease, and may be related to enhanced viral lethality and/or development of cerebral encephalopathy. Recently, we obtained preliminary evidence of peroxidation products in serum samples from a patient with RS (not shown).

The second period during the course of chemical/viral treatment when abnormalities in serum lipid profiles were once again apparent was four days following viral infection, the time when mortality rates in the animals receiving virus and Tox + virus began to increase (Fig. 1). Significant decreases were observed in all classes of lipids in the sera of mice that received the combined Tox + virus treatment, whereas reductions in the virus-treated animals were limited to the neutral glyceride and FFA fractions (Fig. 3). In an earlier human study, Pollack et al. (1) found that the mean content of serum total lipid was significantly reduced in 22 patients with RS. Unlike the situation with the mice, human hypopanlipidemia was due to a selective loss in the cholesterol fraction. Significant reductions in this fraction were also observed by Chaves-Carballo et al. (15). These authors reported that low and high density lipoprotein cholesterol were particularly low in RS sera, and continued to decline in patients who eventually did not survive. This pattern was evident even when a poor outcome was not expected clinically; accordingly, they suggested that low serum cholesterol could be of prognostic value in identifying high risk patients.

One feature of the mouse serum data which appears to distinguish this animal model from RS patients is the absence of hyperfreefattyacidemia. High levels of circulating nonesterified fatty acid (1,2) with disproportionate increases in short, medium and/or long chain acids (3,4,28) have in the past been considered a classic feature of RS. However, close scrutiny of the literature data raises several questions regarding the significance of elevated FFA. First, Ogburn et al. (28) demonstrated that among patients who were admitted to the hospital with a diagnosis of RS, the mean initial levels of serum FFA (i.e., before exchange transfusion) were significantly lower in those who did not survive than in those who did. Furthermore, in three out of four of the nonsurvivors, values obtained at death were also very low. These authors did not present serum data from control individuals. This raises the question of the choice of appropriate controls for the evaluation of RS serum data. Viral infections are known to affect lipid metabolism (29-31), and yet "control" serum samples have always been taken from either healthy age-matched children (3) or from age-matched children hospitalized for reasons other than viral illness (1). We suggest that more appropriate control values would be those obtained from patients with influenza infections.

There are several possible explanations for the absence

of hyperfreefattyacidemia in the mouse model. First, it is generally presumed that elevated serum FFA is a consequence of fatty acid mobilization from adipose tissue due to a stimulation in adipocyte lipolysis (7). A net increase in lipolysis (resulting from either an increase in hormone-sensitive lipase activity or a decrease in lipoprotein lipase activity) may require specific virus/host combinations. The mouse-adapted FluB (which is not a native mouse pathogen) used in this study may not be appropriate for reproducing this particular feature of the human disease in mice. On the other hand, serum lipids in mice are very susceptible to genetic variation (36,37), and the effects of experimental insults (e.g., viral infection, Tox + virus treatment) on these profiles almost certainly depend on genetic disposition. Cuendet et al. (38) demonstrated that obese mice are able to mobilize fat to meet caloric demands during times of stress and have a better ability to survive experimental insult than do lean animals. Genetic factors are presumed also to play a role in the susceptibility of children to RS; however, whether there is a relationship between body fat status and RS has not to our knowledge been examined.

As a final point, mention should be made of the nutritional status of the mice during the course of chemical/viral treatment. Animals that received combined Tox + virus treatment were clearly more ill than those in any other group, and it would not be unexpected if these animals were in a fasting state. However, the body weights in these animals were not significantly lower than those in the other treatment groups. Moreover, upon autopsy, all mice were found to have milk-laden stomachs (the animals were breastfed throughout the experiment), which suggests that the Tox-dependent viral enhancement observed in the mouse model cannot be attributed solely to effects on feeding status.

In summary, the chemical/viral treatment course followed in our mouse model for RS reproduces some but not all of the serum lipid changes seen in the human disease. Perhaps one most significant observation is that reductions in serum cholesterol, which appear to be consistent and potentially important features of RS, appear in the mice even before the animals are infected with virus. The mechanisms leading to this and other lipid alterations are not yet known, nor are the roles of these changes in the etiology and outcome of RS. Only through studies of the evolution of metabolic abnormalities will we develop an understanding of this very complex disease process.

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Autoxidation of Cholesterol in Tallows Heated under Deep Frying Conditions: Evaluation of Oxysterols by GLC and TLC-FID

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The products of cholesterol autoxidation (oxysterols) in heated animal food fat were determined qualitatively and quantitatively to evaluate their toxicity and those of the foods in which they occur. Samples of beef tallow were taken from deep-fat fryers while they were in use. The oxysterols were identified and assayed by gas liquid chromatography and thin layer chromatography on Chromarods with flame ionization detection (TLC-FID). The two methods were compared and the TLC-FID method was found more convenient for a rapid estimation of autoxidation. Of the original cholesterol, 25% was destroyed during cooking and partly transformed into 3β-5-6β-trihydroxy-5α-cholestane, 7α-hydroxy-, 7β-hydroxy-, 7-oxo-cholesterol, 7-oxo-cholesta-3-5-diene and cholesterol epoxides. Certain other oxysterols were present in smaller quantities.

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Although the toxicity for humans of the predominant oxysterols (OS) (Fig. 1) which form rapidly by heating free (1) or esterified cholesterol (CHOL) (2) has not been clearly established (3), these compounds are considered to be potentially atherogenic. Some of their biological properties, including cytotoxic (4,5), mutagenic (6,7) and enzyme-inhibiting capacities (8), can be directly or indirectly related to atherogenesis (9,10). An atherogenic effect on an animal species (white New Zealand rabbit) has been demonstrated (11,12) with large doses of 5 mg/kg/day per os. Several recent works have directly or indirectly shown that certain of these OS are easily absorbed by the intestinal mucosa. This applies to 25-hydroxycholesterol (13) and 5,6α-epoxy-5α-cholesta-3β-ol (Bascoul, J., Domergue, N., Olle, M., and Crastes de Paulet, A., unpublished data). In addition, the heating of CHOL during cooking favors autoxidation, because of the presence of oxygen, high temperatures, the length of heating time (1) and the lipid medium (14), all of which encourage the appearance of peroxides.

For these reasons, we made a qualitative and quantitative evaluation of the OS formed during the heating of beef tallow. The samples were collected by the French government's anti-fraud department (Service de la Repression des Fraudes) from stainless steel deep-fat frying tanks used by restaurants for making "french fries."

MATERIALS AND METHODS

Saponification of food fat. About 5 g of tallow, weighed exactly, was added to 100 ml of 1 N KOH in methanol with 1 mg of dehydroepiandrosterone (DHEA) or cholestenone dissolved in methanol. These compounds were used as internal standards in assaying CHOL and OS, respectively, by means of gas liquid chromatography (GLC) or thin layer chromatography-flame ionization detection (TLC-FID). In spite of the susceptibility of DHEA and cholestenone to enolization and the possibility of partial decomposition under these basic conditions, the ratios CHOL/DHEA and CHOL/cholestenone were not significantly modified during saponifications, as shown by reference compounds derivatized and scanned without treatment. The mixture was refluxed for 2 hr. This long heating period is necessary to hydrolyze the very hydrophobic cholesteryl esters (e.g., cholesteryl stearate). Water (200 ml) was added. The unsaponifiable matter was extracted with two 250-ml portions of CH_2Cl_2. The non-aqueous phase was washed several times with 1 N NaOH, then with water to pH 7, and finally dried. About 10 mg of unsaponifiables were isolated per 5 g of tallow. The recommended hydrolytic conditions and the rigorous washing of the organic phase were necessary to obtain unsaponifiables which could be directly subjected to GLC or TLC-FID without first being purified by TLC. Under these conditions, autoxidation of CHOL is negligible during the saponification. An overload of 20 mg of CHOL in 5 g of tallow does not modify the quantities of OS.

Qualitative analysis by TLC. Chromatography was performed on a thin layer of silica gel (Kieselgel 60 F 254; Merck, Darmstadt, Federal Republic of Germany). Visualization was obtained by spraying with a solution of 50% H_2SO_4 and heating for several minutes at 180 C.

Quantitative analysis by TLC-FID. The absorbent consisted of a uniform quartz rod coated with a layer of silica

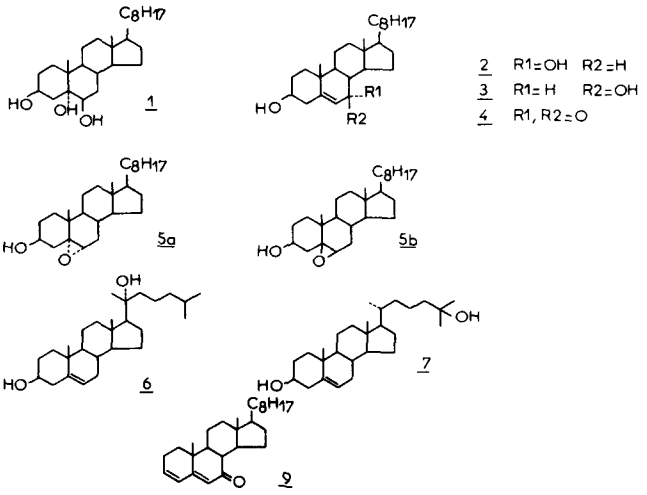

FIG. 1. Predominant oxysterols formed during the rapid autoxidation of cholesterol. The compounds are numbered according to the increasing Rfs measured by TLC (see Materials and Methods). (1) 3β-5-6β-trihydroxy-5α-cholestane: triol; (2) 3β-7α-dihydroxy-cholesta-5-ène: 7α-hydroxy CHOL; (3) 3β-7β-dihydroxy-cholesta-5-ène: 7β-hydroxy CHOL; (4) 3β-hydroxy-7-oxo-cholesta-5-ène: 7-oxo-CHOL; (5a) 5-6α-epoxy-5α-cholesta-3β-ol: α-oxide; (5b) 5-6β-epoxy-5β-cholesta-3β-ol: β-oxide; (6) 3β-20-dihydroxy-(20S)-cholesta-5-ène: 20α-hydroxy CHOL; (7) 3β-25-dihydroxy-cholesta-5-ène: 25-hydroxy CHOL; (8) (cholesterol) formula not represented here: CHOL; (9) 7-oxo-cholesta-3-5-diène: Δ^{35}-7-oxo-CHOL.

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gel and held in place by a soft glass frit (Chromarod-SII) (15). The Chromarods used were chosen to have the same response coefficient toward a mixture constituting equal amounts of the oxysterols studied. One μg of unsaponifiable in a methylene chloride solution (1 $\mu g/\mu l$) was deposited on the Chromarod. Development was carried out in a cyclohexane/ethyl ether mixture (4:6, v/v). After migration, the products were located and quantitatively evaluated by means of flame ionization using an Iatroscan apparatus. The quantitative evaluations were done with a Shimadzu ICR1 integrating calculator. DHEA was used as an internal standard in assaying CHOL and OS by TLC-FID. The standard curve for CHOL (Fig. 2a) was plotted for ratios of CHOL/DHEA ranging from 1:1 to 10:1. The CHOL/DHEA mixtures were subjected to hydrolysis under conditions analogous to those used with the tallow samples. Peaks with equal areas had mass ratios (CHOL/DHEA) of 0.73.

Analysis by GLC. The analysis was carried out on SE-54 capillary columns at 274 C. Derivatization (methyloxime [Mox] in the case of carbonyls; trimethylsilyether [TMS] in the case of alcohols) was performed according to a previously described method (16). Cholestenone was used as the internal standard. The CHOL-cholestenone mixtures were hydrolyzed and derived in the same way as the fat samples. The two *syn*- and *anti*-isomers of methyloxime were clearly resolved. The standardization consisted of comparing the sum of the two peaks of the derivatized cholestenone with the peak size of the CHOL and the OS and gave the following results for CHOL: for ratios between 1:1 and 10:1, we obtained the line shown in Figure 2b. Peaks with equal sizes, as measured by GLC, had a CHOL/cholestenone mass ratio of 0.82.

Assay of polar components. We used the technique recommended by Waltking and Wessels (17), which consists of separating the polar and nonpolar compounds contained in the oxidized fat samples by chromatography on a silica gel column (particles 0.063 to 0.20 mm in diameter, 5% water). The dimensions of the column were standardized: interior diameter, 2.1 cm; length, 45 cm. For packing, the silica was suspended in a mixture of ethyl ether and petroleum ether (87:13, v/v). The fat to be examined (about 1 g, precisely weighed) was dissolved in 10 ml of the above-mentioned mixture. The solution was applied to the top of the column. The nonpolar compounds were eluted in the same solvent system (150 ml) at a flow-rate of about 150 ml/hr. The eluent was collected in a preweighed flask. The solvent was then evaporated at 60 C under a nitrogen stream. The nonpolar compounds were weighed; the polar compounds were left on the column and calculated by subtraction. Satisfactory separation was verified by TLC.

RESULTS

Of the numerous OS formed by autoxidation in a liquid state, those which appear most rapidly and in the greatest quantities are listed in Figure 1. Very small quantities of 20- and 25-hydroxycholesterols are present. They form essentially during autoxidation in the solid state (in proportions of the order of 10^3 ppm [2]); oxidation in the side chain is favored by the crystal structure of cholesterol (18,19), which has its aliphatic chain on the outside, and a rapid propagation of radicals in the solid state. Because of their small quantity, these two compounds were not assayed in the samples used in this work.

Qualitative and quantitative evaluation of cholesterol and OS by GLC. The fat temperatures at the times of sampling are given in Table 1. The heating was done in stainless steel deep-fat frying tanks used for making "french fries." The heating time reported by the users ranged from 56 to 70 hr. The OS were evaluated by comparing their GLC retention time with that of a sample, and in some cases by GLC-MS on a LKB 2091 at 780 eV. In the quantitative evaluations, cholestenone was used as the internal standard. The chromatographic profile of a synthetic mixture of the predominant OS cited (Fig. 1) is shown in Figure 3a.

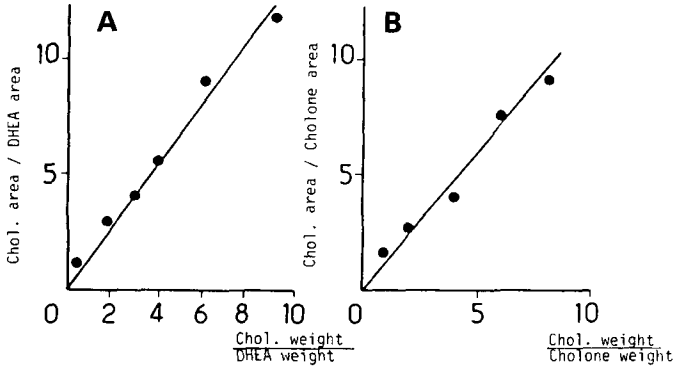

FIG. 2. (A) Standard curves for TLC-FID used in the assay of cholesterol (Chol) and oxysterols. Internal standard: dehydroepiandrosterone (DHEA). (B) Standard curves for GLC used in the assay of the same compounds. Internal standard: cholestenone (Cholone).

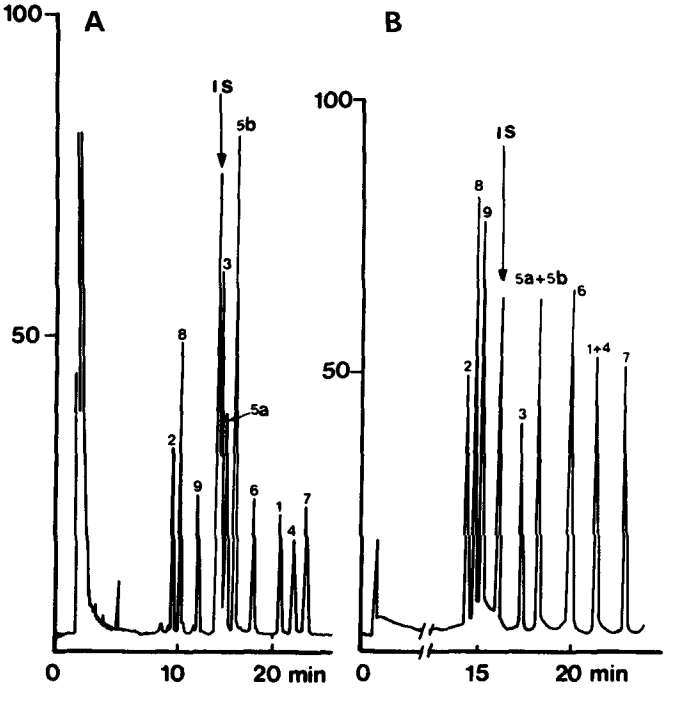

FIG. 3. (A) GLC of a mixture of derivatized oxysterol and cholestenone (internal standard: IS) at 274 C on an SE-54 column (Spiral; Dijon, France). See Fig. 1 for numbering of compounds. 7β-HydroxyCHOL arises with the first peak of cholestenone methyloxime. (B) The same mixture on a column coated with OV-101 (Spiral).

TABLE 1

Cholesterol and Polar Compounds in Fats Before and After Heating

	Sample														Mean value	
	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b	7a	7b	8a	8b	Unheated sample	Heated sample
Temperature (C)	—	142	—	171	—	173	—	146	—	184	—	171	—	147	—	182		164± 17
Cholesterol (ppm)	1705	1300	1918	1635	1874	1300	1336	910	1306	1175	1592	1143	1773	1313	1456	1169	1620± 236	1243± 205
Cholesterol loss after heating																		
ppm	405		283		574		426		131		449		460		287		376±137	
%	24		15		31		32		10		28		26		20		23±8	
Polar compounds (%)			0.4	14	0.6	19	3	30	0.8	21	0.2	16	1.1	6.7	0.8	20	1±0.7	18±7
Polar compounds formed by heating (%)			13.6		18.4		27		20.2		17.8		5.6		19.2		17±7	
Intensity of oxysterols on TLC plates	low		low		high		high		medium		high		medium		high			

The results obtained with the OS were very close whether the column packing was coated with SE-54 (Fig. 3a) or with OV-101 (17) (Fig. 3b). However, with SE-54, we observed a clear separation of 7-oxocholesterol (4) and triol (1), of cholesterol epoxides with α- and β-configurations and of *syn*- and *anti*- methyloxime isomers of cholestenone (cholestenone Mox).

In contrast, 7β-hydroxycholesterol is superimposed with the predominant peak of cholestenone Mox in SE-54, whereas it is separated on OV-101 from the single peak of cholestenone Mox. Nevertheless, the use of cholestenone as the internal standard was still possible; the quantities of 7α- and 7β-hydroxycholesterol formed during autoxidation were approximately equal, which allowed us to make the correction necessitated by the superimposition of the 7β-hydroxycholesterol/cholestenone Mox peaks. The results of cholesterol assay before and after heating, as well as the polar components levels, are listed in Table 1. The mean cholesterol level before heating was 1620 ± 236 ppm. After heating, there were only 1243 ± 205 ppm. The mean loss was 367 ± 137 ppm, or about 23%. The mean level of oxidized triglycerides formed by heating was 18 ± 7%.

The results of quantitative evaluation of OS by GLC and TLC-FID are shown in Figure 4. The predominant compounds were triol (only assayed by TLC-FID), 7α-, 7β-hydroxy, 7-oxo-CHOL and Δ^{35}-7-oxo-CHOL. The two epoxides of cholesterol were present in small quantities.

The sum of the quantities of individual polar products separated by GLC in selected samples is shown in the Table 2 mean value of 124 ± 50 ppm. The integration of the whole series of polar peaks obtained by means of TLC-FID (from the starting point to the less polar epoxide peak) in the same samples is also indicated in the Table 2

TABLE 2

Levels (ppm) of Polar Oxysterols (Triol, 7α-, 7β-, and 7-Oxo-cholesterol) Measured by GLC and TLC-FID

Sample	TLC-FID	GLC
3b	350	136
4b	375	156
5b	230	182
6b	350	98
7b	400	42
8b	—	134
Mean	341 ± 65	124 ± 50

mean value of 341 ± 65 ppm. This value is greater than the former since it corresponds to the whole polar portion of the unsaponifiables, comprising all the OS with an Rf smaller than that of epoxides. It is closer to the CHOL loss during heating (Table 1) than the evaluation of polar OS by GLC.

TLC on a Chromarod SII and flame ionization detection. The samples whose OS visualization was the clearest, using TLC on a plate of silica gel, were analyzed by TLC-FID (samples 3b, 4b, 5b, 6b, 7b and 8b). Figure 4 gives an example of the separation of the principal OS (cf. Fig. 1) under these conditions.

Quantitatively, we observed a predominance of triol which was difficult to detect by GLC (Fig. 5). The results concerning 7α- and 7β-hydroxyCHOL were analogous to those obtained by GLC. On the other hand, the epoxides were difficult to detect by this method.

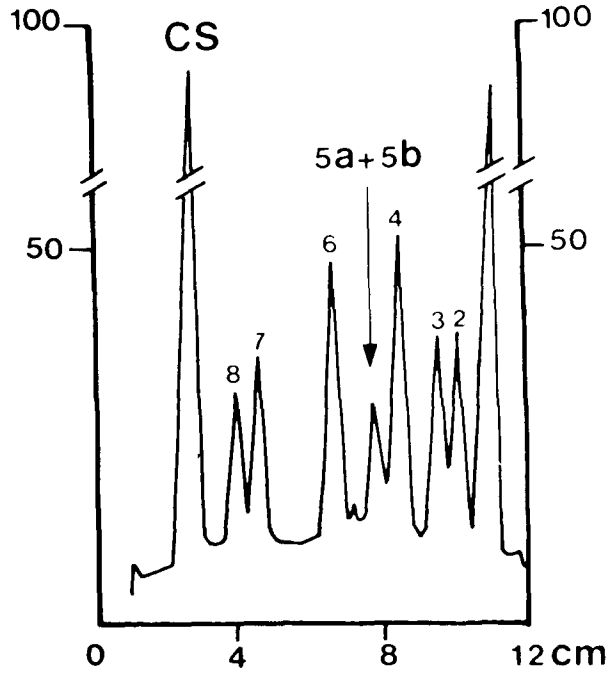

FIG. 4. TLC-FID of a mixture of derivatized oxysterols and cholesteryl stearate (CS) on an SII Chromarod. Migration solvent: cyclohexane/ethyl ether (4:6, v/v). See Fig. 1 for numbering of compounds. The front of the solvent moved to 2.5 cm and the start was at 12 cm from the top of the rod.

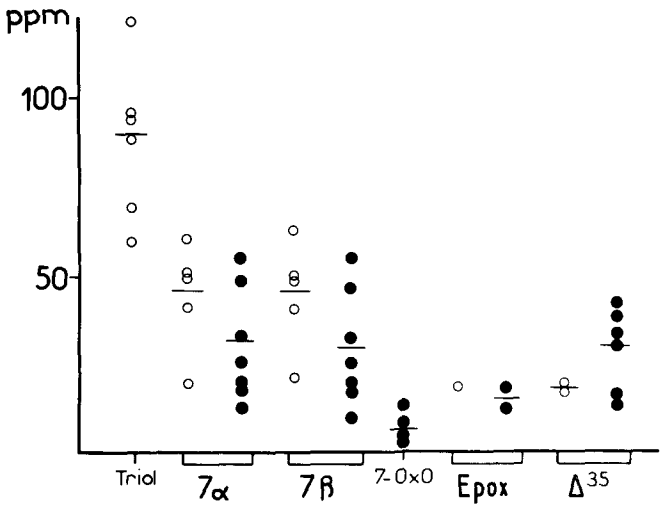

FIG. 5. Quantities (ppm) of the different oxycholesterols assayed by GLC (●) and TLC-FID (○).

DISCUSSION

The CHOL fraction which disappears during the heating of an animal fat used for deep-fat frying in restaurants (in this case, beef tallow) is on the order of 25%, starting with new fat heated for about 60 hr.

The major portion of the missing 25% of CHOL (376 ± 137 ppm) (Table 1) was transformed into polar products (341 ± 65 ppm, 90%) constituting the set of polar peaks observed by TLC-FID (Table 2). From the two types of analyses (GLC, Fig. 3; TLC-FID, Fig. 4) it can be seen that the OS formed were, in decreasing order of magnitude: triol (1), 90 ppm; 7α- and 7β-hydroxyCHOL (2 and 3), 40 ppm; and Δ^{35}-7-oxo-CHOL (8), 20 ppm. β-Oxide (5b; accompanied by about 5% of the isomer, 5a) was formed in smaller quantities (15 ppm). Lastly, 7-oxo-CHOL (4) was evaluated at about 10 ppm. The 20- and 25-hydroxyCHOL were not directly perceptible. Preliminary isolation with preparative TLC would be required before they could be observed.

This evaluation shows that, under the conditions described here, about 25% of the CHOL is transformed into identified OS. The GLC and TLC-FID techniques used for identification and assay yield complementary results. GLC provides a very good resolution of the mixture of autoxidation products (Fig. 3), but it underestimates the results for OS compared to TLC-FID. The latter method can be performed more rapidly and is more appropriate for routine assays. Its resolution is not as good, but the form of its results allows a better evaluation of the CHOL autoxidation profile. The whole group of polar products, situated between the starting point and the Rf of the epoxide, appears at once, and its integration can provide a better and quicker measurement of the CHOL oxidation level. GLC is a better method for specific evaluation of the autoxidation products because of its better resolution. The underestimation of OS quantities observed in this study could have several origins, such as incomplete derivatization of certain compounds, irreversible absorption of highly polar compounds in the GLC column and thermal destruction (265 C) of certain OS.

In conclusion, the detection of relatively large quantities of OS (100-300 ppm) in beef tallow used for deep-fat frying, currently suspected of being highly atherogenic, should lead to an examination of the nutritional risks involved in impregnating food with these heated fats. In this respect, systematic evaluation of the total OS in heated food fats could be a more significant element in testing the "quality" of these fats than the measurement of polar compounds, which are neither atherogenic nor mutagenic.

ACKNOWLEDGMENTS

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cis-Vaccenic Acid in Mango Pulp Lipids

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A peak corresponding to a methyl octadecenoate other than oleate has been detected on the capillary gas chromatogram of the methyl esters of mango pulp fatty acids. This octadecenoate was isolated by silica gel and argentation column chromatography, high performance liquid chromatography and argentation thin layer chromatography, and then analyzed by infrared, nuclear magnetic resonance and gas chromatography-mass spectrometry, chromatographic separations and oxidative degradation. These analytical data proved that the octadecenoic acid was *cis*-vaccenic acid (*cis*-11-octadecenoic acid). The concentration of this acid in total octadecenoic acids ranged from 35% to 50% in the pulp of mangoes from Fiji, Mexico, the Philippines and Taiwan. *cis*-Vaccenic acid was revealed to be one of the major component fatty acids of non-polar lipids (mainly triacylglycerols), glycolipids and phospholipids in mango pulp. The glycolipids contained *cis*-vaccenic acid (ca. 20%) in higher concentration than oleic acid (ca. 15%). A trace amount of *cis*-vaccenic acid (0.5%) was detected in the total lipids of mango seeds. Profile of fatty acid composition of mango pulp lipids (0.2–0.3 wt% of wet pulp) was characterized by the presence of n-7 acid isomers, *cis*-vaccenic acid and palmitoleic acid, and unusual mono- and dienoic positional isomers. *Lipids 21,* 388–394 (1986).

cis-Vaccenic acid (*cis*-11-octadecenoic acid) (1) is well known to be a component fatty acid of microorganisms, animal tissues and human tissues, and also of dietary fats obtained by partial catalytic hydrogenation of vegetable oils and marine oils. The metabolism and incorporation of *cis*-vaccenic acid in the living materials mentioned above and the formation of this acid in the dietary fats have been well investigated by many researchers (2–7).

In the field of higher plant biochemistry, Chisholm and Hopkins (8,9) first reported the presence of *cis*-vaccenic acid in milkweed *(Asclepias syriaca)* seeds, but only a few papers have been published (10–16). The lack of interest in *cis*-vaccenic acid in higher plant lipids was probably due to its low concentration in common vegetable oils, and some difficulties and time consumption in analytical procedures.

Recent progress in gas chromatography (GC) using high resolution capillary columns has made it possible to detect and determine *cis*-vaccenic acid together with other isomeric monoenoic acids in the lipids of several kinds of plants (17–19). Recently Kleiman and Payne-Wahl (20) also reported the presence of *cis*-vaccenic acid in seed oils from three species of the genus *Entandraphragma* (Meliaceae family). We proposed new analytical procedures for estimation of *cis*-vaccenic acid in plant lipids by gas chromatography-mass spectrometry (GC-MS) as its pyrrolidine- (21) and bis(methylthio)-derivatives (22). By use of this system, we revealed the content of *cis*-vaccenic acid in triacylglycerols of seven kinds of common vegetable oils (21) and in total lipids of parsley, soybean and maturing *Mallotus japonicus* (Euphorbiaceae family) seeds and also of chlorella (22).

In the course of our screening of *cis*-vaccenic acid in the higher plants available for food, we have found that the pulp lipids of edible fruits always contained *cis*-vaccenic acid as well as oleic acid as a component of octadecenoic acids. In this paper, we prove the presence of *cis*-vaccenic acid in the pulp lipids of mango *(Mangifera indica),* and describe the estimation of this acid.

MATERIALS AND METHODS

Reference substances and other chemicals. The sources of reference substances were as follows: palmitoleic, *cis*-vaccenic and elaidic acids from P.L. Biochemicals Inc. (Milwaukee, Wisconsin); oleic, linoleic and linolenic acids from Research Laboratory of Nippon Oil and Fats Co. Ltd. (Amagasaki, Japan); and henicosanoic acid (purity 99.8%) from Nakarai Chemicals Ltd. (Kyoto, Japan). These acids were methylated and purified by thin layer chromatography (TLC). *trans*-Vaccenate was prepared by the geometric isomerization of *cis*-vaccenate with *p*-toluenesulfinic acid (23), followed by purification using argentation TLC (AgTLC). The purity of monoenoic acids used was more than 99.5% by capillary GC analyses of their methyl esters and GC-MS analyses as their dimethyl disulfide adducts (22). All other chemicals were analytical reagent grade, and all organic solvents were distilled in glassware prior to use.

Materials and extraction of total lipids. Four market types of mangoes (grown in Fiji, Mexico, the Philippines and Taiwan) were used. Each sample, five fully ripe mangoes having uniformity based on weight and color, was peeled and divided into pulp and seed parts. The two parts were separately immersed in boiling water for 5 min to inactivate the enzymes (24). The pulp part was homogenized in chloroform/methanol (2:1, v/v) with a Waring blender. The homogenate was filtered and the residue was extracted twice with the same solvent mixture. The combined extracts were washed with water and dried over anhydrous Na_2SO_4. After removal of Na_2SO_4 and insoluble nonlipid contaminants by filtration, the solvent was evaporated nearly to dryness by a rotary evaporator below 35 C. The total lipids thus obtained were redissolved in a definite volume of chloroform and stored under an atmosphere of nitrogen at -20 C until analysis. The seed total lipids were obtained and stored in the same manner.

Chemical treatments: derivatization. Fatty acid methyl esters (FAME) of total lipids and nonpolar lipids containing free fatty acids were prepared by treating the lipids with 0.5 N KOH/methanol at 80 C for 20 min in a reaction vial connected to a reflux condenser and successively for 10 min with BF_3-etherate (ca. 47%) which was added directly to the vial through the condenser to ca. 10% concentration in the final mixture. Free fatty acids were methylated with 14% BF_3/methanol (25). Other lipid classes were converted to FAME with 0.5% (w/v) sodium methoxide at 80 C for 20 min. Trimethylsilyloxy

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derivatives of FAME were prepared according to the method given in the literature (26), except N,O-bis(trimethylsilyl)acetamide/pyridine was used for a silylating reagent in this study, and pyrrolidine derivatives were obtained as previously described (21). Dimethyl disulfide adducts of methyl monoenoates demonstrated by Francis (27) were prepared by our rapid procedure (28) on a reduced scale. As a standard procedure for the single-step preparation of adduct, methyl monoenoate (1 μmol) was incubated with 1 ml of dimethyl disulfide containing 13 mg of I_2 at 35 C for 30 min. After reduction of I_2 with 30% (w/v) $NaHSO_3$ aqueous solution, 0.5 ml of hexane/ether (1:1, v/v) was added to the resulting mixture. Three μl of the upper phase of the mixture was introduced directly to GC-MS. In case of quantitative analysis, a mixture of FAME (1 μmol equivalent for monoenoates) was subjected to reaction and treatments in the same manner. After removal of contaminating by-products (originated from polyenoates) in the hexane/ether layer by silica gel TLC, the purified fraction of methyl monoenoate adducts was analyzed by GC-MS.

Chemical treatments: oxidative degradation. To determine the double bond positions in FAME, oxidative fission was carried out with $KMnO_4/NaIO_4$ in *t*-butanol, followed by pyrolysis methylation of the resulting monocarboxylic acid and half ester of dicarboxylic acid with tetramethylammonium hydroxide in the injection port of a gas chromatograph (29).

Chemical treatments: hydrogenation. FAME were hydrogenated in hexane in the presence of platinum black as a catalyst at room temperature for 40 min.

Column chromatography. Preparative column chromatography was performed on a 40 × 1.2 cm i.d. glass column packed with 20 g of silica gel (E. Merck, Darmstadt, Federal Republic of Germany, No. 7734) to purify a large quantity of FAME. The solvent system was hexane/ether (90:10, v/v). Group separation of total lipids into three lipid classes was done on the same packed-column by a successive elution with 500 ml of chloroform for separating non-polar lipids, 500 ml of acetone for glycolipids and 500 ml of methanol for phospholipids. Preparative argentation column chromatography was run by using a stepwise elution with hexane/ether (99:1, 98:2, 97:3, v/v, 400 ml each) on a 40 × 2 cm i.d. glass column packed with 60 g of silica gel (E. Merck, No. 7734) and 12 g of $AgNO_3$.

High performance liquid chromatography (HPLC). Preparative group separation of FAME according to chain length was performed with a Shimadzu LC-4A liquid chromatograph equipped with an auto sampler (Shimadzu SIL-2AS), a 30 cm × 6 mm i.d. YMC-Packed Column ODS (5 μm particle size) (Yamamura Laboratory Co. Ltd., Kyoto, Japan), and a refractometer (Shimadzu RID-2AS). The mobile phase was methanol/water (90:10, v/v) at a flow rate of 1.0 ml/min. The eluent was traced with a Shimadzu Chromatopac C-R2AX and collected in test tubes by a fraction collector linked to the sampler.

TLC and AgTLC. Commercially precoated silica gel plates (E. Merck, No. 11845) were used for nonpolar lipid class separation with hexane/ether/acetic acid (80:30:1, v/v/v) as a developing solvent, and for purification of a small amount of FAME and dimethyl disulfide adducts of FAME with hexane/ether/acetic acid (90:10:1, v/v/v) system.

For impregnation of silica gel plates with $AgNO_3$, the plates (E. Merck, No. 11845) were developed with 20% (w/v) $AgNO_3$/acetonitrile from bottom to top, air-dried in the dark, and finally activated by heating at 110 C for 60 min before use. Benzene/ether (90:10, v/v) was used to separate FAME according to the number of double bonds and to the *cis-trans* configuration (system I). The toluene multiple development at −25 C (30) was adopted to separate monoenoates according to the position of double bond on the aliphatic chain (system II). The spots were located on the plate under ultraviolet light (254 nm) after spraying of 0.02% 2′,7′-dichlorofluoroscein in methanol.

GC. Packed-column GC was carried out with a Hitachi 163 gas chromatograph equipped with a flame-ionization detector (FID). Operating conditions were column, 3 m × 3 mm i.d. glass column packed with 5% Shinchrom E-71 (medium polarity) on 80–100 mesh Shimalite AW (Shinwakako Co. Ltd., Kyoto, Japan); carrier gas, nitrogen (40 ml/min); column temperature, 150 C or 205 C. Capillary GC was carried out with a Hitachi 263-30 gas chromatograph equipped with an FID and a glass-lined splitter. Operating conditions were column, 50 m × 0.24 mm i.d. SS-10 (medium polarity) fused silica capillary column (Shinwakako Co. Ltd.); carrier gas, helium (2.0 ml/min); split ratio, 1/10; column temperature, 95 C to 190 C at 3 C/min. Data processor was a Hitachi Chromato-integrator D-2000.

GC-MS. GC-MS analysis was performed on a Hitachi 663-30 gas chromatograph coupled to a Hitachi M-80A double focusing mass spectrometer with an M-003 minicomputer on-line system. Operating conditions were column, 1 m × 3 mm i.d. glass column packed with 2% OV-101 on 100–120 mesh Chromosorb WHP; carrier gas, helium (40 ml/min); column temperature, 120 C, 180 C or 240 C; injector and separator temperatures, 260 C; ionizing voltage, 20 eV; emission current, 100 μA; accelerating voltage, 3.0 kV; ion source temperature, 160 C; scan range, 0–500 m/z (3.1 sec/cycle).

Other spectral analysis. Infrared (IR) spectra were measured as liquid films or 5% solution in hexane with a Hitachi 285 IR spectrophotometer. Nuclear magnetic resonance (NMR) spectra in carbon tetrachloride were recorded on a Hitachi R-24 NMR spectrometer (60 MHz) with tetramethylsilane as an internal standard.

RESULTS

Figure 1 shows a typical gas chromatogram of FAME from total lipids of mango pulp. The pulp total lipids of four market types of mangoes examined gave essentially a similar profile of fatty acids with two characteristic peaks in the elution range of methyl octadecenoates. Peak area ratio of the faster peak (compound I) to the later one (compound II) is ca. 52:48 (Fig. 1). The retention times of compounds I and II agreed very closely with those of oleate and *cis*-vaccenate standards, respectively. We, however, tried to isolate the isomeric octadecenoates to identify them more accurately and to examine any possible existence of further unusual isomers.

Isolation and identification of isomeric methyl octadecenoates. FAME (ca. 1 g) prepared from the pulp total lipids of mango (the Philippines, 1985) were purified by preparative silica gel column chromatography and roughly separated into saturated, mono- and polyenoic

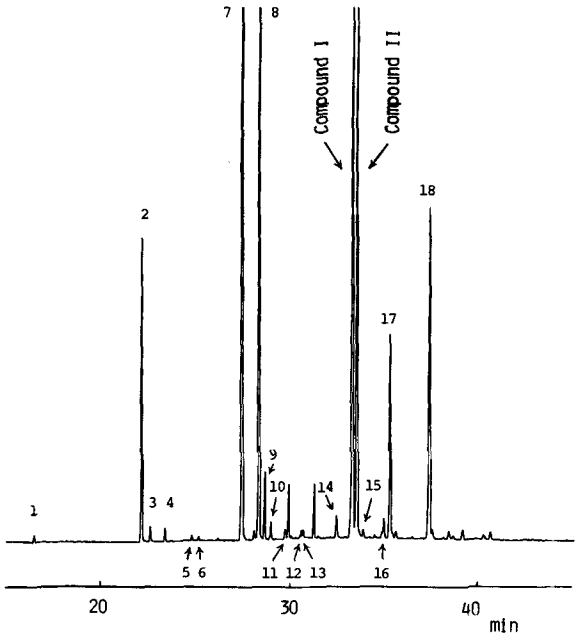

FIG. 1. Gas chromatogram of fatty acid methyl esters from total lipids of mango pulp. The mango was grown in the Philippines in 1985. Peak area ratio of compounds I to II is ca. 52:48. Peak numbers are the same as those in text and in Table 2. Column, SS-10 fused silica capillary column (50 m × 0.24 mm i.d.).

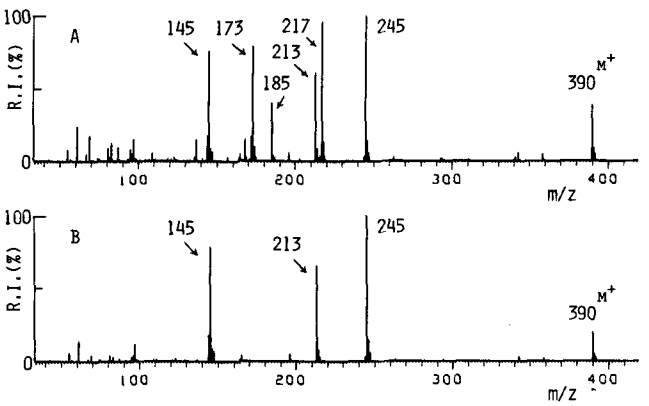

FIG. 2. Mass spectra of dimethyl disulfide adducts of the methyl *cis*-octadecenoates (A) isolated from the pulp total lipids of mango grown in the Philippines in 1985, and of compound II (B) isolated from the esters. Spectrum A is taken at the peak top of the adducts. Therefore, intensities of characteristic fragment ions do not represent the proportion of the isomers. Operating conditions are ionizing voltage, 20 eV; column, 1 m × 3 mm i.d. glass column packed with 2% OV-101 on 100–120 mesh Chromosorb WHP; column temperature, 240 C. Other conditions are given in text.

ester fractions by preparative argentation column chromatography. Predominant methyl monoenoates accompanied with small amounts of saturated and dienoic esters were recovered from a 400-ml fraction of hexane/ether (98:2, v/v). Mass chromatography of FAME from the other two fractions (99:1 and 97:3, v/v eluates) showed no molecular ion at m/z 296. Therefore, the hexane/ether (98:2, v/v) fraction should contain positional and/or geometrical isomers of methyl octadecenoates.

The crude methyl monoenoates were then fractionated according to chain length by HPLC, and the eluents corresponding to C_{18} FAME were combined, concentrated to dryness and dissolved in a small volume of hexane. IR spectrum of the C_{18} monoene sample (FAME) showed no absorption at the region of 960–970 cm^{-1}, indicating no *trans* double bond. After hydrogenation of an aliquot of the sample (FAME), it gave a single peak (methyl stearate) on the capillary gas chromatogram, and a single molecular ion at m/z 298 by GC-MS. The fragmentation pattern was identical with that of methyl stearate. These results indicated that the isolated FAME had nonbranched C_{18} chain.

To eliminate the contaminating saturated and dienoic esters, the crude methyl octadecenoates were purified by AgTLC with solvent system I many times, and a band corresponding to standard *cis*-monoenoate was recovered. No band of *trans*-monoenoate was observed. The purified methyl *cis*-octadecenoates thus obtained showed two major peaks whose retention times were identical with those of compounds I and II by capillary GC. The peak area ratio of compounds I to II (ca. 52:48) was the same as the ratio given in Figure 1. A minor peak which eluted after compound II corresponded to peak no. 15 in Figure 1, whose peak area was ca. 1/100 of compound II.

The methyl *cis*-octadecenoates were separated according to the position of double bond by AgTLC with solvent system II. Two spots were detected on the plate: the higher spot (R_f = 0.70) corresponded to that of standard *cis*-vaccenate and the lower one (R_f = 0.58) to that of standard oleate. Esters from the R_f 0.70 fraction (ca. 5 mg) and R_f 0.58 fraction (ca. 5 mg) were separately recovered by the repeated AgTLC separation. The esters of the R_f 0.70 and R_f 0.58 fractions gave a single peak corresponding to those of compound II and compound I by capillary GC, respectively. NMR spectra of these esters showed multiplet signals at δ 5.25 ppm (2H, olefinic protons) (31), and the pattern was identical with that of standard oleate or *cis*-vaccenate.

The chromatographic behaviors plus the IR and NMR spectra of compounds I and II suggested that these compounds were methyl *cis*-octadecenoates, namely oleate and *cis*-vaccenate, respectively. The double bond positions of these compounds, however, were not evident except for the capillary GC data.

Double bond positions of compounds I and II. Figure 2 shows mass spectra of dimethyl disulfide adducts of the purified methyl *cis*-octadecenoates before AgTLC (solvent system II) separation (A), and of dimethyl disulfide adduct of compound II isolated from the purified esters by AgTLC (solvent system II) (B). These adducts gave recognizable molecular ions at m/z 390 and a series of characteristic key fragment ions showing the original double bond positions in the molecules. In spectrum A (Fig. 2), sets of fragment ions at m/z 145 and 245 were produced from the cleavage between the methylthio-substituted carbons of C-11 and C-12, and fragment ions at m/z 173 and 217 from the carbons of C-9 and C-10 (27). Fragment ions at m/z 213 and 185 were yielded due to loss of methanol from the ions at m/z 245 and 217, respectively (27). These assignments were also supported by our high resolution MS observations previously reported (28).

In spectrum B (Fig. 2), only the three key fragment ions were recorded. The ions at m/z 145 and 245 corresponded to the cleavage between the carbons of C-11 and C-12, and

the ion at m/z 213 was due to loss of methanol from the ion at m/z 245. This observation indicated that the adduct was methyl 11,12-bis(methylthio)octadecanoate (27,28). Dimethyl disulfide adduct of compound I was identified as methyl 9,10-bis(methylthio)octadecanoate in the same manner (27,28).

Trimethylsilyloxy derivative of compound I gave a mass spectrum identical with that of standard oleate, where fragment ions at m/z 215 and 259 were useful for deciding that the original double bond was at the C-9 carbon (26). In the spectrum of the derivative of compound II, it was observed that significant fragment ions were shifted to m/z 187 and 287 with 28 mass unit interval from the corresponding ions (at m/z 215 and 259) of the derivative of compound I. The observation represented the cleavage occurring between the trimethylsilyloxy-substituted carbons, C-11 and C-12. The mass spectrum was the same as that of methyl 11,12-bis(trimethylsilyloxy)octadecanoate prepared from standard *cis*-vaccenate.

Pyrrolidine derivatives of compounds I and II gave a series of key fragment ions: m/z 196, 208, 222 and 236 (compound I), and m/z 196, 210, 224 and 236 (compound II). Therefore, by use of the rule for location of double bonds reported by Andersson and Holman (32), the derivative of compound I was identified as N-octadec-9-enoylpyrrolidine and that of compound II as N-octadec-11-enoylpyrrolidine.

On oxidative degradation of compounds I and II for the location of the double bond, compound I gave methyl nonanoate and nonanedioate, whereas compound II gave methyl heptanoate and undecanedioate. These mono- and dicarboxylic acid esters were identified by comparing their retention times with those of the fission products of standard oleate and *cis*-vaccenate, and also by GC-MS.

The mass spectral and chemical degradation data obtained here proved that the double bond position of compound I was at the C-9 carbon and that of compound II was at the C-11 carbon. Therefore, these results led to the conclusion that mango pulp lipids contained *cis*-vaccenic acid as well as oleic acid.

Other monoenoic positional isomers and some minor fatty acids. A minor peak corresponding to the peak of no. 15 in Figure 1 was detected on the gas chromatogram of the purified methyl *cis*-octadecenoates. As complete separation of the isomers according to the double bond position by AgTLC needed a small sample size on the plate, recovery of the unknown isomer failed after separation by AgTLC (solvent system II) because of its low content in the methyl octadecenoate mixture. However, mass chromatography of dimethyl disulfide adducts of the purified methyl *cis*-octadecenoates (see Fig. 2A) showed that a mass spectrum taken at the peak tailing of the adducts (7 scan after the peak top) gave a series of minor ions at m/z 117, 241 and 273 due to the fragmentation of methyl 13,14-bis(methylthio)octadecanoate. Consequently, the unknown isomer was identified as *cis*-13-octadecenoic acid on the basis of the mass spectrum and the elution position by capillary GC.

By GC-MS of saturated FAME isolated from total FAME, minor saturated fatty acids were identified as follows (cf. Fig. 1): peak no's. 1, 12:0; 5, 15:0; 11, 17:0. The following structures of minor isomeric C_{14}-C_{17} chain monoenoic acids were presumed from GC-MS of their dimethyl disulfide adducts (cf. Fig. 1): peak no's. 3, 14:1(n-10); 4, 14:1(n-5); 6, 15:1(n-10); 10, 16:1(n-3); 12, 17:1(n-9); 13, 17:1(n-8). Further confirmation of these monoenoic positional isomers and the identification of other unknown peaks (Fig. 1) are under way in our laboratory.

TABLE 1

Profile of Pulp Lipids of Mango Grown in the Philippines in 1985[a]

Lipid class (O-acyl type)	Fatty acid distribution (wt %)[b]
Nonpolar lipids	
Triacylglycerols	34.6
Others[d]	11.0
Glycolipids	24.2
Phospholipids	30.2
Total[d]	100.0

[a]Each value is an average of three determinations.

[b]Determined by packed-column GC of fatty acid methyl esters derived from each lipid class. To each lipid class was added a known amount of 21:0 methyl ester as an internal standard before esterification.

[c]Including steryl esters, diacylglycerols, monoacylglycerols and free fatty acids.

[d]Total crude lipids containing the O-acyl lipids, free fatty acids, pigments, sterols and other lipids amounted to 0.3 wt % of the wet pulp by weighing method.

Profile of mango pulp lipids. The pulps from four market types of mangoes examined contained 0.2–0.3 wt % (wet basis) total lipids including O-acyl lipids, free fatty acids, sterols, pigments and other lipids. The contents of the total lipids were lower as reported previously with mangoes of India growth (33–35) than those of common edible oil seeds. The amount of pulp total lipids in mango from the Philippines was ca. 0.9 g per fruit and their lipid profile is shown in Table 1. The ratio of triacylglycerols/glycolipids/phospholipids was ca. 1.00:0.70:0.87 based on the weight of their component fatty acids. A similar profile was observed in the mangoes from different sources examined in this study.

Fatty acid composition and cis*-vaccenic acid content.* Fatty acid composition of mango pulp lipids was clearly different from those of common vegetable oils (soybean, safflower, olive, etc.) with respect to the variety of fatty acids, especially their positional isomers. In the analyses of four types of mangoes by capillary GC, more than 30 peaks (including unidentified peaks) usually appeared in their gas chromatograms. No marked difference in the composition of major fatty acids has been observed among different mango samples. Fatty acid composition of pulp lipids of mango from the Philippines is shown in Table 2, for example.

Major component fatty acids were palmitic, palmitoleic, oleic and *cis*-vaccenic acids in nonpolar lipids, glycolipids and phospholipids, and these four acids constituted more than 65% of total acids in these lipids. Myristic acid was somewhat concentrated in nonpolar lipids as was linolenic acid in glycolipids. Linoleic acid showed very low content in mango pulp lipids, whereas an abnormal amount of octadecadienoic acid could be detected with 3–8% of total acids in these lipids. Its structure was presumed to be

TABLE 2

Major Fatty Acid Composition and *cis*-Vaccenic Acid Content in Pulp Lipids of Mango Grown in the Philippines in 1985[a]

Peak no.[c]	Fatty acid	Lipid class[b] (wt %)				
		Nonpolar lipids	Triacylglycerols	Glycolipids	Phospholipids	Total lipids
2	14:0	9.5	8.6	1.3	3.5	5.7
7	16:0	23.9	28.2	17.3	24.5	22.2
8	16:1(n-7)	16.3	16.7	14.4	16.7	17.3
9	16:1(n-5)	1.3	0.7	1.5	0.8	1.3
14	18:0	1.2	1.6	0.4	0.3	0.5
(I)	18:1(n-9)	14.1	13.5	15.3	21.4	15.8
(II)	18:1(n-7)	14.3	14.3	21.5	13.9	14.5
16	18:2(9,12)	0.4	0.8	0.9	0.3	0.6
17	18:2(9,15)?	4.6	4.4	3.1	8.1	5.4
18	18:3(9,12,15)	8.5	3.8	15.6	6.3	9.7
	Others[d]	5.9	7.4	8.7	4.2	7.0
cis-Vaccenic / *cis*-Vaccenic + oleic[e]		50.4	51.4	58.4	39.4	47.9

[a]Determined by capillary GC. Each value is an average of three determinations.

[b]Corresponding to those in Table 1.

[c]Corresponding to those in Fig. 1.

[d]Including minor fatty acids (below 0.2%) and unidentified fatty acids.

[e]Calculated from the data by capillary GC, followed by confirming the values by mass chromatography as their dimethyl disulfide adducts.

cis-9,*cis*-15-octadecadienoic acid based on the mass spectrum of its trimethylsilyloxy derivative, IR and ultraviolet spectra and chromatographic behaviors. The details will be reported elsewhere.

cis-Vaccenic acid amounted to about half of the octadecenoic acids in total lipids and in nonpolar lipids of the pulp from mango grown in the Philippines in 1985. The proportion of *cis*-vaccenic acid to octadecenoic acids was slightly higher in glycolipids than in phospholipids. The lower proportion of this acid was observed only in the steryl ester fraction (data not shown), but the other nonpolar lipids (free fatty acids, diacylglycerols and monoacylglycerols) gave similar proportions to those shown in Table 2. The contents of *cis*-vaccenic acid in the pulp total lipids of mangoes from different sources are summarized in Table 3. The pulps of mangoes from four countries and of different harvested years always contained *cis*-vaccenic acid, with the contents varying in the range of 35–50% of octadecenoic acids. These results indicate that *cis*-vaccenic acid is one of the major fatty acids in mango pulp.

On the contrary, a trace amount of *cis*-vaccenic acid (0.5% of total fatty acids) could be detected in mango seed lipids by capillary GC. Fatty acid composition of mango seed lipids was quite different from that of pulp lipids.

In this paper, the content of *cis*-vaccenic acid (Tables 2 and 3) was determined by capillary GC, followed by confirming the proportion by mass chromatography as their dimethyl disulfide adducts (22). Figure 3 shows a typical mass chromatogram taken in the course of estimation of *cis*-vaccenic acid in various samples. Peak areas of m/z 145 due to the fragmentation of methyl 11,12-bis(methylthio)octadecanoate and m/z 173 due to that of methyl 9,10-bis(methylthio)octadecanoate (from scan no's. 62 to 90) were substituted into the equation as previously described (22). From the plot of the calculated value against a calibration curve (22), the proportion of *cis*-vaccenic acid in this sample was determined as 46.4%. Thus, the proportion determined by mass chromatography was almost equal to the calculated proportion (47.9% in Table 2) by capillary GC. To enhance the reliability of analytical value, two independent systems for estimation of *cis*-vaccenic acid were used in this study.

TABLE 3

***cis*-Vaccenic Acid Content in Pulp Total Lipids of Mangoes from Different Sources[a]**

Source and harvested year	Octadecenoic acids of total fatty acids[b] (wt %)	*cis*-Vaccenic / *cis*-Vaccenic + oleic[c] (wt %)
Fiji		
1984	32.1	37.8
Mexico		
1983	26.5	50.7
1984	27.2	36.0
The Philippines		
1983	36.5	49.4
1984	26.2	36.2
1985	30.3	47.9
Taiwan		
1985	25.0	46.3

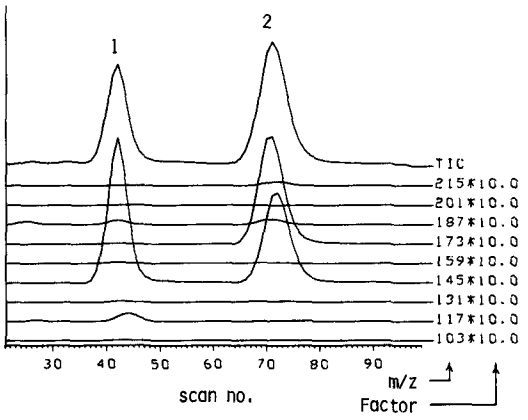

FIG. 3. Mass chromatogram of dimethyl disulfide adducts of fatty acid methyl esters from the pulp total lipids of mango grown in the Philippines in 1985. 1, Dimethyl disulfide adducts of methyl hexadecenoates; 2, dimethyl disulfide adducts of methyl octadecenoates. Adducts were prepared directly from a mixture of fatty acid methyl esters shown in Fig. 1 without prefractionation. Fragment ions in the figure indicate the original double bond positions from the terminal methyl group in the starting esters (e.g., m/z 103 for n-4 and m/z 117 for n-5 isomers). Operating conditions are the same as in Fig. 2.

DISCUSSION

The present study unequivocally demonstrates the presence of *cis*-vaccenic acid in the pulp lipids of mango (from the Philippines in 1985) by chromatographic, spectrometric and chemical methods. Furthermore, a series of detections of this acid in the pulp lipids of other mangoes from different countries and of different harvested years (Table 3) emphasizes that *cis*-vaccenic acid is the common component fatty acid of "mango pulp lipids."

In previous papers on pulp lipids from Indian (33,34) and South African (36) mangoes, the term "oleic acid" was applied to the compound corresponding to the "18:1" peak on gas chromatogram. The application of the term seemed inadequate because packed-column GC could not separate methyl *cis*-vaccenate from methyl oleate under the conventional conditions. Therefore, reexamination should be made on the previous data concerning "oleic acid" in the cases of mango and other plant lipids. Previous workers (33–35) observed a correlation between the intensity of aroma and flavor characteristics of mango pulp and the ratio of palmitic acid to palmitoleic acid. Evidence of an abundance of *cis*-vaccenic acid in the mango pulp lipids may give further information on the correlation, because both palmitoleic and *cis*-vaccenic acids have n-7 structure relating to the double bond.

A small amount of *cis*-vaccenic acid usually could be detected in common vegetable seed oils (10–12,14,16,21, 37). However, mango, one of the most popular fruits in the world, contains *cis*-vaccenic acid as a high percentage of total fatty acids in the lipids of edible parts. Other popular fruits also contain *cis*-vaccenic acid in their pulp lipids, though the contents of this acid vary (Shibahara, A., et al., unpublished data). Consequently, the effect of *cis*-vaccenic acid in pulp lipids of fruits on human nutrition should be considered because pulps are consumed as table fruit, fruit salad, fruit juice, etc.

Seher and his coworkers detected *cis*-vaccenic acid in several plant samples by using capillary GC (17–19). In their results, the content of *cis*-vaccenic acid in total fatty acids was low in seed lipids except for *Asclepias incarnata* and *Asclepias syriaca* seed lipids (18), of which the latter had been proven a rich source of *cis*-vaccenic acid by Chisholm and Hopkins (8,9). We are interested in their observations that *cis*-vaccenic acid always occurred in higher concentration than oleic acid in the lipids of edible parts (leaves, flowers, sprouts, etc.) of vegetables belonging to the Cruciferae family, whereas the proportion between these acids was reversed in the seed lipids of the vegetables (19). The same tendency is observed between the pulp lipids and seed lipids of mango in this study. Appelqvist (38,39) observed by using capillary GC that fatty acid compositions were strikingly different between individual embryonic tissues of rapeseed, and that the monoenoic n-7 positional isomers including *cis*-vaccenic acid in seed coat were equal to or dominated over the monoenoic n-9 positional isomers in comparison with other morphological tissues, cotyledon and hypocotyl. These observations indicate that metabolism of *cis*-vaccenic acid and of oleic acid may be quite different in individual parts of a plant and also in individual tissues of a part.

cis-Vaccenic acid is revealed to be one of the major component fatty acids of nonpolar lipids, glycolipids and phospholipids in mango pulp (Table 2). *cis*-5-Octadecenoic acid of *Ephedra sinica* seed (40) and petroselinic acid of parsley seed (41) are localized on nonpolar lipid fractions. In the present experiments, *cis*-vaccenic acid of mango pulp is widely distributed among main lipid classes. From these findings on the presence of *cis*-vaccenic acid in glycolipid and phospholipid fractions, we consider that this acid has some unknown physiological or biochemical functions due to its n-7 structure in these membrane lipids, or it acts merely as a hydrophobic material of cell membranes of mango pulp.

Biosynthetic pathway of *cis*-vaccenic acid is not confirmed in higher plants (41–44). In our preliminary experiments with mango pulp slices and [U-^{14}C]acetate, monoenoic acid fraction in the pulp lipids of mango was proven to be labeled, but the determination of isotopically labeled *cis*-vaccenic acid failed because of its low radioactivity and the difficulty of separating this acid. Thus, in addition to radioisotopic experiments, we are trying a GC-MS detection method (45) using deuterated fatty acid tracers to find the biosynthetic pathway of *cis*-vaccenic acid in mango pulp.

In the future we will report on the fatty acids in pulp lipids of other commonly available fruits.

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Influence of Dietary Fat on Metabolism of (14-^{14}C)Erucic Acid in the Perfused Rat Liver. Distribution of Metabolites in Lipid Classes

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Two groups of rats were fed diets containing 20% by weight of either partially hydrogenated marine oil supplemented with sunflower seed oil (PHMO) or palm oil (PO) for 8 wk. Using a liver perfusion system, the effect of dietary long chain monoenoic fatty acids on the uptake and metabolism of [14-^{14}C]erucic acid was studied. The perfusion times were 15 and 60 min, respectively. The two groups showed equal ability for erucic acid uptake in the liver but differed in the channeling of the fatty acids into various metabolic pathways. A higher metabolic turnover of 22:1 in the PHMO livers relative to the PO livers was demonstrated by an increased recovery of total [^{14}C]labeling in the triglyceride (TG) and phospholipid (PL) fractions, already evident after 15 min of perfusion. The chain-shortening capacity was highest in the PHMO group, reflected by a higher [^{14}C]18:1 incorporation in both TG and PL, and increasing from 15 to 60 min of perfusion. The amount of [^{14}C]18:1 found in PL and TG after 60 min of perfusion of livers from rats fed PO corresponded to that shown for the PHMO group after 15 min. The PL demonstrated a discrimination against 22:1 compared to TG, and, when available, 18:1 was highly preferred for PL-synthesis.

The total fatty acid distribution in the TG, as determined by gas liquid chromatography (GLC), reflected the composition of the dietary fats. In the total liver PL, 22:1 and 20:1 were present in negligible amounts, although the PHMO diet contained 12–13% of both 22:1 and 20:1. In the free fatty acid fraction (FFA), the major part of the radioactivity (~80%) was [14-^{14}C]erucic acid, and only small amounts of [^{14}C]18:1 (<2%) were present, even after 60 min of perfusion. The shortened-chain 18:1 was readily removed from the FFA pool and preferentially used for lipid esterification.

Lipids 21, 395–400 (1986).

Partially hydrogenated marine oils, used for margarine production in northern Europe, as well as natural marine oils taken as a dietary supply of n-3 fatty acids are rich in monoenoic fatty acids with chain lengths 20 and 22 (1,2). It is, therefore, of interest to study the metabolism of such fatty acids in the liver.

The use of liver perfusion for studying the liver uptake and metabolism of fatty acids is well established (3–5). Such studies have, however, been performed with animals fed laboratory pellet diets of varying compositions that may introduce different metabolic conditions and give contradicting results. Experiments with very long chain fatty acids with 20 and 22 carbon atoms require attention to the pre-experimental conditions, as it is known that such fatty acids induce an additional cellular oxidation pathway through the peroxisomes (6–9).

In the present study, liver perfusions with [14-^{14}C]erucic acid were used to examine the cellular metabolism of long chain monoenoic fatty acids. The two perfusion times were chosen to give information on the influx and metabolism after a short availability of the fatty acid (15 min) and after a longer period (60 min). Rats adapted to long chain fatty acid metabolism by feeding partially hydrogenated marine oil were compared with rats fed a palm oil diet; the diets were adjusted to the same content of linoleic acid.

The distribution of erucic acid and its shortened-chain metabolites in various liver lipid classes is given, and the influence of dietary fatty acids on the deposition will be discussed.

MATERIALS AND METHODS

Animal experiments. Male weanling rats (Wistar, specific pathogen-free) obtained from Møllegård's Breeding Laboratory Aps. (Ll. Skensved, Denmark) were for 8 wk fed experimental diets containing, by weight, 20% fat, 20% casein, 40% corn starch, 10% sucrose, 0.5% choline chloride, 4% cellulose powder and appropriate amounts of vitamins and trace elements as described previously (10). Two dietary fats were used, either PHMO containing relatively high amounts of 22:1 and 20:1 isomers as well as 18:1 and 16:1, or PO with 18:1(n-9) as the major monoenoic fatty acid. To assure the same adequate amount of essential fatty acids in the two dietary groups, a supplement of sunflower seed oil was added to the PHMO diet. The composition of the dietary fats is shown in Table 1. Diet and water were given ad libitum. The rats were kept in a room temperature of 25 C and a relative

TABLE 1

Fatty Acid Composition (%) of Dietary Fats (Determined by GLC)[a]

Fatty acid	Dietary fat	
	PHMO	PO
12:0	0.1	0.3
14:0	5.6	1.2
16:0	12.4	42.8
16:1	8.5	0.3
18:0	3.1	5.4
18:1	16.6	38.6
18:2, c,t + t,c	1.6	—
18:2, c,c	10.5	10.8
20:0	1.4	0.3
20:1 c + t	13.4	—
20:2 c,t + t,c	3.5	0.2
20:2 c,c + 20:3	2.2	—
22:0	1.2	—
22:1 c + t	12.2	—
22:2 c,t + t,c	2.2	—
22:2 c,c + 22:3	3.4	—

[a]PHMO, partially hydrogenated marine oil + sunflower seed oil (87:13, v/v). PO, palm oil.

humidity of 45%. All animals were fasted overnight (16-18 hr) before liver perfusions were performed.

Liver perfusion. The perfusions were carried out essentially as described by Seglen (11). The perfusion medium consisted of 50 ml Krebs-Henseleit bicarbonate buffer, pH 7.5, 0.5% glucose and 0.028% $CaCl_2$; an erucic acid-albumin complex (12) was added to give a final concentration of 1 mM and 14.1 mg/ml, respectively. The labeled erucic acid was measured to 0.05 μCi/ml. The perfusion medium was continuously gassed with 95% O_2-5% CO_2 and recirculated through the liver at a flow rate of 35 ml/min for 15 or 60 min. At the termination of the perfusion the liver was rinsed with Krebs-Henseleit buffer to remove residual perfusate. Then the liver was blotted, inspected for adhering nonhepatic tissue and weighed.

Chemicals. All chemicals were of analytical grade, and all solvents were redistilled before use. [14-^{14}C]Erucic acid (50 mCi/mmol) was obtained from Centre d'Etude Nucléaires (Gif-sur-Yvette, Desclay, France).

Bovine serum albumin (essentially fatty acid-free) and erucic acid (99% purity) were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Di-methyl-di-chlorosilane (DMCS) for impregnating silica gel plates was delivered by Machery-Nagel (Düren, Federal Republic of Germany). Lipid standards for thin layer chromatography (TLC) and GLC were from Nu-Chek-Prep (Elysian, Minnesota).

Analytical methods. Total liver lipids were extracted with chloroform/methanol (2:1, v/v) as reported earlier (13).

Lipid classes were separated by TLC on silica gel G in hexane/diethyl ether/acetic acid (80:20:1, v/v/v) (14). Fractions were scraped off and used either for quantitation of radioactivity by scintillation counting in a Beckman LS 250 (10 ml toluene with 0.4% PPO and 0.01% dimethyl POPOP as scintillation fluid) or for the preparation of methyl esters (14) for further analysis including determination of the total fatty acid pattern by GLC. The GLC-analyses were performed on a glass column (9′ × 2 mm) with 10% SP 2330 on 100/120 Chromosorb W, AW (Supelco, Bellefonte, Pennsylvania), operated from 170 to 240 C, 2 C per minute. The internal standard for quantitation, pentadecanoic acid (15:0), was added to the silica gel scrapings.

The distribution of radioactivity in the various unsaturated fatty acids (me-esters) from triglycerides, free fatty acids and phospholipids was measured on fractions separated by argentation TLC (12.5% $AgNO_3$ in silica gel G, 0.25 mm; solvent system of benzene/hexane [6:4, v/v], two developments). This system was also used to isolate monoene fractions, which after extraction with diethyl ether/water (99:1, v/v) were separated on DMCS-treated silica gel G plates (0.25 mm) according to chain length (solvent system of acetonitrile/methanol/water [5:5:1, v/v/v], two developments). The bands were visualized with iodine vapor. Scintillation counting was made on scrapings when iodine had evaporated from the plate.

Statistical methods. The results are expressed as mean ± SD. The level of significance was evaluated by Student's t-test.

RESULTS

Use of [14-^{14}C]erucic acid by the liver. The total uptake of erucic acid and the distribution of radioactivity in rat liver after perfusion with [14-^{14}C]erucic acid is shown in Table 2. The use of [14-^{14}C]labeling instead of [1-^{14}C]labeling allowed a tracing of the incorporated fatty acid backbone in various lipid classes, even after 60 min of perfusion. The total deposition of erucic acid in the liver tissue (nmol/g) was equal for both perfusion times, and no difference between the two dietary groups was observed. The observations after 15 min resemble those just after a high fat meal, when the fatty acids have been taken up by the liver, whereas the 60 min of perfusion represents a steady state of metabolism. Most of the radioactivity was as expected, more than 90%, in the lipid soluble fraction. Only 6-9% of the radioactivity was

TABLE 2

Total Uptake of Erucic Acid and Distribution of Radioactivity in Rat Liver Fractions after Perfusion with [14-^{14}C]Erucic Acid

Dietary fat	Perfusion time (min)	Erucic acid uptake (nmol/g)	Distribution of tissue radioactivity (%)		Distribution of tissue lipid radioactivity (%)				
			Water-soluble	Lipid-soluble	TG	PL	FFA	CHE	DG
PHMO	15	1635 ± 300	7.0 ± 1.5	93.0 ± 1.5	62.3 ± 4.0^{c}	14.3 ± 0.6^{a,c}	16.0 ± 2.7^{c}	1.8 ± 0.8	5.6 ± 1.0^{a}
PHMO	60	1720 ± 470	8.8 ± 1.9	91.2 ± 1.9	61.3 ± 8.9	18.7 ± 3.3^{d}	14.7 ± 7.7	2.9 ± 1.4	3.0 ± 0.4
PO	15	1490 ± 325	6.4 ± 1.3	93.6 ± 1.3	53.5 ± 4.3^{a}	11.7 ± 0.4^{a}	27.5 ± 5.4^{a}	1.4 ± 0.2^{b}	6.0 ± 2.0^{a}
PO	60	1895 ± 280	5.7 ± 1.1	94.4 ± 1.1	68.7 ± 6.7	14.4 ± 1.5	10.8 ± 3.7	2.5 ± 1.4	3.6 ± 0.4

Data are presented as mean ± SD of four determinations. Tissue uptake is calculated as nmol fatty acids/g liver (wet wt) after 15 and 60 min of perfusion with 1 mM erucic acid (initial radioactivity in the perfusate was 59 × 10^5 cpm).

Abbreviations: PHMO, partially hydrogenated marine oil + sunflower seed oil (87:13, v/v); PO, palm oil; TG, triglycerides; PL, phospholipids; FFA, free fatty acids; CHE, cholesterol esters; DG, diglycerides. (Trace of cholesterol may be included).

$^{a}p < 0.01$, 15 min vs 60 min.

$^{b}p < 0.05$, 15 min vs 60 min.

$^{c}p < 0.01$, PHMO vs PO.

$^{d}p < 0.05$, PHMO vs PO.

found in the water-soluble fraction, representing fatty acid CoA-esters, ketone bodies and other short chain metabolites. After 60 min of perfusion, a significant difference was observed between the two dietary groups in the water-soluble fractions. The higher value (9%) for the PHMO group indicates a greater oxidation rate than for the group fed PO (6%). The difference in the metabolic state between the two perfusion times is more clearly illustrated in the distribution of labeling among lipid classes (Table 2). For both dietary groups and both perfusion times the major part (65–80%) of the radioactivity was present in the TG. Only 10–25% of the labeling was found in the FFA fractions and 11–19% in the PL.

The group fed PO showed a significantly higher value (28%) for FFA after 15 min compared to the PHMO group (16%); further, a substantial decrease was observed after 60 min (11%) in the PO group, but not in the PHMO group. This decrease was counterbalanced by increased values mainly in the TG, but also in the PL. In the PHMO group a small increase was found only in the PL. The differences between the two groups reflect the different metabolism of long chain fatty acids, indicating a higher turnover rate of erucic acid in the PHMO livers already adapted to long chain monoenoic fatty acid metabolism.

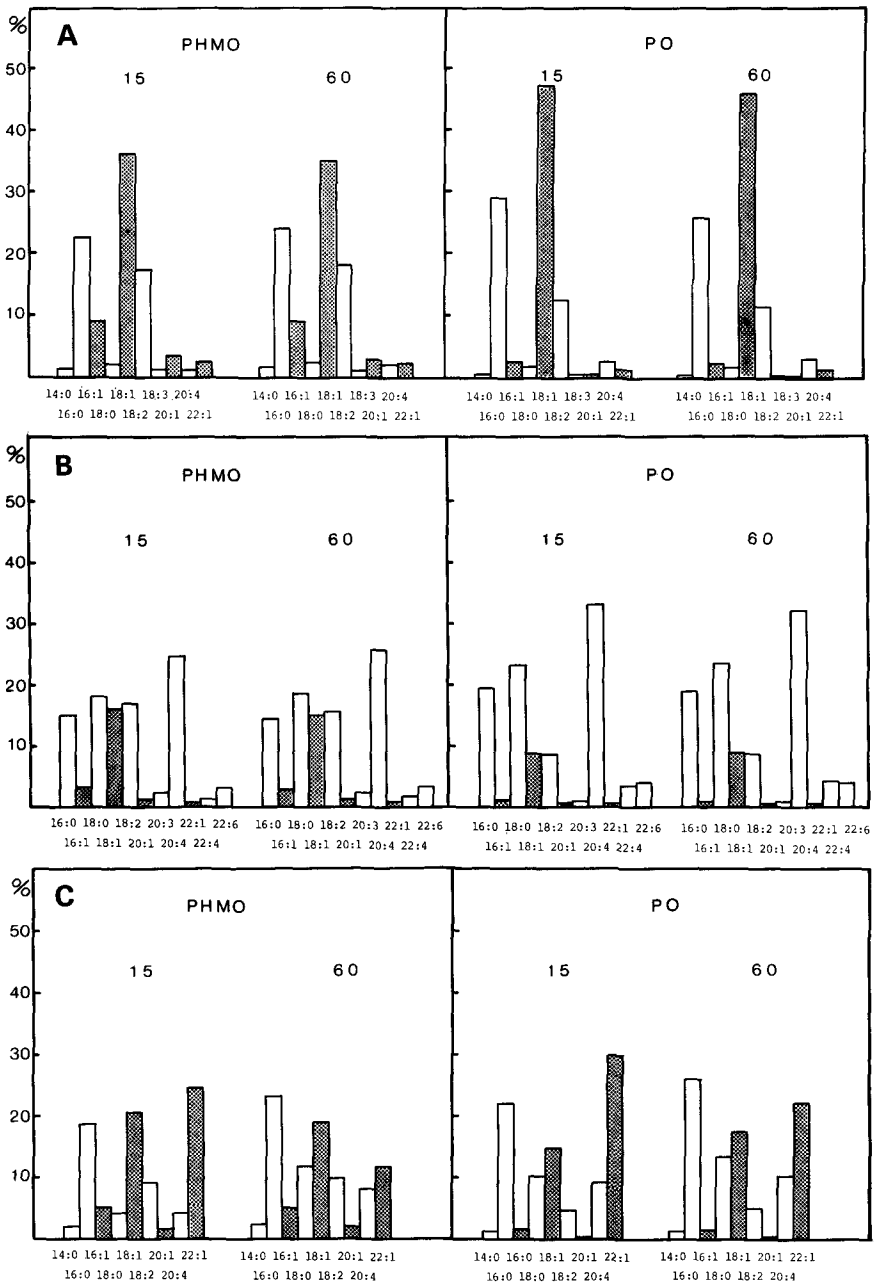

FIG. 1. The fatty acid pattern in liver lipid classes from rats fed diets containing PHMO or PO. (A) Triglycerides; (B) phospholipids; (C) free fatty acids. Perfusion times were 15 and 60 min. Shaded vertical bars indicate monoenoic fatty acids. For experimental conditions see Materials and Methods.

The labeling in the remaining lipid classes, cholesterol-esters and diglycerides was much lower, and equal for the two groups.

Fatty acid distribution in liver lipid classes. The fatty acid patterns of TG, PL and FFA as determined by GLC are shown in Figure 1. The TG (Fig. 1A) reflected the composition of the dietary fats, and there was no difference between 15 and 60 min of perfusion. The perfusion fatty acid, erucic acid, only slightly influenced the total distribution. A deposition of 1.5% 22:1 was found in the rats fed palm oil, but despite the presence of 11% 22:1 in the PHMO, only about 2.5% was deposited in the liver TG.

For the PL (Fig. 1B), both dietary groups showed only a negligible amount of 22:1, although 11-20% of the total radioactivity was found in this lipid class. Shortened-chain products and, possibly, small amounts of de novo synthesized fatty acids account for the labeling. As previously reported (13,15), a decreased conversion of linoleic acid to arachidonic acid in rats fed PHMO was observed.

The total FFA in the liver tissue was rather small as normally found, whereas the radioactivity in this fraction was rather high (Table 2), as would be expected because of a high percentage of labeled 22:1 (Fig. 1C). Even after 60 min of perfusion the 22:1 accounted for a considerable part of the fatty acid pool available for biosynthesis. The lower values for 22:1 in the PHMO group compared to the PO group, for both 15 and 60 min of perfusion, support the findings of induced chain-shortening activity in the liver when partially hydrogenated marine oils are fed to rats. The presence of relatively lower amounts of arachidonic acid in the PHMO group and higher 18:2 compared to the PO group is consistent with the polyunsaturated fatty acid (PUFA) deposition in PL mentioned above, reflecting a decreased desaturation in the FFA pools before esterification.

Incorporation of monoenoic fatty acids in liver lipid classes after perfusion with erucic acid. The total fatty acids from various lipid classes were separated according to unsaturation by Ag^+-TLC. For TG, PL and FFA, the major part of the radioactivity (80-95%) was found in the monoenoic fatty acids for both perfusion times (data not shown). The remaining part was either present in saturated fatty acids, formed by de novo synthesis from labeled acetyl-CoA, or in polyunsaturated fatty acids as a result of chain elongation and desaturation.

The major part of the infused labeling was always found in the TG and the highest values in livers from rats fed PO after 60 min of perfusion. A significant increase in the total radioactivity of PL was observed for both dietary groups from 15 to 60 min.

The monoenoic fatty acids were subsequently analyzed on reversed-phase TLC according to chain length (Table 3).

In the TG, the radioactivity was still present mainly as [14-^{14}C]erucic acid; this acid therefore is not discriminated in TG synthesis. After 60 min of perfusion, a significantly lower deposition was observed in the

TABLE 3

Incorporation of Monoenoic Fatty Acids in Triglycerides (TG), Phospholipids (PL) and Free Fatty Acids (FFA) from Rat Livers Perfused with 1 mM Erucic Acid

	PHMO			PO		
	TG	PL	FFA	TG	PL	FFA
15-Min perfusion						
Monoenoic fatty acids incorporated (nmol/g liver)	945 ± 60[a]	175 ± 5[a]	205 ± 10[a]	715 ± 5[a]	135 ± 5[a]	325 ± 15[a]
Fatty acid, % of monoenoic						
<16:1	trace	trace	2.5 ± 0.9	trace	trace	3.3 ± 1.7
16:1	1.4 ± 0.5	2.4 ± 1.0	4.2 ± 3.6	0.8 ± 0.4	2.5 ± 0.4	3.2 ± 3.2
18:1	21.0 ± 1.9[a]	43.9 ± 7.3[a]	1.7 ± 0.6	18.1 ± 3.8	38.8 ± 13.3	1.9 ± 0.8
20:1	5.1 ± 1.3	10.6 ± 0.9[c]	5.2 ± 2.9	3.4 ± 1.3	7.4 ± 0.7	8.3 ± 4.3
22:1	72.9 ± 1.2[a]	43.1 ± 6.3[a]	86.2 ± 0.8[a]	77.7 ± 4.9[b]	51.5 ± 14.4	83.4 ± 6.8
60-Min perfusion						
Monoenoic fatty acids incorporated (nmol/g liver)	745 ± 30	235 ± 5	185 ± 10	1130 ± 40	210 ± 5	160 ± 15
Fatty acid, % of monoenoic						
<16:1	trace	trace	3.6 ± 0.9	trace	trace	3.8 ± 0.2
16:1	0.9 ± 0.6	3.3 ± 0.6	2.0 ± 1.0	0.5 ± 0.4	3.1 ± 0.9	5.3 ± 3.5
18:1	33.8 ± 11.0[c]	64.8 ± 2.2[c]	1.7 ± 1.6	20.8 ± 2.8	44.5 ± 3.1	1.5 ± 1.5
20:1	6.2 ± 1.6	11.5 ± 1.7	8.8 ± 2.4	4.9 ± 1.2	9.7 ± 1.7	4.6 ± 1.7
22:1	59.2 ± 8.7[c]	20.4 ± 1.8[c]	82.6 ± 1.0	72.8 ± 1.7	42.8 ± 1.9	85.0 ± 5.0

Total monoenoic fatty acids derived from erucic acid are expressed as nmol fatty acids/g liver. Distribution according to chain length is given as percentages of total labeled monoenoic fatty acids. For details, see Materials and Methods. Data are presented as mean ± SD of four determinations. For abbreviations, see Table 2.

[a] $p < 0.01$, 15 min vs 60 min.

[b] $p <$ 15 min vs 60 min.

[c] $p < 0.001$, PHMO vs PO.

PHMO group compared to PO-fed rat liver due to peroxisomal activity. The main shortened-chain product was 18:1, and remarkably small amounts of 20:1 were present. Other labeled monoenoic acids were practically absent in the TG.

For PL, the major radioactive fatty acids were oleic (18:1) and erucic (22:1) acids, but 20:1 contributed relatively more in this lipid class. Other labeled monoenoic acids were present only in minor quantities. The chain-shortening capacity was again higher in the PHMO group, reflected by a major amount (40–45%) of 18:1(n-9) after 15 min, increasing to about 65% after 60 min of perfusion. The amount of 18:1 found in the PO group for both perfusion times corresponded to that shown for the PHMO-fed rats after 15 min. When available, oleic acid is highly preferred for PL synthesis.

For FFA, the major part of the radioactivity (about 80%) was present as erucic acid, even after 60 min of perfusion. A striking difference from the other lipid classes analyzed was the near absence of 18:1. Shortened-chain 18:1 is rapidly removed either for esterification or further degradation. The latter is supported by the presence of appreciable amounts of acids with shorter chain lengths (16:1 and <16:1). In other lipid classes chain lengths of <16:1 were almost absent.

DISCUSSION

Perfusion studies elucidating fatty acid metabolism have commonly been carried out with organs from rats fed laboratory pellet diets (3–5), which may introduce metabolic capacities different for specific fatty acids. Thus it is known that high fat diets and especially those containing very long chain monoenoic fatty acids induce a peroxisomal oxidative degradation pathway (6–9).

In the present studies the metabolism of [14-^{14}C]erucic acid in rats adapted to a high fat diet containing long chain fatty acids (PHMO) was compared to rats fed PO, without such fatty acids. The use of a [14-^{14}C]- instead of a [1-^{14}C]labeled erucic acid allowed a better tracing of shortened-chain products, and the scattering of radioactivity in saturated and polyenoic fatty acids was also kept at a minimum. The rats were fasted overnight. Perfusion times were chosen to simulate the primary influx in the liver after a fat-rich meal (15 min) and the fed state with ample supply of 22:1 (60 min).

Christiansen et al. (7) made similar dietary experiments with rats but focused on the distribution of TG in the liver and the exported very low density lipoprotein (VLDL)-TG after 3 hr of perfusion. It was found that the accumulated labeled VLDL-TG pool was less than 5% after 3 hr and only about 2% after 1 hr. The labeling of the liver lipids under the present experimental conditions (15 and 60 min) should, therefore, reflect the metabolism of [14-^{14}C]erucic acid. After 15 min of perfusion the major part of the radioactivity was esterified in TG mainly as [14-^{14}C]erucic acid, with slightly higher values for shortened-chain products obtained in the group adapted to long chain fatty acid metabolism. Carbon chains shorter than 16:1 were present only in negligible amounts.

For the phospholipids, much lower total incorporation was apparent, with a slightly higher value for the group fed PHMO. This shows the low affinity of 22:1 for PL synthesis, whereas the 18:1 metabolite, resulting from increased peroxisomal oxidation in the adapted (PHMO) rat, is readily incorporated.

The FFA fraction represents mainly the recent uptake of [14-^{14}C]erucic acid for both dietary groups (83–86%), but in contrast to the above-mentioned lipid classes, very small amounts of [14-^{14}C]18:1 (1.7–1.9%) were present, showing a rapid esterification of this shortened-chain metabolite into TG and especially into PL. Metabolites with chain lengths shorter than 16:1 were present in this fraction in contrast to other lipid classes, indicating the extent of the oxidative degradation of the infused fatty acid.

The increased amount of labeled FFA after 15 min in the livers of rats fed PO shows the lower peroxisomal oxidation rate for the transformation of [14-^{14}C]erucic acid into 18:1 in this group.

The incorporation of labeling in TG after 60 min of perfusion was still the highest among the lipid classes, but redistribution among chain lengths was observed for the PHMO-fed rats, showing that in the TG also the preferential form of fatty acid storage was as 18:1. This change was even more pronounced for the PL and the chain-shortening capacity was highest in the PHMO-adapted rats, so that the PO group after 60 min was comparable to the PHMO group after 15 min.

The total fatty acid composition of PL from the liver of rats fed PHMO showed only minor amounts of 22:1 and 20:1, although the diet contained 12–13% of both. This confirms that the liver is capable of degrading the long chain monoenoic fatty acid to the preferred 18-chain between meals, consistent with the finding of much lower [14-^{14}C]22:1 incorporated after 60 min of perfusion.

These results stress the importance of dietary background for the metabolism of specific fatty acids. Adaptation to diets is one of the factors governing the rates of oxidation and esterification of fatty acids in the liver cell. The present study also confirms the importance of the chain shortening of long chain monoenoic fatty acids before use in the general liver metabolism of lipids.

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Effect of Epinephrine on the Oxidative Desaturation of Fatty Acids in the Rat Adrenal Gland

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Delta-6 and Δ5 desaturation activity of rat adrenal gland microsomes was studied to determine the effect of microsomal protein and the substrate saturation curves. This tissue has a very active Δ6 desaturase for linoleic and α-linolenic acids and a Δ5 desaturase for eicosa-8,11,14-trienoic acid. The administration of epinephrine (1 mg/kg body weight) 12 hr before killing, produced approximately a 50% decrease in desaturation of [1-^{14}C]linoleic acid to γ-linolenic acid, [1-^{14}C]α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid and [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid. A 30% decrease in Δ5 desaturation activity was also shown after 7 hr of epinephrine treatment. The changes on the oxidative desaturation of the same fatty acids in liver microsomes were similar. No changes were observed in the total fatty acid composition of adrenal microsomes 12 hr after epinephrine treatment. Mechanisms of action of the hormone on the biosynthesis of polyunsaturated fatty acids in the adrenal gland are discussed.
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The biosynthesis of polyunsaturated fatty acids and their regulation have been extensively studied in liver of mammalian animals, where linoleic and arachidonic acids are the major components of essential fatty acids. In certain tissues, such as the adrenal gland, polyunsaturated acids other than arachidonic acid predominate or are present in significant concentrations. The adrenal gland of the rat contains large amounts of docosa-7,10,13,16-tetraenoic acid (adrenoic acid) belonging to the linoleic acid family (1-3). Another interesting observation is that in this tissue in normal animals, the aforementioned acids as well as arachidonic acid are characteristic of the cholesterol ester fraction and not of phospholipids, the most usual source of tissue polyunsaturated fatty acids (4-6).

It is also known that the biosynthesis of unsaturated fatty acids is under hormonal control (7). In this respect, it was demonstrated that epinephrine decreases Δ6 and Δ5 desaturation activity in liver microsomes (8,9). This effect was considered to be either a direct consequence of an increase of the intracellular concentration of cyclic AMP or an indirect effect evoked probably by a product liberated to the circulation by the action of the hormone.

In view of the aforementioned evidences, our experiments were designed, first, to find the best laboratory conditions to measure Δ6 and Δ5 desaturation activity in adrenal gland microsomes of rats and, second, to study the influence of the administration of epinephrine on the oxidative desaturation of linoleic, α-linolenic and eicosa-8,11,14-trienoic acids and the fatty acid composition of the rat adrenal microsomes.

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MATERIALS AND METHODS

[1-^{14}C]Linoleic (52.6 mCi/mmol, 99% radiochemical purity), [1-^{14}C]eicosa-8,11,14-trienoic (54.9 mCi/mmol, 99% radiochemical purity) and [1-^{14}C]α-linolenic (51.0 mCi/mol, 98% radiochemical purity) acids were purchased from New England Nuclear Corp. (Boston, Massachusetts). NADH, ATP, CoA and other factors were provided by Sigma Chemical Co. (St. Louis, Missouri). All other chemicals used were of analytical grade.

Animals and treatment of animals. One-hundred and eighty female Wistar rats (200-250 g) fed on a commercial diet (Cargill type C) and water ad libitum, were used. The rats were fasted for 24 hr and refed with commercial diet for two hr. They were killed 7 or 12 hr after the refeeding period. To test the epinephrine effect, the rats were grouped in lots of 20 animals each. At the end of the aforementioned refeeding period, two groups of rats were injected subcutaneously with epinephrine (1 mg/kg body weight); one group was killed 7 hr and the other 12 hr after the injection. For each period of treatment the rats used as controls were treated identically except for the substitution of 0.9% saline for epinephrine.

Isolation of microsomes. The rats were killed by cervical dislocation. Liver and adrenal glands were immediately removed and placed in ice-cold homogenizing solution containing 0.25 M sucrose, 62 mM phosphate buffer pH 7.0, 0.15 M KCl, 5 mM $MgCl_2$ and 100 μM EDTA. The adrenal glands were trimmed of surrounding fat and were decapsulated. Livers or adrenal glands of each group of rats were pooled separately, weighed after blotting on filter paper and gently homogenized in 1:3 (w/v) homogenizing solution.

The microsomal fractions from pooled organs were separated by differential centrifugation at 100,000 g as described previously (10). Protein content was measured by the method of Lowry et al. (11).

In vitro enzyme assays. The desaturation of the fatty acids by liver and adrenal microsomes was measured by estimation of the percentage conversion of [1-^{14}C]linoleic, [1-^{14}C]α-linolenic and [1-^{14}C]eicosa-8,11,14-trienoic acids to their corresponding products.

The desaturation activity of the microsomes was determined at 37 C in air, incubating the microsomal protein with the labeled substrate diluted with unlabeled fatty acid to maintain 250,000 cpm in each tube. The medium contained 4 μmol ATP, 0.1 μmol CoA, 1.25 μmol NADH, 5 μmol $MgCl_2$, 2.25 μmol glutathione, 62.5 μmol NaF, 0.5 μmol nicotinamide and 62.5 μmol phosphate buffer (pH 7) in a total volume of 1.5 ml of 0.15 M KCl, 0.25 M sucrose solution. Incubations were routinely performed in triplicate.

The tubes containing substrate and cofactors were placed in a metabolic shaker for 60 sec at 37 C; then the reaction was started by addition of the microsomal suspension to the incubation medium. The incubation was carried out for 10 min and then the reaction was stopped by the addition of 2 ml of 10% KOH in ethanol. After 45

min of saponification at 85 C under nitrogen, the acidified solution was extracted with petroleum ether (bp 30–40 C). The fatty acids were esterified with methanolic 3 M HCl (3 hr at 68 C) and the distribution of the radioactivity between substrate and product was determined by gas liquid radiochromatography in an apparatus equipped with a Packard proportional counter. The column was packed with 15% EGSS-X (ethylene glycol succinate) coated on Chromosorb WHP (80–100 mesh), Supelco, Inc. (Bellefonte, Pennsylvania). Percentage of conversion was calculated from the distribution of radioactivity between substrate and product measured directly on the radiochromatograms. The specific activity expressed as nmol of product per mg of microsomal protein per min was calculated from these data. The fatty acid methyl esters were identified by comparison with authentic standards.

Experiments performed. Experiments 1 and 2 were designed to find out the best conditions for the estimation in vitro of the oxidative desaturation of fatty acids at 6 and 5 positions in adrenal microsomes. Similar assays were done simultaneously with liver microsomes. The microsomal fractions of liver and adrenal glands used in the first and second experiments were obtained from a pool of 100 rats. In experiment 1 different weights of liver and adrenal microsomal protein (0.5 to 2.5 mg) were incubated with 10 nmol (6.7 μM) of [1-^{14}C]linoleic and [1-^{14}C]eicosa-8,11,14-trienoic acids. In experiment 2 the substrate saturation curves for different fatty acids were determined. In this case, to measure Δ6 desaturation activity, 1.5 mg of microsomal protein and increasing amounts of linoleic acid were used. To determine Δ5 desaturation activity, different amounts of eicosa-8,11,14-trienoic acid were incubated in the presence of 1 mg microsomal protein.

Experiment 3 was designed to show the effect of 12 hr of epinephrine administration on the oxidative desaturation of linoleic, α-linolenic and eicosa-8,11,14-trienoic acids by adrenal and liver microsomes. Adrenal microsomal Δ6 desaturation was measured incubating 1.5 mg protein and 20 nmol (13.4 μM) of labeled linoleic or α-linolenic acids.

The Δ5 desaturation activity was measured with 1 mg adrenal microsomal protein and 15 nmol of labeled eicosa-8,11,14-trienoic acid.

The amount of fatty acid incubated was calculated from the corresponding substrate saturation curves found in experiment 2, so that the speed of the reaction was independent of the substrate concentration.

In experiment 4 the effect of 7 hr of epinephrine treatment on Δ5 desaturation activity was determined. The conditions of the assay used were the same as those in the third experiment.

In the third and fourth experiments, liver microsomal Δ6 and Δ5 desaturation activity was measured simultaneously with 50 nmol (33.5 μM) of the radioactive substrates and 2.5 mg protein. The amounts of substrate and product used in liver microsomal measurements were similar to those used in previous experiments where the effect of epinephrine was first described (8).

Total fatty acid microsomal analysis. Microsomal lipids were extracted with chloroform/methanol (2:1, v/v) following the procedure of Folch et al. (12). The fatty acids were converted to methyl esters and analyzed in a Hewlett-Packard, model 5840 A, gas liquid chromatograph equipped with a flame ionization detector. The column was packed with 10% SP 2330 coated on Chromosorb WAW 100–200 mesh, Supelco Inc. (Bellefonte, Pennsylvania). The oven temperature was programmed from 140–220 C at 3 C/min to separate methyl esters ranging from 12 to 22:6ω3. Retention time and peak areas were determined electronically using a Hewlett-Packard Reporting Integrator. Identification of methyl esters was made by comparison with known methyl ester standards.

RESULTS

Figure 1 shows the nmol of substrate converted to products per minute by liver and adrenal gland as a function of the amount of microsomal protein added to the incubation medium. Since analyses of pooled adrenal and liver samples from 100 animals were used in the first and second experiments, the statistical significance was not determined. This figure shows that the adrenal gland has enzymes able to desaturate linoleic to γ-linolenic acid and eicosa-8,11,14-trienoic acid to arachidonic acid. The measured enzyme activity was protein-dependent and at the same protein level Δ5 desaturase was more active than Δ6 desaturase. In these conditions, Δ6 and Δ5 desaturase activities measured in the liver were similar.

The substrate saturation curves for the liver and adrenal gland desaturation enzyme are shown in Figure 2. As the concentration of substrate is increased the initial velocity of both enzymes is also increased. The behavior of each enzyme was similar in liver and in adrenals. The Δ5 desaturase seems to be completely saturated at 6.7 μM of eicosatrienoic acid, the lowest concentration substrate

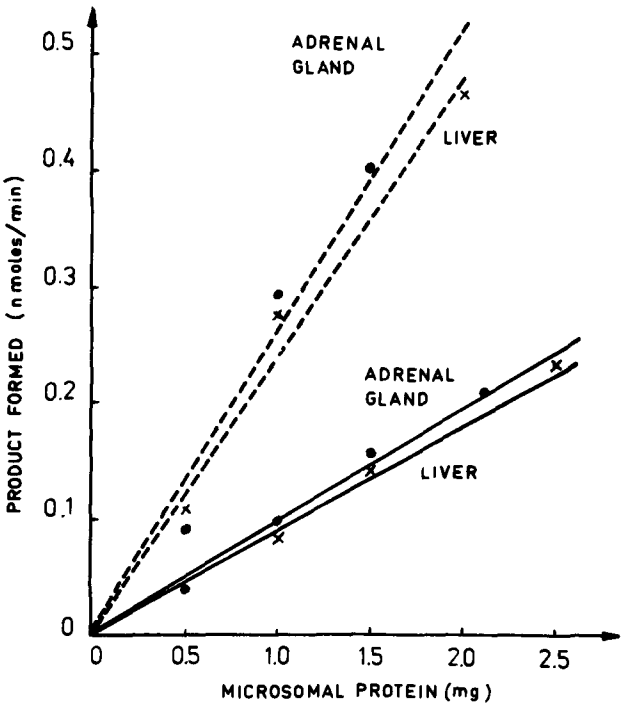

FIG. 1. Effect of the amount of microsomal protein upon the oxidative desaturation of [1-^{14}C]linoleic acid (——) and [1-^{14}C]eicosa-8,11,14-trienoic acid (— — —) of adrenal gland and liver. Results are the mean of triplicate determinations made for each point. Technical details are described in the text.

TABLE 1

Effect of Epinephrine Administration (1 mg/kg Body Weight) on Δ6 and Δ5 Desaturation Activity[a] in Adrenal Microsomes

	Time of treatment (hr)	Δ6 Desaturation		Δ5 Desaturation, eicosatrienoic acid
		Linoleic acid	α-Linolenic acid	
Control	12	0.313 ± 0.018	0.318 ± 0.020	0.520 ± 0.025
Epinephrine	12	0.157 ± 0.014	0.133 ± 0.028	0.260 ± 0.013
Control	7	—	—	0.335 ± 0.029
Epinephrine	7	—	—	0.240 ± 0.010

Results are the mean of pooled adrenal glands of 20 rats analyzed in triplicate ±1 S.D. Technical details are described in the text.

[a]nmol of substrates converted/mg of protein/min.

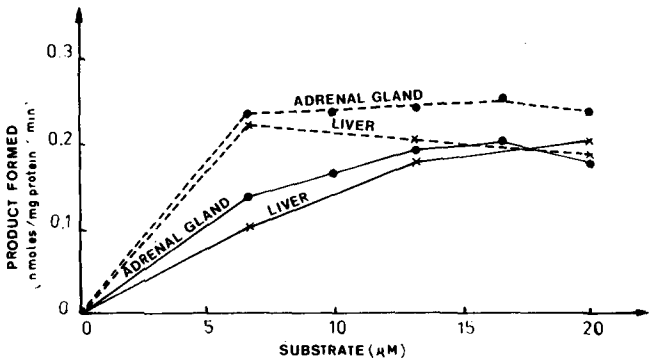

FIG. 2. Substrate saturation curves for the desaturation of [1-^{14}C]linoleic acid (——) and [1-^{14}C]eicosa-8,11,14-trienoic acid (– – – –) in adrenal gland and liver microsomes. Microsomal protein, 1.5 and 1 mg, was incubated to measure Δ6 and Δ5 desaturation activity, respectively, at 37 C for 10 min under conditions described in the text. Triplicate determinations were made for each point.

TABLE 2

Comparative Fatty Acid Composition of Total Lipids (Wt %) of Adrenal Microsomes of 12-Hr Epinephrine-treated and Control Rats

Fatty acids	Control	Epinephrine-treated
16:0	7.0 ± 0.1[a]	7.0 ± 0.1[a]
18:0	32.5 ± 0.2	33.0 ± 0.06
18:1	8.0 ± 0.3	8.0 ± 0.3
18:2	4.0 ± 0.2	4.0 ± 0.2
20:3ω6	2.0 ± 0.1	2.0 ± 0.05
20:4ω6	39.0 ± 0.3	38.5 ± 0.1
22:4ω6	4.5 ± 0.2	4.5 ± 0.3
22:5ω6	1.0 ± 0.1	1.0 ± 0.25
22:5ω3	1.0 ± 0.05	1.0 ± 0.2
22:6ω3	1.0 ± 0.1	1.0 ± 0.1

Only fatty acids over 1% were tabulated.

[a]Results are the mean of pooled adrenal glands of 20 rats analyzed in triplicate ±1 S.D.

used, while Δ6 desaturase reaches the plateau at about 13.4 μM linoleic acid concentration.

The effect of epinephrine administration on the specific activity of Δ6 and Δ5 desaturases of rat adrenal gland is shown in Table 1. The data represent analyses of pooled adrenal samples from 20 animals in each group. They also demonstrate that the adrenal gland has a very active Δ6 desaturase that converts α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid. Twelve hours of epinephrine administration produced approximately a 50% decrease on the conversion of linoleic to γ-linolenic acid, α-linolenic to octadeca-6,9,12,15-tetraenoic acid and eicosa-8,11,14-trienoic to arachidonic acid. A 30% inhibition on Δ5 desaturation activity was also shown after 7 hr of epinephrine treatment. A similar inhibition on Δ6 and Δ5 desaturase activity was also shown in liver microsomes of the rats treated with the hormone.

Table 2 presents the total fatty acid composition of adrenal gland microsomes in control animals and in rats treated with epinephrine. Minor components have been omitted for the sake of clarity. The major fatty acids of adrenal microsomes of control rats and rats treated with epinephrine were oleic and arachidonic acids. Docosa-7,10,13,16-tetraenoic acid is specifically present in a significant concentration in the adrenal microsomes. The administration of a pulse of epinephrine produced no changes on the total fatty acid composition of the adrenal microsomes 12 hr after the injection.

DISCUSSION

Under normal dietary conditions rat liver lipids contain high levels of both linoleate and arachidonate but only low levels or trace amounts of the other acids derived from dietary linoleate. In the linoleate metabolic sequence, linoleate is desaturated to the 6,9,12 -18:3 acid, which is rapidly converted by chain elongation to 8,11,14-20:3 and then desaturated to arachidonate. Although arachidonate is chain-elongated to 7,10,13,16-22:4 at a reasonable rate in vitro (13), this acid and 4,7,10,13,16-22:5 are not found in large amounts in liver lipids. Besides, when rats are fed with 7,10,13,16-22:4 this acid is not accumulated in liver lipids (13) but is retroconverted to arachidonate, which is then incorporated into the lipids

(14). However, adrenoic acid is an important component of adrenals.

From the results shown in Figure 1, it is possible to establish that adrenal gland microsomes possess very active Δ6 and Δ5 desaturases. The existence of a measurable conversion of linoleic to γ-linolenic acid was already demonstrated in a previous preliminary work (15). However, the present work proves that the gland also desaturates α-linolenic and eicosa-8,11,14-trienoic acids. The behavior of the adrenal Δ6 and Δ5 desaturases is similar to that found in the liver of the same animals, indicating a similar mechanism for arachidonic acid biosynthesis. The substrate saturation curves shown in Figure 2 reveal that the initial velocity of the enzyme was independent of substrate concentration, from 6.7 μM to 20 μM of eicosa-8,11,14-trienoic acid and from 13.4 μM to 20 μM of linoleic acid. Therefore, 13.4 μM of linoleic acid and 6.7 μM of eicosatrienoic acid were chosen for the enzyme activity assays. A similar concentration of 13.4 μM α-linolenic acid was used because of the similarity of linoleic and α-linolenic acid saturation curves.

Several studies carried out in our laboratories have demonstrated the hormonal regulation of Δ6 and Δ5 desaturating activity in rat liver. Epinephrine decreases the activity of both desaturases not only in liver (8,9) but also in the adrenal microsomes (Table 1), demonstrating that is a general function of the hormone not restricted only to liver. The conversion of all the fatty acids studied—linoleic, linolenic and eicosa-8,11,14-trienoic—into their higher homologs decreased about 50% when the animals were treated for 12 hr with epinephrine. A similar decrease was observed when the desaturation of the same acids was measured comparatively in liver microsomes. In this tissue epinephrine had already depressed Δ5 desaturation activity after 3 hr of the administration of the hormone, an effect that was enhanced after 12 hr (9). The adrenal gland has similar behavior—a 30% decrease on the conversion of eicosa-8,11,14-trienoic acid to arachidonic acid was already observed after 7 hr of epinephrine treatment.

The effect of epinephrine on liver Δ6 desaturating activity was postulated to be mediated through an enhancement of the intracellular cyclic AMP levels, since it was mimicked by pharmacological activators of β-receptors and inhibited by β-blockers (8,16). Besides, dibutyryl cAMP administration to intact rats also produced a significant decrease in Δ6 and Δ5 desaturation activity (9,17). Therefore, cyclic AMP levels would apparently trigger the effect produced by the hormone since an increase of the nucleotide was shown 30 min after the administration of the hormone with a return to control levels after 3 to 12 hr. Moreover, the enhancement of cAMP precedes the decreasing of the desaturase activities (8,9).

The existence of adenyl cyclase activity in the plasma membrane of the adrenal gland sensitive to ACTH has been described (18,19). The presence of a cytosolic receptor for cyclic AMP also has been recognized in this tissue (20). Therefore, it is possible that the mechanism of epinephrine action postulated in a previous work (8) may also operate in adrenal glands. A decrease of oleyl-CoA (Δ12) desaturase activity over the first 2 hr after the administration of epinephrine and isoproterenol also was shown by Umeki and Nozawa (21) in Tetrahymena. These authors, like us, suggest that β-adrenergic agonists may modulate the microsomal fatty acyl-CoA desaturase system by acting through the increase of intracellular cyclic AMP content.

Therefore, epinephrine administration would modify the dynamics of polyunsaturated fatty acid synthesis without changing the fatty acid composition of adrenal gland microsomes in the period studied.

ACKNOWLEDGMENTS

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Mechanism of Anesthesia: The Potency of Four Derivatives of Octane Corresponds to their Hydrogen Bonding Capacity

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The anesthetic potency of four derivatives of n-octane was measured by tadpole righting reflex and expressed as effective millimolar concentration of drug in membrane, ED_{50}^{M}. Potency diminished (ED_{50} increased) in this order: 1-octanol, $ED_{50}^{M} = 5.5$; 1-(2-methoxyethoxy)octane, $ED_{50}^{M} = 28$; 1-methoxyoctane, $ED_{50}^{M} = 61$; and 1-chlorooctane, $ED_{50}^{M} > 100$. Since the aliphatic chain length was kept constant it is concluded that the differences in anesthetic potency are a consequence of the differences in head group structure. This result is predicted by a theory (*Lipids 17,* 1001–1003 [1982]) which holds that anesthesia is the result of a drug-induced restructuring of the hydrogen belts, those strata of the membrane that contain the hydrogen bond receiving and donating CO and OH groups of the membrane lipids and the adjoining proteins. The Meyer-Overton rule for anesthetics should be modified: chemicals induce anesthesia at equimolar in-membrane concentration provided their hydrogen-bonding parts are identical.
Lipids 21, 405–408 (1986).

The Meyer-Overton rule of anesthesia affirms that the general anesthetic potency of any compound correlates with its lipid-water partition coefficient, and that anesthesia occurs when a certain drug concentration, equal for all compounds, is reached in the lipids of the neuronal cell (1,2). From studies with alcohols of different chain length it has been determined that "concentration" should be interpreted as *molarity* of the drug in the lipid of the cell membrane (3–6). Numerous lipid theories of anesthesia have sprouted from the rule, most of them agreeing on the following (5,7–14): the drug is taken up by the neuronal cell membrane where it causes a perturbation in the lipid bilayer which then causes a perturbation in the proteins of the membrane, eventually changing the conformation of a protein essential for conductance (e.g., an ion channel protein) so as to inactivate it. Variant theories have the drug interact directly with a membrane protein without mediation by membrane lipid (15–18). If we accept the concept of perturbation we must ask: what is the nature of the perturbation of the bilayer, and what is the mechanism of the lipid-protein (or drug-protein) interaction? At this point, changes in membrane "fluidity," caused by the drug, have often been invoked; but fluidity is a vague and amorphous condition and does not go far as an explanation; neither do presumed changes in bilayer thickness, volume, phase transitions or phase separations, all of which have been proposed (5,19–21). The correlation of one or the other of these factors with anesthesia has frequently been demonstrated, but cause and effect relationships have not been established.

In a theory advanced by us (22), all physical effects of anesthetics on membranes are assumed to be only accidental and unessential. The essential, and universal, property of anesthetics is their ability to restructure the "hydrogen belts" of the neuronal membrane. Lipid theories of anesthesia have, in general, treated the cell membrane as a simple entity, at best divided in two regions, a hydrophobic core and a polar region on each side. In Figure 1 we have given attention also to a region between these layers which is neither hydrophobic nor polar but capable of hydrogen bonding, a zone we call "hydrogen belt" because it is occupied by hydrogen bond acceptors (CO groups of phospholipids) and donors (OH groups of cholesterol, sphingosin, and—we postulate—proteins) (23,24). Normally, donors and acceptors in the membrane are balanced (i.e., each is bonded to another hydrogen belt occupant, including water), so that the hydrogen belt is organized in a distinct pattern. Anesthetic molecules form and break hydrogen bonds in the belt and restructure this pattern. This will affect hydrogen-bonding allosteric sites on the membrane proteins (or the drug may itself interact with such sites). A more thorough exposition of the theory has been given elsewhere (22).

Our theory predicts that variations of the hydrophobic part of an anesthetic molecule, such as the aliphatic chain of an alcohol, will have little influence on its potency; this has, in fact, repeatedly been shown (3–6). The hydrophobic part of the drug serves, we believe, merely as an anchor. On the other hand, changes in the hydrogen-bonding region of a molecule should affect its anesthetic action; this is what we set out to demonstrate in this study.

MATERIALS AND METHODS

Octanol-3,4-$^3H_2$ was prepared by tritiation (Amersham Corp., Arlington Heights, Illinois) from *cis*-3-octen-1-ol (ICN Pharmaceuticals, Plainview, New York). Synthesis

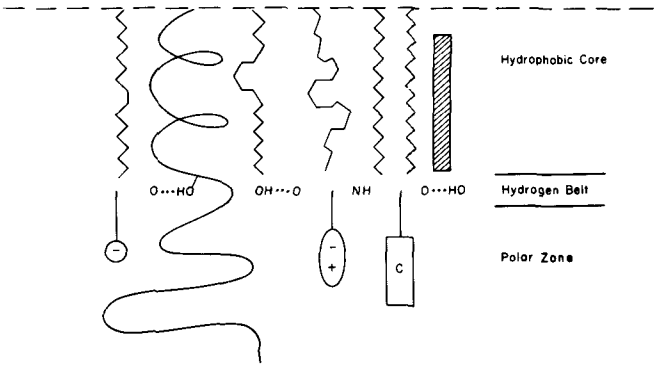

FIG. 1. One-half of a biological membrane, with stratification indicated. Zigzag lines are aliphatic chains; the cross-hatched rectangle is cholesterol; C is carbohydrate, + and − are charged phospholipid heads. In the hydrogen belt, O groups are those of CO of phospho- and sphingolipids; OH groups are from cholesterol, sphingosin and proteins. The helix stands for a membrane protein, a hydroxyl group of which is bonded to a phospholipid carbonyl O group. This kind of hydrogen bonding is thought to be disrupted and restructured by the anesthetic. Adapted from ref. 22.

of the other octane derivatives started from the ^{3}H-octanol so obtained: methoxyoctane via octane-ONa (NaH, tetrahydrofurane) + methyl iodide (25); methoxyethoxyoctane from methoxyethanol → methoxyethyl tosilate → methoxyethyliodide, + octane-ONa (26); chlorooctane from octanol, $SOCl_2$, pyridine (27). The compounds were purified by silicic acid chromatography (hexane/ether) and checked for purity by thin layer chromatography and radioactivity scanning (TLC Scanner, Berthold LB 2760, Wildbad, Federal Republic of Germany); all were >95% pure. Aqueous solutions were prepared by shaking an excess of each compound with nitrogen-purged distilled water overnight and filtering through diatomaceous earth. From these solutions, dilutions were prepared to which the tadpoles were exposed; the precise molarities of these solutions during the experiments were determined by taking samples at 30 min after addition of the tadpoles, measuring the radioactivity, and calculating the molarity from the previously determined specific activity of the compound.

Membrane-water partition coefficients were determined with erythrocyte ghosts as the membrane phase (4,28). It has been shown that erythrocyte ghosts can stand in correctly for neuronal membranes in partitioning studies (4). The volume of the membrane phase was calculated from the protein concentration with the protein content taken as 40% (29) and the density as 1.17. The partition coefficients obtained are dimensionless (concentration/concentration).

Xenopus frogs were obtained from Carolina Biological Supply Co. (Burlington, North Carolina) and bred according to the instructions supplied by this company. The tadpoles were used at the age of 8–10 days after spawning, with 10 animals each in six or seven graded aqueous solutions (100 ml) of the chemicals. Anesthesia was defined as the loss of the righting reflex after prodding with a glass tip, followed by recovery of the tadpoles after transfer to distilled water. The number of anesthesized animals (after equilibration, which was found to be complete after 1 hr) was plotted against in-membrane drug concentration (aqueous concentration × partition coefficient) (Fig. 2). Effective doses in-membrane, ED^M_{50}, were calculated from the data by the method of Waud (30).

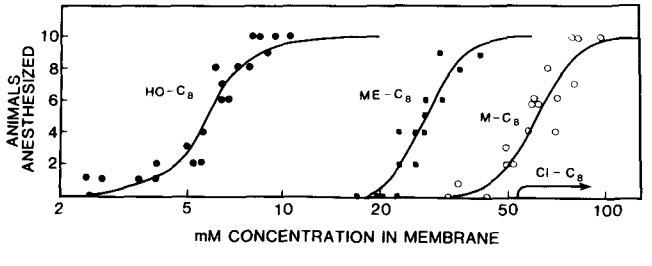

FIG. 2. Dose-response curves of anesthetic action, measured as loss of righting reflex of tadpoles in dependence of in-membrane drug concentration, of four derivatives of octane: HO-C_8, octan-1-ol; ME-C_8, 1-(2-methoxyethoxy)octane; M-C_8, 1-methoxyoctane; Cl-C_8, 1-chlorooctane. The arrow with Cl-C_8 indicates that anesthetic response could not be obtained even at saturation of the compound in water.

RESULTS

Data collected from three to four experiments, for each compound, are plotted as dose-response curves in Figure 2. With chlorooctane no anesthesia occurred, in four experiments, at the highest obtainable concentration, 350 M in water, or 53 mM in-membrane. The arrow emanating from this value on the abscissa is meant to indicate that the anesthetic concentration for chlorooctane lies beyond its solubility limit, and its ED^M_{50}, considering the slope of the other compounds, must be above 100 mM.

Table 1 presents the ED^W_{50}, in mmolar aqueous concentration, for each drug; the erythrocyte membrane-water partition factor; and the ED^M_{50} in-membrane, calculated from ED^W_{50} × partition factor. It can be seen how the anesthetic potency decreases (ED^M_{50} increases) from octanol to chlorooctane. Methoxyethoxyoctane is about five times, methoxyoctane about 10 times, chlorooctane at least 20 times less potent than octanol. The anesthetic power diminishes in parallel to the decline in the capacity of the compound to engage in hydrogen bonding (31–33). The alcohol octanol is a potent donor but can accept a proton as well (serve simultaneously as acid and base) (32). The ether group is a weaker base only. Not unexpectedly, the diether is twice as potent as the monoether. The chloro group, finally, is a base so weak that carbon tetrachloride is commonly used as an inert solvent in hydrogen bonding studies (31,32). (A more quantitative evaluation of hydrogen bond strength follows.)

DISCUSSION

Changes of hydrogen bond structures in the membrane, occasioned by the drug, have repeatedly been proposed as the mechanism underlying anesthesia (34–37). However, the idea has found no foothold among current theories, although its case can be argued persuasively from available evidence. For example, aliphatic alcohols from ethanol to dodecanol have been found to be of nearly equal potency at equal molar in-membrane concentration (3–6). Since the effective hydrophobic chain length (38) in the sequence varies from 0 to 10, and the volume of the hydrophobic moiety and its area of contact with

TABLE 1

Anesthetic Doses, ED_{50}, for Four Derivatives of Octane, in Water and in Membrane, Measured as Loss of Righting Reflex of Tadpoles

	ED^W_{50} (mM)	Membrane-water partition coefficient	ED^M_{50} (mM)
Octanol	0.05	109 ± 11	5.5 ± 0.7
Methoxyethoxyoctane	0.18	155 ± 13	28.3 ± 3.2
Methoxyoctane	0.35	175 ± 11	61.3 ± 5.9
Chlorooctane	>0.7	151 ± 10	>100

ED^W_{50}, in water; ED^M_{50}, in membrane, equals ED^W_{50} × partition coefficient. For the determination of the coefficient, erythrocyte ghosts were used as representative membrane and quantitated by protein assay. Ghost were assumed to contain 40% protein and have a density of 1.17 (29). Equilibration of ghosts with buffer containing the radioactive compound was at 37 C for 1 hr (28).

the membrane vary accordingly, it is clear that this part of the molecule cannot be responsible for anesthesia. This leaves the hydroxyl group as the only candidate, and structure and volume of that group do remain constant through the sequence. Similarly, aliphatic acids are more potent by an order of magnitude than the corresponding aliphatic alcohols (5), and barbiturates, with their five hydrogen bonding sites, are still more potent (5). All these drugs are amphiphilic; their hydrogen-bonding parts must reside in the region of the hydrogen belts, and they must interact in some fashion with corresponding hydrogen bonding sites of the membrane.

Plausible as the case for hydrogen bonding may be, it is by no means generally accepted or appreciated. For this reason, we present new evidence of the fundamental importance of such bonding. We chose aliphatic anesthetics and kept the chain length constant, for greater assurance.

The interpretation of the results (Table 1) calls first for an analysis of their accuracy; second, for some quantitative assessment of the hydrogen bonding capacity of the polar groups. For the purposes of our study, accuracy of the values of Table 1 relative to each other only is required, and this is assured by the identity of procedures for determining both P_c and ED_{50} for all four compounds. The chlorooctane, having but little polarity, might have been expected to show a higher membrane-water partition coefficient; perhaps the polarity of the membrane may curb its solubility in it. (A higher P_c for chlorooctane would, by the way, raise ED_{50}^M and so not threaten but further endorse our results.) For octanol, P_c and ED_{50}^M have been measured before, with erythrocyte ghosts as membrane, by Pringle et al. (6): $P_c = 152$; ED_{50}^M(mmolar) = 9. The coefficient compares reasonably well with ours ($P_c = 109 \pm 11$), as does the ED_{50}^M ($= 5.5 \pm 0.7$). If our ED_{50}^M is calculated with the P_c of Pringle et al. (6), it becomes $ED_{50}^M = 7.7 \pm 1.0$. Agreement is also good between the tadpole tests, as shown by the ED_{50} (i.e., in water) for octanol: Pringle et al. (6), 0.06 mM, against our 0.05 mM.

The quantitation of hydrogen bond capacity poses a problem because both base and acid contribute to bonding strength, so that no isolated value can be measured for any group; but relative, and quite quantitative, assessments can be made, and they are sufficient to establish our gradient. For chlorohexane (in CCl_4, C_6H_5OH as donor), a free energy change $-\Delta G$ (standing for bond strength) of -0.5 kcal/mol has been measured (ref. 32, p. 325); for ethers (same conditions), $-\Delta G$ is 1.3 kcal/mol (ref. 32, p. 317). We can assume that this difference (though not the respective different values) will be maintained in aqueous solution, so that we can expect a difference in hydrogen bonding strength ($-\Delta G$) between chlorides and ethers of ca. 2 kcal/mol. If chlorides do not form hydrogen bonds (or form extremely weak ones), we find a partial bond strength for ethers of ca. 2 kcal, a value plausible for a weak base, common hydrogen bond strengths being 3–6 kcal/mol (39). For alcohols, no comparable data are available, but if we assign to the ether function of the OH group again a value of 2 and add the $-\Delta G$ expected for a strong donor (i.e., the proton), we estimate a combined hydrogen bond strength of 6–8 kcal/mol. These values cannot be far from reality, and the gradation of Table 1 is thus certainly established.

The results of Figure 2 and Table 1 clearly support our contention that it is the hydrogen-bonding regions of the drug and of the membrane that govern anesthesia. The anesthetic potency differs widely between the four octane derivatives. It drops with the decreasing hydrogen-bonding capacity of the compound; however, we should perhaps not extrapolate that exactly this tendency must always prevail. At present, we cannot predict anesthetic potency from chemical structure; the question has been treated elsewhere (22).

The results of this study may stimulate a fresh review of several old problems of the theory of anesthetics:

1. Can the Meyer-Overton rule still be considered valid? Not in its general form. ED_{50}^M is not at all the same for the different compounds of Table 1; it varies 20-fold at least. However, the constancy of the ED_{50}^M in the alcohol series (3–6) suggests a modified rule: Equipotency of drugs is obtained at equal in-membrane molarity, provided the hydrogen-bonding part of the molecules is the same.

2. Does the drug molecule restructure the lipid bilayer first (as suggested by lipid theories of anesthesia) or the protein directly? Two sequences of events might lead to anesthesia (the asterisk stands for "restructured"):

A) drug + bilayer $\rightleftharpoons$ *bilayer
*bilayer + protein $\rightleftharpoons$ *protein

B) drug + bilayer $\rightleftharpoons$ drug-in-bilayer
drug-in-bilayer + protein $\rightleftharpoons$ *protein

Sequence A may be valid, i.e., the drug may restructure the hydrogen belt by interacting with the hydrogen bonding pattern of the lipids; then, protein-lipid hydrogen bonding becomes affected. On the other hand, there is no reason why a drug could not interact as in sequence B with the protein directly, the lipid bilayer serving as a mere solvent for both molecules. This sequence has, in fact, the advantage of simplicity over sequence A. The Meyer-Overton rule is satisfied in B as well as in A: both *bilayer concentration and drug-in-bilayer concentration are functions of the bilayer/water partition of the drug. Possibly, the situation is described better by A in some cases, by B in others. In the case of direct drug-protein interaction (B), however, our theory insists that this also will take place in the hydrogen belt of the membrane, and that it is characterized by hydrogen bond interaction between the drug and an allosteric site on the protein.

3. Is there evidence for membrane lipid-protein hydrogen bonding? Evidence is emerging lately for such bonding; two instances are well documented. Glucose-6-phosphatase has been shown to be sensitive to hydrogen bonding by lipids: lysophosphatidylcholine inactivates the enzyme; 1-ether-2-deoxylysophosphatidylcholine partially activates it; cholesterol reverses the deactivation by lysoPC; OH-blocked cholesterol, on the other hand, does not (40). These results can be understood only as a consequence of lipid-protein interactions in the hydrogen belts of the membrane. Furthermore, just as cholesterol modulates, in this example, the action of lysophosphatidylcholine, it has been found to modulate the action of anesthetics in membranes (41). Protein kinase C must be hydrogen-bound to the *sn*-1 ester CO of diacylglycerol for activation (42), and there must be additional such bonding sites since there is steric specificity for *sn*-1,2-diacylglycerol (43). Clearly, more instances of lipid-protein hydrogen bonding will be found in the future.

4. Perturbation or restructuring of the membrane? "Perturbation" implies the creation of disorder and leads to the inference that all lipid-soluble chemicals will produce the same kind of disorganization (fluidization) in the membrane; this is not likely to be true. Rather, every chemical will modify the hydrogen belt in its own particular manner. "Restructuring" is more appropriate; it opens the door to an understanding of the great variety of action of anesthetic drugs. The matter is discussed elsewhere (22), together with our interpretation of some other puzzles, such as the anesthetic activity of alkanes and rare gases and the influence of pressure on anesthesia.

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Effect of Pyridoxine Deficiency on Phospholipid Methylation in Rat Liver Microsomes

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The effect of altered methionine metabolism during pyridoxine deficiency on the activity of phosphatidylethanolamine methyltransferase (EC 2.1.1.17) and the levels of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) has been evaluated in rat liver microsomes. Animals fed a pyridoxine-deficient diet for 7 wk displayed a fivefold increase in the hepatic tissue level of S-adenosylhomocysteine when compared to either control or pair-fed animal counterparts. When PE methyltransferase was assayed in vitro, a significant increase in specific activity was observed using enzyme preparations from either pair-fed or pyridoxine-deficient rats. On the other hand, phospholipid levels did not conform to the measured enzyme activity. The level of PC in microsomes from either pyridoxine-deficient or pair-fed animal groups was significantly lower than that determined for the control group of rodents. However, the level of PC was noticeably lower in microsomes from pyridoxine-deficient animals than that from pair-fed animals, which received 45% of the feed intake of the control animals. In addition, the level of PE in microsomes from pair-fed and pyridoxine-deficient animals was significantly higher than that analyzed from the control animals, further confirming decreased methylation of substrate to product. It is concluded that pyridoxine deficiency may alter the methylation of phospholipid in the endoplasmic reticulum above and beyond that produced by feed restriction alone. *Lipids 21,* 409–412 (1986).

Studies using cell-free enzyme preparations (1–3) have revealed that S-adenosylhomocysteine (SAH) is a potent, competitive inhibitor of phosphatidylethanolamine (PE) methyltransferase. SAH apparently has a greater affinity than S-adenosylmethionine (SAM) for the binding site on the rat liver enzyme (3), which may explain the decreased rate of phospholipid methylation in vivo, either when the intracellular level of SAH in liver is elevated (4) or when SAH analogues are administered to animals (5,6). Thus, these observations would suggest that the absolute molar ratio of SAM to SAH in hepatocytes may regulate PEMT and phospholipid methylation.

Recent work (7) has demonstrated that a methyl-deficient diet fed to rats is capable of promoting a reduction in the SAM:SAH ratio, accompanied by a reduction in the methylation status of phospholipid. As such, it is conceivable that other dietary factors altering methionine metabolism may also affect phospholipid metabolism. Previously, altered methionine metabolism was observed in rats fed a pyridoxine-deficient diet, as indicated by an accumulation of SAH in liver tissue and accordingly an overall decrease in the SAM:SAH ratio (8). However, the effect of pyridoxine deficiency on transmethylation has not been delineated to our knowledge. This need has been partially addressed in the present work. We wish to report that a pyridoxine deficiency, as well as restricted feed intake, decreases phospholipid methylation in rat liver microsomes.

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MATERIALS AND METHODS

Materials. [^{14}C-Methyl]-S-adenosyl-L-methionine (sp act 49 mCi/mmol), as well as [8-^{14}C]adenosine (52 mCi/mmol) needed for the synthesis and purification of S-[8-^{14}C]adenosylhomocysteine (8), was obtained from ICN Chemical and Radioisotope Division (Irvine, California). Certified ACS grade organic solvents and Silica Gel G (Redi-Plate) TLC plates were purchased from Fisher Scientific Co. (Pittsburgh, Pennsylvania). All other chemicals were provided by the Sigma Chemical Co. (St. Louis, Missouri). Dietary components for preparing the diets were supplied by ICN Nutritional Biochemicals (Cleveland, Ohio).

Animals and treatment. Male Sprague-Dawley rats (45–60 g initial bwt) from Taconic Farms (Germantown, New York) were housed individually in wire-bottom cages with an automated light cycle from 6 a.m. to 6 p.m. Three groups of five animals each were fed their respective diets for 7 wk. One group of animals was fed a pyridoxine-deficient diet (9) ad libitum, while a second group was pair-fed the same diet supplemented with pyridoxine hydrochloride (22 mg/kg prepared diet). The third group received the latter diet ad libitum and served as the control. Water was freely available to all animals, and their feeding bowls were changed daily with clean counterparts containing fresh food.

Tissue preparation. At the end of their dietary regimes, animals were stunned by a blow to the back of the head and decapitated. The liver was rapidly excised and sectioned into two pieces. One piece was placed immediately in ice-cold 10% TCA and a 1:5 (w/v) homogenate prepared for quantitative analyses of SAM and SAH as cited below. The other piece of tissue was homogenized in ice-cold 5 mM potassium phosphate buffer, pH 7.5, at a concentration of 1:9 (w/v). Following centrifugation at 10,000 × g for 10 min at 2 C, the supernatant was then centrifuged at 100,000 × g for 60 min. The resultant microsomal pellet was resuspended in the buffer to give a protein concentration of 1–2 mg/ml.

Determination of hepatic tissue levels of SAM and SAH. Quantitative analyses of SAM and SAH were performed on the ether-extracted TCA supernatant, with [^{14}C-methyl]-S-adenosyl-L-methionine and S-[8-^{14}C]adenosylhomocysteine as internal standards, using the method of Eloranta et al. (8). The levels were expressed as nmol/g wet tissue weight.

Enzyme assay. Using the microsomal suspension, the activity of PE methyltransferase was assayed using the radiochemical procedure described by Hoffman and Cornatzer (10), except that exogenous phosphatidylcholine (PC) and detergent were omitted from the reaction mixture. The assay is based on the methylation of endogenous

PE to PC, with [^{14}C-methyl]-S-adenosyl-L-methionine as the methyl donor. Apparently, exogenous PE does not appear to significantly increase the rate of transmethylation (10,11). After lipid extraction of the terminated incubation mixture, 1 ml of the organic phase containing the radiolabeled product was counted for radioactivity using a scintillation cocktail consisting of 10 ml 2,5-diphenyloxazole in toluene (12 g/l) and 6 ml ethylene glycol monoethyl ether. Close to 90% of the radioactivity in the organic phase is apparently present as $^{14}CH_3$-labeled phospholipid, and 95% of this radioactivity is accounted for by PC (10). Protein determination was performed using a modification of the Lowry method (12) with bovine serum albumin as the standard.

Determination of microsomal phospholipid. Extraction and purification of lipid from microsomes were based on the method of Bligh and Dyer (13). To 3.2 ml of the microsomal suspension was added 12.0 ml methanol/chloroform (2:1, v/v). After mixing in 4.0 ml chloroform and 4.0 ml water, the mixture was filtered through a glass-sintered crucible with mechanical vacuum and centrifuged. The top aqueous methanol layer was suctioned off along with the thin, denatured protein layer present. Three ml of the lipid-containing chloroform phase was removed and evaporated to dryness with nitrogen gas. The residue was resuspended in 0.1 ml chloroform. Quantitative analyses of the PE and PC contained in the concentrated lipid extract were performed by thin layer chromatography as detailed by Parker and Peterson (14), but using commercial silica gel G plates. Phospholipid was identified against known standards using iodine vapor, with the levels expressed as μg phospholipid phosphorus per mg microsomal protein.

Statistics. Statistical differences were evaluated using ANOVA and Duncan's New Multiple Range test (15).

RESULTS

In addition to dermatosis of the paws and nose, other symptoms of vitamin deficiency were observed in the pyridoxine-deficient group of animals. As shown in Table 1, rats fed the pyridoxine-deficient diet for the 7-wk period consumed only 45% of the total feed intake of animals in the control group receiving the same diet supplemented with pyridoxine. Moreover, pyridoxine-deficient and pair-fed rats had a total weight gain of only 25 and 35%, respectively, of that experienced by control animals. The significantly lower feed efficiency ratio ascertained for the pyridoxine-deficient diet confirms the inability of this diet to support growth to the same extent, as in the presence of pyridoxine.

The data in Figure 1 substantiate that the metabolism of methionine in liver is altered during pyridoxine deficiency (8). Although the tissue levels of SAM did not differ among the three groups of animals, pyridoxine-deficient rats had a concentration of SAH of about fivefold higher than that for both the pair-fed and control animals. Thus, the absolute molar ratio of SAM to SAH dropped from about 4.4 in both the control and pair-fed rats to 0.9 in the pyridoxine-deficient ones.

In order to evaluate the effect of altered methionine metabolism during pyridoxine deficiency on phospholipid methylation in rat liver microsomes, the specific activity of PE methyltransferase was initially measured in tissue preparations from all animals. A significant increase in enzyme activity was seen in preparations from both pair-fed and pyridoxine-deficient rats over that obtained from control animals (Table 2). As shown in Table 3, phospholipid analyses revealed that liver microsomes from either pair-fed or pyridoxine-deficient rats contained a significantly higher level of PE than that present in microsomes from control rats. On the other hand, the PC level was lower in microsomes from both pair-fed and pyridoxine-deficient rats than that seen in microsomes from control animals. However, there was a significantly lower level of PC in microsomes from pyridoxine-deficient rats than microsomes from pair-fed rats.

TABLE 1

Total Feed Intake and Weight Gain of Control, Pair-fed and Pyridoxine-deficient Animals Fed Dietary Regimes for Seven Wk

Animal group	Feed intake* (g)	Wt gain (g)	Feed efficiency ratio of diet†
Control	867.3 ± 12.7[a,‡]	342 ± 8[a]	0.394 ± 0.005[a]
Pair-fed	383.4 ± 11.8[b]	121 ± 8[b]	0.314 ± 0.011[b]
Pyridoxine-deficient	392.1 ± 13.1[b]	87 ± 3[c]	0.222 ± 0.007[c]

*Values are the average ± SEM for five animals.

†Total weight gain ÷ total food intake.

‡Values in a column not followed by the same superscript letter are significantly different, $p < 0.05$.

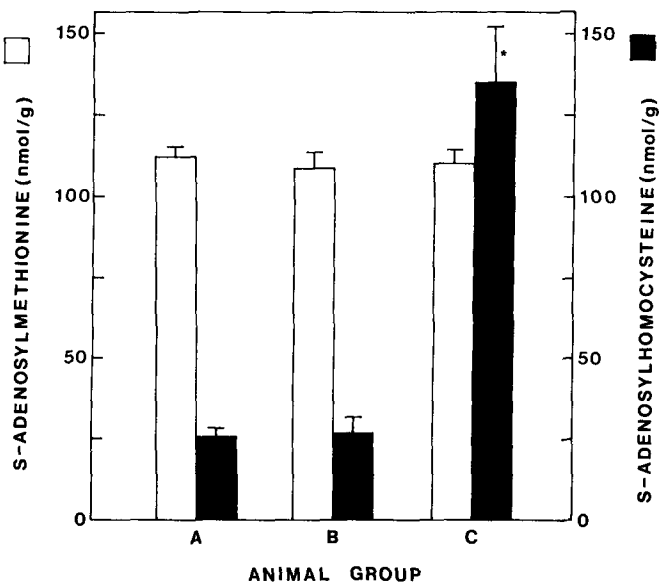

FIG. 1. Rat liver tissue levels (wet wt) of S-adenosylmethionine and S-adenosylhomocysteine during pyridoxine deficiency. Animals (45–60 g initial bwt) were maintained on their respective diets for seven wk. Values represent the average ± SEM for five rats in either the control (A), pair-fed (B) or pyridoxine-deficient (C) animal groups. *Significantly different from control and pair-fed groups ($p < 0.01$).

TABLE 2

Rat Liver Microsomal Phosphatidylethanolamine Methyltransferase Activity in Control, Pair-fed and Pyridoxine-deficient Animals

Animal group	Phosphatidylethanolamine methyltransferase (cpm × 10^{-3}/mg protein)
Control	0.40 ± 0.04[a]
Pair-fed	0.55 ± 0.02[b]
Pyridoxine-deficient	0.58 ± 0.01[b]

Animals (45-60 g initial bwt) were maintained on respective diets for seven wk. Values represent the average ± SEM for five rats. Data sharing a common superscript in a column are not significantly different ($p > 0.05$).

TABLE 3

Rat Liver Microsomal Phospholipid in Control, Pair-fed and Pyridoxine-deficient Animals Fed Dietary Regimes for Seven Wk

Animal group	Phosphatidylethanolamine (μg phospholipid phosphorus/mg protein)	Phosphatidylcholine (μg phospholipid phosphorus/mg protein)
Control	2.11 ± 0.11[a]	8.01 ± 0.23[a]
Pair-fed	3.28 ± 0.07[b]	7.19 ± 0.12[b]
Pyridoxine-deficient	2.90 ± 0.22[b]	6.25 ± 0.19[c]

Animals (45-60 g initial bwt) were maintained on their respective diets for seven wk. Values represent the average ± SEM for five rats. Data sharing a common superscript in a column are not significantly different ($p > 0.05$).

DISCUSSION

Pyridoxine deficiency is known to interfere with the metabolism of methionine. An accumulation of SAH and an overall decrease in the SAM:SAH ratio in liver have been the most noticeable effects (8). Presumably, degradation of SAH is somewhat thwarted during pyridoxine deficiency due to a reduction in the activities of cystathionine synthase (EC 4.2.1.22) and/or cystathionase (EC 4.4.1.1), both pyridoxal phosphate-requiring enzymes in the transsulfuration pathway. Transmethylation reactions would be expected to be rather impeded under such conditions, in view of the enzyme kinetics data showing that SAH has an inhibition constant (k_i) significantly lower than the Michaelis constant (K_m) possessed by SAM for a number of methyltransferases (16-20), including PE methyltransferase (3). Indeed, lowering the SAM:SAH ratio in vivo can result in decreased methylation of nucleic acid (4,21), biogenic amines (22), protein (4,23) and also phospholipid (4,23). Although a fivefold increase in the hepatic tissue level of SAH, without a concurrent change in SAM, was obtained in pyridoxine-deficient rats in the present study, the marked reduction of the SAM:SAH ratio was not accompanied by any inhibition of PE methyltransferase, as measured in vitro using microsomal preparations. Instead, an increase in specific activity was observed for the enzyme, which was also the case using liver microsomes prepared from pair-fed animal counterparts. The tissue level of SAH, as well as SAM, in liver was not altered in pair-fed rats in relation to values obtained for control animals. On the other hand, decreased methylation of phospholipid was indicated by an accumulation of PE and a reduction of PC in liver microsomes from both pyridoxine-deficient and pair-fed animals.

A similar disparity between PE methyltransferase activity in vitro and phospholipid methylation in vivo has been described. Even though the conversion of PE into PC was inhibited by about 50% when isolated, intact hepatocytes were incubated in the presence of 0.5 mM chlorophenylthio-cAMP, PE methyltransferase in microsomes from these cells displayed a twofold stimulation of enzyme activity (24). In addition, when rats were fed a methyl-deficient diet resulting in a reduction of the SAM:SAH ratio in liver, phospholipid methylation in vivo was depressed, as indicated by a reduction of radiolabeled PC in microsomes after administration of [1,2-^{14}C]-ethanolamine (7). Once again, however, the activity of PE methyltransferase as measured in vitro did not conform, since an increase in enzyme activity occurred concurrently with an increase and decrease in the microsomal levels of PE and PC, respectively. As pointed out in both of these studies (7,24), the endogenous level of PE in microsomes may determine the activity of PE methyltransferase in vitro. This situation may indeed be valid, since exogenous PE does not appear to significantly stimulate the enzyme (10,11). In other words, PE methyltransferase activity may serve as an indication of the level of PE in microsomes. Therefore, endogenous accumulation of PE may stimulate PE methyltransferase activity in vitro.

While pyridoxine deficiency can negatively influence phospholipid methylation, it appears that chronic feed restriction is also capable of exerting the same effect to a significant extent. However, this finding is not readily explainable. The effect seems to occur in the absence of disturbed methionine metabolism, since SAM and SAH concentrations are not altered in pair-fed rat livers when compared to those values seen in control rat livers. Yet, it is rather interesting that the alternative pathway for the synthesis of PC involving choline phosphotransferase (EC 2.7.8.2) was not able to compensate for the drop in PC in liver microsomes from the pair-fed or the pyridoxine-deficient animals, in spite of adequate choline in the diet actually consumed. Although the activity of rat liver choline phosphotransferase was not measured in the present study, apparently the activity of the enzyme decreases during methyl group deficiency (1,7).

In conclusion, it would appear that pyridoxine deficiency decreases phospholipid methylation in the endoplasmic reticulum above and beyond that produced by feed restriction alone. The effect of pyridoxine deficiency on other transmethylations is currently under investigation.

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METHODS

High Performance Liquid Chromatographic Separation of Monoacylglycerol Enantiomers on a Chiral Stationary Phase

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High performance liquid chromatographic separation of monoacylglycerol enantiomers as di-3,5-dinitrophenylurethane derivatives was carried out on a chiral stationary phase, N-(*S*)-2-(4-chlorophenyl)isovaleroyl-D-phenylglycine chemically bonded to *v*-aminopropyl silanized silica. Complete separation of the urethane derivatives of racemic monoacylglycerols with saturated acyl groups of C_{12}-C_{18} was achieved using a stainless steel column (25 cm long) packed with the 5μ particles, an isocratic elution at ambient temperature with a mixture of hexane/ethylene dichloride/ethanol as a mobile phase, and a UV detector. The *sn*-1 enantiomers were eluted ahead of the corresponding *sn*-3 enantiomers. Complete separation of the *sn*-2 isomers from the corresponding enantiomers and partial separation of the enantiomer homologues differing by two acyl carbons also were observed.
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Enantiomer separation of monoacylglycerols has not thus far been achieved by chromatographic means (1,2), although it would be useful as a fundamental technology in the stereochemical study of acylglycerols and a simple method for determination of optical purity.

Recently, Ôi and Kitahara (3) reported the direct enantiomer separation of some chiral alcohols by high performance liquid chromatography (HPLC) on chiral stationary phases, (*S*)-2-(4-chlorophenyl)isovaleric acid and its amide derivatives bonded to silanized silica, after conversion of the alcohols into their 3,5-dinitrophenylurethane derivatives. This result prompted us to study the direct HPLC separation of monoacylglycerol enantiomers on the chiral stationary phases, and a preliminary communication was reported (4).

This paper describes the detailed HPLC behavior of saturated monoacylglycerol enantiomers as di-3,5-dinitrophenylurethane derivatives on a chiral stationary phase, N-(*S*)-2-(4-chlorophenyl)isovaleroyl-D-phenylglycine bonded to silanized silica.

MATERIALS AND METHODS

Monoacylglycerols. Optically active 3-palmitoyl-*sn*-glycerol (*sn*-3-16:0) and *sn*-3-18:0, and racemic 1-palmitoylglycerol (*rac*-16:0) and *rac*-18:0 were obtained from Fluka AG (Buchs, Switzerland). *Rac*-12:0 and *rac*-14:0 were from Sigma Chemical Co. (St. Louis, Missouri). These samples were used without further purification because gas chromatography of them as trimethylsilyl ether derivatives (5) showed purity of more than 98% and the absence of the corresponding *sn*-2 isomers. Thin layer chromatography (TLC) on silica gel G impregnated with boric acid (5) also showed the absence of the *sn*-2 isomers. Optically inactive *sn*-2-16:0 (Serdary Research Lab., London, Ontario, Canada) purified by the TLC was used additionally.

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Preparation of derivatives. Di-3,5-dinitrophenylurethane derivatives of monoacylglycerols were prepared on the basis of the procedure of Ôi and Kitahara described on the derivatives of chiral alcohols (3). A monoacylglycerol of 20 μmol was dissolved in 450 μl of dry toluene and 45 μmol of 3,5-dinitrophenyl isocyanate (Sumitomo Chemical Co., Osaka, Japan) was added. The mixture was heated at 70 C in a 0.5 ml glass vial with a Teflon linked screw cap for 1 hr (or 3 hr without heating) in the presence of 45 μl of dry pyridine with occasional shaking. After cooling, the solvent was removed at 40 C under reduced pressure. The resulting urethane derivatives were purified by TLC on a silica gel GF plate (20 × 20 cm, 0.25 mm thick) containing fluorescence indicator (Analtech Inc., Newark, Delaware). Prior to use, the plate was developed with chloroform and was activated at 110–120 C for 1 hr. The reaction mixture dissolved in 0.5 ml of chloroform was spotted and developed twice using a mixture of hexane/ethylene dichloride/ethanol (40:15:3, v/v/v) as a developing solvent. The band of the urethane derivatives (R_f 0.6–0.7) was marked under UV irradiation and extracted from the adsorbent with ether.

UV spectra of the urethane derivatives were taken in ethanol and in the same solvent as the mobile phase used in HPLC (described below) on a Shimadzu UV-160 spectrophotometer (Shimadzu Co., Kyoto, Japan).

HPLC. HPLC separation was carried out with a Shimadzu LC-6A instrument equipped with a chiral column (stainless steel, 25 cm × 4 mm i.d.) packed with 5μ particles of N-(*S*)-2-(4-chlorophenyl)isovaleroyl-D-phenylglycine chemically bonded to *v*-aminopropyl silanized silica, Sumipax OA-2100 (Sumitomo Chemical Co.). A Guard-pac precolumn module with a silica insert (Millipore Co., Milford, Massachusetts) was attached to the inlet of the chiral column. The analysis was done isocratically using a mixture of HPLC grade hexane/ethylene dichloride/ethanol (40:12:3, v/v/v) as a mobile phase at a constant flow rate of 1 ml/min at ambient temperature. The samples dissolved in chloroform were injected into installation through a Rheodyne Model 7125 loop (20 μl) injector. Peaks were monitored at 0.32 AUFS with a Shimadzu SPD-6A variable wavelength (195–350 nm) UV detector having an 8 μl flow cell. Peak area percentages and retention times were measured with a Shimadzu integrator, Chromatopac C-R2AX.

RESULTS AND DISCUSSION

Derivatives. The reaction of monoacylglycerols with 3,5-dinitrophenyl isocyanate proceeded readily in toluene solution under the existence of pyridine, and the purification of the resulting di-3,5-dinitrophenylurethanes by TLC gave better HPLC chromatograms. The urethane derivatives (R_f 0.6–0.7) were separated clearly from a main by-product (R_f 0.3–0.4), which appeared as a yellow band without UV irradiation, derived from the reagent

isocyanate by double development TLC using a mixture of hexane/ethylene dichloride/ethanol (40:15:3, v/v/v) as a development solvent (91% yield for *rac*-12:0). Elemental analysis for *rac*-12:0 urethane derivatives (97% purity): found, C 50.59, H 5.38, N 11.74%; calcd for $C_{29}H_{36}O_{14}N_6$, C 50.29, H 5.24, N 12.13%. The formation of mono-3,5-dinitrophenylurethanes as by-products could not be confirmed under the conditions used. The urethane groups are more stable than the ester groups, and it seems to be difficult to recover the original monoacylglycerols without the hydrolysis of the ester groups.

Figure 1 shows the UV spectrum of *rac*-12:0 as di-3,5-dinitrophenylurethane derivatives in the same solvent as the mobile phase used in HPLC. The urethane derivatives have a strong absorption at 226 nm (λ_{max}) as indicated by the ε value of 60,000 in ethanol and also have good absorption at 254 nm (ε 31,000), which is fixed in universal UV detectors. Moreover, the derivatives have the ε value of 4,000 at 340 nm (λ_{max}). Thus, the urethane derivatives have a sufficient sensitivity for HPLC detection over the wide range of wavelengths in UV.

Separation. Figure 2 shows the typical chromatograms of optically active and inactive monoacylglycerols as di-3,5-dinitrophenylurethane derivatives on a chiral column, OA-2100. The retention time of the later peak of *rac*-16:0 in Figure 2A was in agreement with that of *sn*-3-16:0 in Figure 2B. The agreement of the retention time was also confirmed by co-injection of *rac*-16:0 and *sn*-3-16:0 (Fig. 2D). Racemic monoacylglycerol samples used were not separated by HPLC on a usual silica column, e.g., Cosmosil 5SL (5μ particles, 20 cm × 4.6 mm i.d.) from Nakarai Chemical Co. (Kyoto, Japan). The chromatograms in Figure 2 are characterized by complete separation of isomeric *sn*-1, *sn*-2 and *sn*-3 monoacylglycerols, sharp and symmetrical peaks within moderate retention times and stronger retention of *sn*-3 enantiomers than the corresponding *sn*-1 enantiomers, which suggests stronger diastereomeric interactions with the stationary phase. The faster elution of the *sn*-2 isomer on the chiral silica column (Figs. 2C and 2D) probably is due to the lesser polarity. A similar phenomenon for monoacylglycerols was observed in TLC on silica gel (2). The OA-2100 column used in this study showed 8,700 theoretical plates for *sn*-3-16:0 peak. This value reduced to 7,600 after 100 injections, but the enantiomers were still separated completely. Complete separation on this chiral column was also achieved for the corresponding monoalkylglycerol enantiomers (Takagi, T., and Itabashi, Y., submitted for publication).

Table 1 gives the chromatographic data of racemic monoacylglycerols of saturated acyl groups of C_{12}-C_{18} on the OA-2100 column. Good and nearly identical values of the separation factors and peak resolution were obtained for all racemic monoacylglycerols used. The separation factor 1.19 and the capacity ratio 3.44 of *sn*-1-14:0 were approximately equal to those of 2-octanol obtained on the chiral column (3). The retention volumes (Vr) of *sn*-1 and

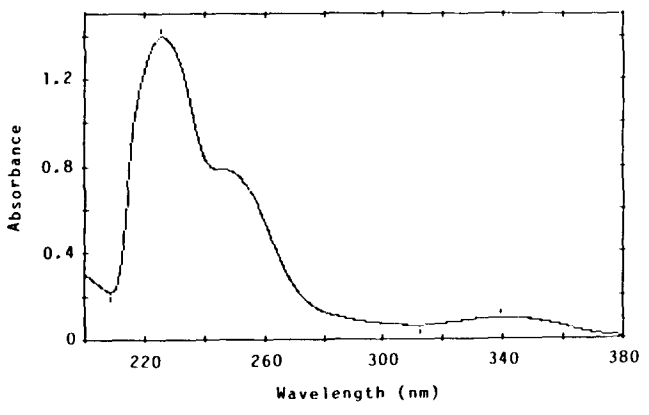

FIG. 1. UV spectrum of di-3,5-dinitrophenylurethane derivatives of racemic 1-monolauroylglycerol (*rac*-12:0) in a mixture of hexane/ethylene dichloride/ethanol (40:12:3, v/v/v) used as a mobile phase in HPLC.

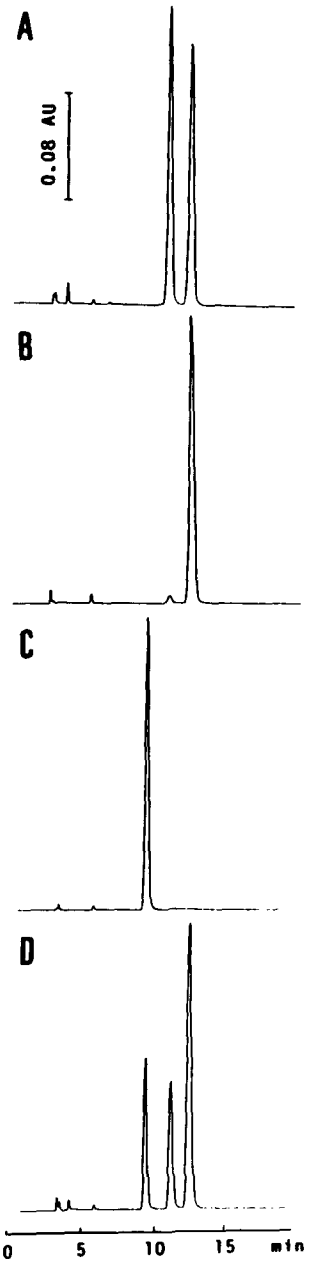

FIG. 2. Separation of isomeric monoacylglycerols as di-3,5-dinitrophenylurethane derivatives on a chiral column, OA-2100. A, *rac*-16:0; B, *sn*-3-16:0; C, *sn*-2-16:0; D, a mixture of A, B and C. Peaks were monitored at 254 nm.

TABLE 1

Chromatographic Data of Racemic Monoacylglycerols on a Chiral Column, OA-2100

Acyl						UV (nm)[e]		
Group	Position	Vr[a]	k'[b]	α[c]	Rs[d]	226	254	340
12:0	*sn*-1	9.35	3.65	1.18	2.47	50.0	50.0	50.0
	sn-3	11.06	4.32			50.0	50.0	50.0
14:0	*sn*-1	8.81	3.44	1.19	2.51	50.0	50.0	50.0
	sn-3	10.44	4.08			50.0	50.0	50.0
16:0	*sn*-1	8.27	3.23	1.19	2.48	49.8	49.8	49.8
	sn-3	9.82	3.84			50.2	50.2	50.2
18:0	*sn*-1	7.78	3.04	1.19	2.47	50.0	49.9	49.9
	sn-3	9.25	3.61			50.0	50.1	50.1

[a]Vr, retention volume (ml) corrected by subtracting the column void volume (2.56 ml).
[b]k', capacity ratio.
[c]α, separation factor (the ratio of the capacity ratios).
[d]Rs, peak resolution. $Rs = 2(t_2-t_1)/(w_1+w_2)$, where t is retention time and w is peak width.
[e]Peak area ratio (%).

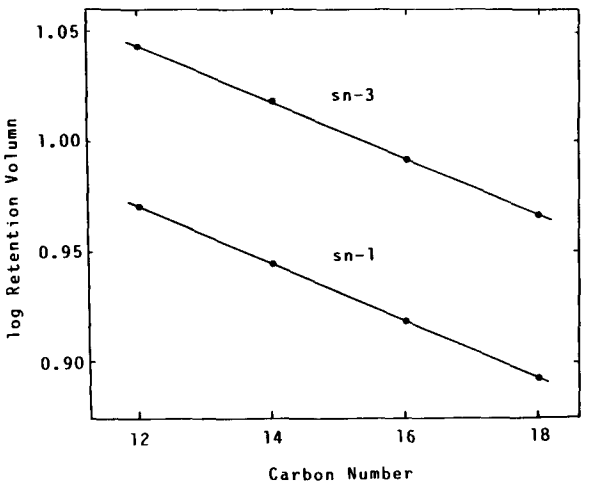

FIG. 3. A plot of log retention volume vs acyl carbon number for monoacylglycerol enantiomers as di-3,5-dinitrophenylurethane derivatives separated by HPLC on a chiral column, OA-2100.

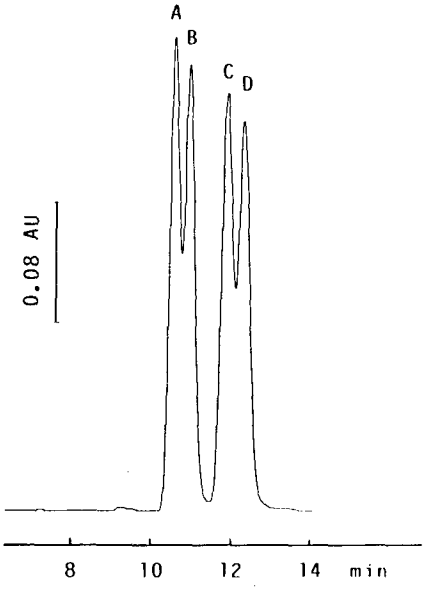

FIG. 4. Carbon number separation of monoacylglycerols as di-3,5-dinitrophenylurethane derivatives on a chiral column, OA-2100. A, *sn*-1-18:0; B, *sn*-1-16:0; C, *sn*-3-18:0; D, *sn*-3-16:0. Ca. 1:1 mixture of *rac*-18:0 and *rac*-16:0 was analyzed. Peaks were monitored at 254 nm.

sn-3 enantiomers decreased with increasing acyl carbons on a normal-phase HPLC using the chiral silica column (Table 1). This can be explained by the lesser polarity of the monoacylglycerols with longer acyl chains. Figure 3 shows the linear relationship in logarithmic Vr against acyl carbon number in each homologous series of enantiomers. The straight lines are approximately parallel. Therefore, a relationship between Vr of *sn*-1 and *sn*-3 enantiomers can be expressed by the following equation under the conditions used:

$$\log Vr(sn\text{-}3) = \log Vr(sn\text{-}1) + 0.074$$

where Vr(*sn*-3) and Vr(*sn*-1) indicate retention volumes of *sn*-3 and *sn*-1 enantiomers with the same acyl group, respectively. The Vr of an *sn*-1 enantiomer is approximately equal to that of an *sn*-3 enantiomer with six more carbons, such as *sn*-1-12:0 and *sn*-3-18:0. These critical pairs could not be separated on the column under the conditions used, but partial separation was observed for other pairs of *sn*-1 and *sn*-3 enantiomers.

Figure 4 shows the partial separation of the enantiomer homologues of monoacylglycerols differing in acyl groups on OA-2100. Apparently, the separation of the homologues differing by two carbons (Rs 0.36) is poor compared to the enantiomer separation. The separation factor of the homologues differing by two carbons was 1.06 in each *sn*-1 and *sn*-3 enantiomer as calculated from Table 1. The separation factor of 1.18–1.19 between enantiomers (Table 1) is approximately equal to that of the homologues differing by six carbons in each *sn*-1 and *sn*-3 enantiomer, such as 1.20 for *sn*-1-12:0/*sn*-1-18:0.

The racemic monoacylglycerols separated on OA-2100 showed nearly the same peak area ratios at different wavelengths in the UV detector (Table 1). This supports the complete separation of racemic monoacylglycerols into the enantiomers and no isomerization during the derivatization procedures and the HPLC analysis. The retention time of a small peak eluted ahead of *sn*-3-16:0 peak (Fig. 2B) was in agreement with that of the corresponding *sn*-1-16:0 peak (Fig. 2A). Thus, the optical purities of *sn*-3-16:0 and *sn*-3-18:0 used in this study were determined as 97.6 and 91.5%, respectively. An almost pure single peak was obtained for *sn*-2-16:0 (Fig. 2C).

Some racemic arylalkylcarbinols as 3,5-dinitrobenzoyl derivatives were separated by HPLC on a chiral stationary phase, 2,2,2-trifluoro-1-(9-anthryl)ethanol bonded to silanized silica (6). The OA-2100 column, however, has no enantioselectivity for 3,5-dinitrobenzoyl derivatives of various chiral alcohols except for some arylalkylcarbinols, and gives effective separations for their 3,5-dinitrophenylurethane derivatives. This may be attributed to the fact that no functional group containing a hydrogen atom is available for hydrogen bonding interaction with the stationary phase (3). The introduction of NH groups as di-3,5-dinitrophenylurethane derivatives into monoacylglycerols contributes to the excellent enantiomer separation achieved in this study.

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COMMUNICATIONS

Plasma Membrane Lipids of Bovine Adrenal Chromaffin Cells

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The lipid composition of plasma membranes isolated from bovine adrenal chromaffin cells has been determined. Choline and ethanolamine phosphatides were predominant; the level of lyso compounds was very low. The amount of cholesterol and the cholesterol/phospholipid molar ratio was low compared to those of the other subcellular fractions of chromaffin cells. A complex pattern of neutral glycolipids was observed in contrast to that of gangliosides.
Lipids 21, 417-419 (1986).

It is generally believed that during exocytosis secretory granules move toward the plasma membrane and fuse with it before releasing the secretory products into extracellular compartments. But the precise mechanism of these events remains elusive partly due to a lack of understanding of the molecular organization of the chromaffin cell plasma membrane. For addressing the question of membrane function, knowledge of the lipid composition is of utmost importance. Membrane lipid distribution is also of special relevance as certain phospholipids have been implicated to be involved in membrane fusion processes (1,2).

The membrane lipid composition of the secretory granules has been extensively studied (3-18), whereas very little is known about the lipid profile of the plasma membrane isolated from chromaffin cells. The only data available (19) pertain to phosphatide compositions. It is with this background in mind that we have analyzed in greater detail the lipids of the plasma membranes isolated from the chromaffin cells of bovine adrenal medulla.

MATERIAL AND METHODS

Plasma membranes of chromaffin cells of bovine adrenal medulla were isolated as described earlier (20). Lipid extraction and partitioning were carried out according to Suzuki (21). Total cholesterol (22), lipid phosphorus (23), phospholipids (24) and "neutral" glycolipids (25) were determined in the Folch lower phase. Alkenyl phospholipids were analyzed according to Horrocks (26). Gangliosides in the Folch upper phase were purified on Sephadex G25 while separation and quantification were achieved by thin layer chromatography (TLC) and densitometry (27,28). Lipid sialic acid was quantified using resorcinol (29). TLC of non-sialylated glycolipids was performed according to established procedures (30). Protein was determined (31) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

As we have reported previously (20), the plasma membranes, which we obtained from the chromaffin cells, were devoid of any mitochondrial or microsomal components.

TABLE 1

Lipids of Chromaffin Cells Plasma Membrane

	Number of determinations	μg/mg Protein
Cholesterol	3	34[a] (27.0-40.1)
Lipid phosphorus	4	18.8 (16.3-21.8)
Cholesterol/phospholipids molar ratio	3	0.14 (0.13-0.15)
Lipid sialic acid	4	4.0 (2.9-5.5)
Neutral glycolipid (as galactose)	2	40.1 (35.1-45.1)

[a]Extreme values are in parentheses.

TABLE 2

Phospholipid Composition of Chromaffin Cells Plasma Membranes

	Percentage of total phospholipids [a]
Total ethanolamine phosphatides	32.2 (32.0-32.5)
Diacyl + alkyl acyl PE	17.7 (16.0-19.1)
Alkenyl acyl PE	14.5 (12.5-15.9)
Serine + inositol phosphatides	10.4 (9.9-11.4)
Total choline phosphatides	48.0 (45.2-50.0)
Diacyl + alkyl acyl PC	42.1 (39.5-44.9)
Alkenyl acyl PC	2.0 (1.6-2.4)
Lysophosphatidylcholine	3.9 (3.7-4.1)
Sphingomyelin	9.3 (7.7-12.2)

[a]Mean value (3 experiments). Extreme values are in parentheses.

The amount of phospholipid found in chromaffin cell plasma membranes (Table 1) is in the range reported for other subcellular fractions (7,10). In contrast, the cholesterol content of the plasma membranes is very low. The cholesterol/phospholipid molar ratio observed for plasma membranes was close to those (0.20-0.30) reported for chromaffin cell endoplasmic reticulum (8) and mitochondria (4). This ratio is notably lower than that for chromaffin granule membranes (0.4-0.6) (9,10). This low requirement for cholesterol could perhaps be attributed to the absence of notable levels of lysophospholipids in plasma membranes, mitochondria or endoplasmic reticulum (Table 2 and refs 3,4,8,15,19). A stoichiometric requirement for cholesterol and lysophosphatidylcholine has

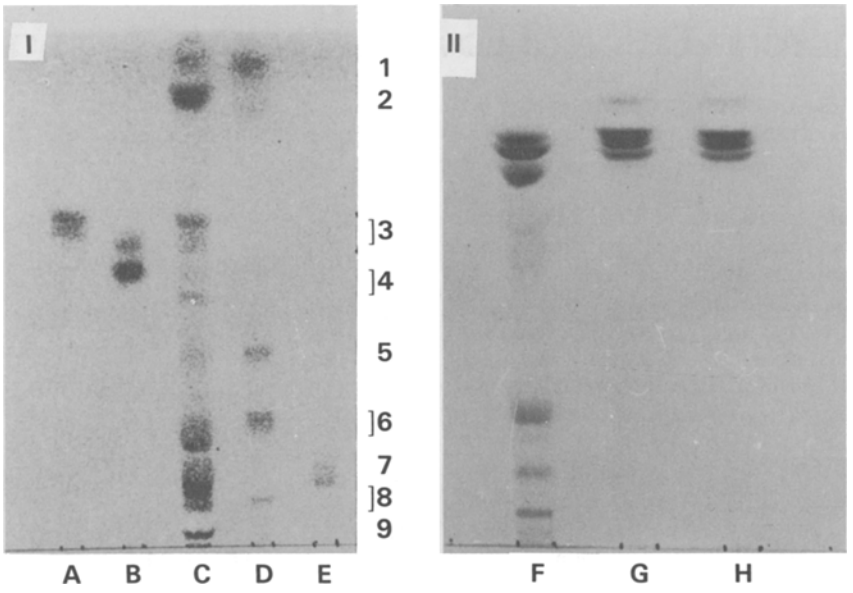

FIG. 1. Thin layer chromatography of glycolipids from chromaffin cell plasma membranes. I: "Neutral" glycolipids. Folch lower phase (21) was submitted to a mild alkaline hydrolysis (47). Alkali-stable lipids were separated on borate-impregnated HPTLC plates (30). A: glucosyl ceramide; B: galactosylceramide; C: plasma membrane glycolipids; D: dihexosylceramide + trihexosylceramide + GM_3 (from kidney); E: sphingomyelin. Spots 2, 4 and 7 were dark yellow, the others were purple after detection with orcinol-sulfuric acid (25). II: Gangliosides. Folch upper phase glycolipids were purified on Sephadex G25 and chromatographed by HPTLC (27). F: From the front to the start—GM_3 (N-acetyl + N-glycosyl) + GD_{1a}, GD_{1b}, GT (from spleen or brain); G, H: plasma membrane gangliosides. The compound ahead of GM_3 gave a brown color with orcinol-HCl (48).

been suggested as an essential parameter for the stabilization of bilayer structures rich in lysolipids (32–34).

The phospholipid pattern (Table 2) is similar to that reported earlier (19). The main discrepancy exists in the slightly lower amount of sphingomyelin found in our plasma membrane preparation, which may be attributed to differences in the preparation method. Our results confirm the very low amount of lysophosphatidylcholine in the plasma membrane found by Nijjar and Hawthorne (19). This is in striking contrast to the high levels of these lysolipids reported in the chromaffin granule membranes (3–5,8–10,12,14). The percentage of lysophosphatides present in plasma membrane is similar to the values found for the other membrane preparations from chromaffin cells, which are not thought to be involved in fusion-mediated secretory processes, such as the Golgi apparatus (9), the microsomes, or the mitochondria (3,4,8,15). However, the value we found for our preparations is within the range reported for plasma membranes from other tissues (see 35–38 for example). The notable difference in the lysophosphatidylcholine content found in membranes capable of fusion and membranes not involved in fusion phenomena could be in favor of the involvement of this lipid in the fusion process. However, this lysophospholipid-mediated process seems to be a characteristic of chromaffin cells since it has not been observed in other secretory cells such as mast or pituitary cells (14). Nearly half of the ethanolamine phospholipids were represented by the alkenyl derivatives. This was in distinction from the chromaffin granules membrane, where alkenylphosphatidylethanolamine accounted for three-quarters of the total ethanolamine phosphatides (10).

A notable part of the glycolipids found in chromaffin cell plasma membrane belongs to the "neutral" glycolipids (Table 1). Fractions migrating together with mono-, di, tri-, tetra- and pentahexosylceramides could be seen (Fig. 1). The pattern observed here is somewhat similar to the one reported in guinea pig adrenals (39). However, results obtained using borate-impregnated thin layer plates showed that in our plasma membrane the main (if not the only) monohexosylceramide was glucosylceramide and not galactosylceramide as found in guinea pig adrenals. Moreover, in contrast to what was observed, the chromaffin cell plasma membrane monohexosylceramide probably accounts for less than 50% of the total neutral lipids. The ganglioside pattern of adrenal medulla has been shown to be species-dependent (39–43). Our results and those of Sekine et al. (39) indicate a species dependence for "neutral" glycolipids also.

Irrespective of the amount chromatographed (up to 5 μg), we have not been able to detect other gangliosides than GM_3 in our membrane preparations (Fig. 1). Similar results were obtained with chromaffin granule membranes where GM_3 represented 95% of the total gangliosides (10,17,18). A triple band was observed after chromatography showing that plasma membrane contained a mixture of N-acetyl and N-glycolyl sialyl lactosylceramide as also found in the secretory granules (18).

Chromaffin cells and neurones have the same embryonic origin. Both develop neurites and synthesize

catecholamines. A very simple ganglioside profile was found in the chromaffin cells while an entirely different pattern exists in adrenergic peripheral neurones, characterized by a high level of polysialogangliosides (44). This shows that a notable amount of polysialogangliosides is not a prerequisite for the maturation of peripheral nerve cells as recently suggested (45,46).

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Diurnal Rhythm of HMG CoA Reductase Activity in Canine Intestine Is Independent of Luminal Contents

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Activity of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34) measured in isolated segments of canine intestinal mucosa showed a distinct diurnal rhythm. Total activity changed over a twofold range with a peak occurring during midday, shortly after feeding. Since the isolated segments had no contact with luminal contents, the rhythm was not directly related to food components or bile salts. Humoral or neural influences must mediate the rhythm. The diurnal rhythm persisted for at least 3–5 mo, but was lost by 10 mo following formation of the isolated segment, possibly because of mucosal involution.
Lipids 21, 420–421 (1986).

A circadian rhythm in the rate of hepatic cholesterol synthesis and in activity of its rate-limiting enzyme, HMG CoA reductase, was initially reported for the mouse and rat in 1969 (1–3). Subsequently, a diurnal rhythm of cholesterogenesis and HMG CoA reductase also has been reported for rat intestinal mucosa (4,5). In rodents, both of these major organs of cholesterol synthesis show peak HMG CoA reductase activity at night—the time of feeding—and nadir activity during the light period (1,4,6,7). Diurnal cycling of reductase activity has also been reported for swine liver and intestine (8). In this species, however, peak activity occurs during the day, six hr after feeding. Indirect evidence, using plasma mevalonate measurements, has also suggested a diurnal rhythm in human cholesterol synthesis (9). Peak mevalonate levels occurred at midnight.

Potential control factors for this rhythm have been reviewed by Rodwell et al. (10). Factors include light/dark cycling, food ingestion and cyclic blood hormone levels. It is known that changing the time of light and dark periods will alter the rhythm of reductase. However, such changes also alter the timing of feeding by the animals and the diurnal rhythms of various hormones (5). Food content can affect the cycle of both cholesterol synthesis and HMG CoA reductase. Cholesterol feeding suppresses both the hepatic and gut rhythms (4,7), bile salts suppress gut activity (11) and cholestyramine increases the hepatic and possibly the gut rhythm (4,8). Hormones also can affect the hepatic cycle, which is blunted or abolished in diabetes (6,12–14). The gut rhythm appears to persist in diabetes (6). Glucagon, cortisone, cyclic AMP and other hormones may also affect activity (14).

We have studied the diurnal changes of canine intestinal HMG CoA reductase in a setting which excludes direct luminal influences of meal time, food content, bile acids and other luminal content.

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MATERIALS AND METHODS

Three healthy, adult mixed-breed dogs fed a diet of Wayne Dog Chow (Allied Mills Inc., Chicago, Illinois) underwent formation of an isolated ileal segment fistula (Thiry-Vella fistula) under pentothal anesthesia (15). A 45-cm ileal segment ca. 30 cm proximal to the ileocecal valve was isolated. Remaining bowel was anastomosed to reestablish luminal continuity. The isolated segment retained vascular relationships but was removed from luminal continuity. Access to the segment was maintained by an enterocutaneous stoma at each end. The segment was perfused with 100 cc saline daily. Animals recovered, gained weight and were studied by being trained to stand unanesthetized for mucosal biopsy from the ileal segment using a Quinton-Rubin suction biopsy tube (15). Biopsy was painless and well tolerated. Biopsies were obtained at least 2–4 wk after formation of the isolated segment but not more than six mo after surgery. The tissue pieces obtained were ca. 4 mm in diameter and consisted of epithelial cells and lamina propria, cleaved at the level of the muscularis mucosa. Animals were studied at six-hr intervals on several separate days.

Total HMG CoA reductase activity was measured in whole mucosal homogenates by a modification of the method of Shapiro et al. (16) as we have previously described (15,17). Conversion of [^{14}C]HMG CoA (New England Nuclear, Boston, Massachusetts) to [^{14}C]-mevalonate was determined during a 15-min incubation and enzyme activity was expressed as pmol mevalonate formed/mg mucosal protein/min. Individual measurements were assayed in duplicate. We have previously validated the conditions and substrate concentrations used in this assay (15,17,18). Values are given as mean ± SEM. Statistics were analyzed by t-test of unpaired variants.

RESULTS AND DISCUSSION

As we have previously reported, mucosal HMG CoA reductase activity increased following formation of the isolated segment (15). Basal midmorning incontinuity mucosal activity for the three animals was 25 ± 5 pmol/mg/min, while activity in the isolated segment was 57 ± 7 pmol/mg/min one week after surgery. Activity then fell over time, to reach lower values by the time of diurnal studies. Figure 1 shows the pattern of total mucosal HMG CoA reductase activity in the isolated loop during the 24-hr period 2–4 wk after surgery. Peak activity occurred during daytime and the twofold difference between 4 p.m. and 10 p.m. was significant at $p < 0.02$. During these studies, animals received a single meal at 1 p.m. However, the diurnal rhythm of total enzyme activity within loop mucosa occurred in the complete absence of luminal content of food material, lipid or bile salt. Thus, the diurnal change must have derived from

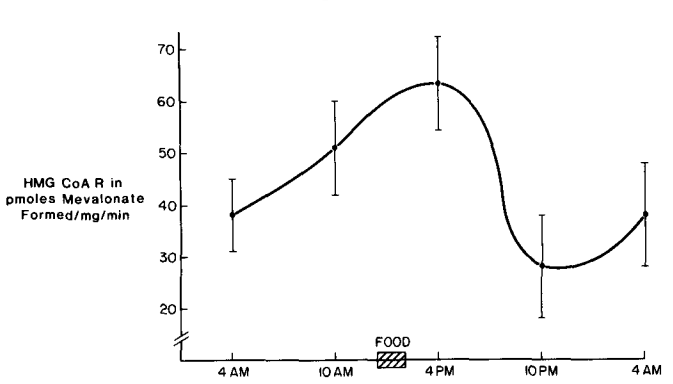

FIG. 1. Total mucosal HMG CoA reductase activity from isolated ileal segments at various times during the 24-hr period. Peak activity occurs between noon and 4 p.m. and is twice the activity at 10 p.m. ($p < 0.02$ by Student's t-test of unpaired variants, $n = 6$–9 biopsies per time point).

a neurohumoral origin and, if it was associated with feeding, a secondary mediator must have been involved. Blood lipids and various hormones are known to be able to affect intestinal cholesterol synthesis and HMG CoA reductase (15,19,20); these may be important. If lipoproteins are involved in the diurnal enzyme rhythm, the mechanism of action must be quite rapid since activity falls by 50% within eight hr of food ingestion. Rapid synthesis and degradation of reductase enzyme is known to occur (10). Since we measured total (dephosphorylated) enzyme activity, changes in phosphorylation state do not play a role.

Over time, the mucosa of isolated Thiry-Vella fistulae undergoes involution (21). The luminal diameter decreases, mucosa thins and villi become atrophic. When tested 9–10 mo following formation of the isolated segments, mucosal biopsies showed a constant, low level of HMG CoA reductase activity. Values were 25.5 ± 2.5 at 10 a.m. ($n = 6$), 22.6 ± 3.5 at 4 p.m. ($n = 6$), 24.0 ± 2.4 at 10 p.m. ($n = 6$) and 29.1 ± 6.7 pmol/mg/min at 4 a.m. ($n = 3$). Mucosa of the chronically isolated segment does not require cholesterol for lipid absorption and, in all likelihood, cell turnover is reduced so that the cholesterol required for membranes is also minimal. Thus, as disuse mucosal involution occurs, cholesterol synthesis falls and the diurnal rhythm is lost.

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Effect of Different Acids with Δ9,12-Dienoic Structures on Δ9 Desaturation Activity in Rat Liver Microsomes

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The effect of oral administration, for 24 or 48 hr, of different octadeca fatty acids containing a 9,12-dienoic structure on the fatty acid composition and Δ9 desaturation activity of liver microsomes of rat fed a fat-free diet was studied. The ethyl esters of linoelaidic and γ-linolenic acids, the methyl ester of linoleic acid and free columbinic acid were administered to rats maintained on a fat-free diet. The supplementation of the fat-free diet with linoelaidate produced no relevant changes in the fatty acid composition pattern of liver microsomes and did not modify the percentage of conversion of palmitic to palmitoleic acid. The addition of linoleate or γ-linolenate to the fat-free diet returned liver microsome Δ9 desaturation activity toward the control and partially restored the liver microsome fatty acid spectrum found in the fat-free diet. Columbinic acid (5-*trans*-9-*cis*,12-*cis*-18:3), which cannot be transformed into arachidonic acid, also decreased the Δ9 desaturation activity enhanced by the fat-free diet and evoked changes in the microsomal fatty acid composition similar to those produced by the ω6 fatty acids. These results suggest that the modulation of Δ9 desaturase activity evoked by dietary administration of unsaturated acids of ω6 series would depend on the *cis* double bond configuration of these acids.
Lipids 21, 425–429 (1986).

It has been known for some time that liver microsomal Δ9 desaturation of fatty acids is affected by different dietary conditions of the animals. The activity of stearyl-CoA desaturase is decreased by starvation and recovered by refeeding (1). Dietary carbohydrate increases stearyl-CoA desaturase (2–4) while a high fat diet inhibits the activity of the enzyme (4). Conversely, the administration of a fat-free diet produces a great increase on the activity of liver microsomal Δ9 desaturase (4,5). The type of fat included in the diet also modifies the activity of this enzyme, which is depressed when rats maintained on glucose, fructose or a fat-free diet are fed linoleic or arachidonic acid (4,6,7).

Moreover, studies have been published on the effect of different fatty acids on Δ9 desaturase activity in isolated and reconstituted enzyme systems (8) or in liver microsomal system (9). However, some aspects of the rapid effects produced on Δ9 desaturase activity by the in vivo administration of unsaturated fatty acid or ω6 series have not been clarified yet.

Since columbinic acid has a special structure in which the *cis,cis*-Δ9,12 double bonds of linoleic acid are present but a *trans* double bond in Δ5 position impedes its desaturation to γ-linolenic acid, a study was undertaken to compare in vivo the effects of octadeca-9,12-dienoic structures of linoleic, γ-linolenic and columbinic acids on Δ9 desaturation of EFA-deficient rats. Animals fed a balanced diet, a fat-free diet and a fat-free diet supplemented with linoelaidic acid were used as controls.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Palmitic acid (56 mCi/mmol, 99% radiochemically pure) was purchased from New England Nuclear Corp. (Boston, Massachusetts). NADH, ATP, CoA, linoelaidic acid ethyl ester and cofactors were provided by Sigma Chemical Co. (St. Louis, Missouri). Ethyl γ-linolenate and unlabeled palmitic acid were purchased from Nu-Chek-Prep Inc. (Elysian, Minnesota). Methyl linoleate was prepared from sunflowerseed oil following the procedure described by Keppler et al. (10). This preparation was 99% pure. Columbinic acid was a gift from U.M.T. Houtsmuller.

Treatment of animals. The experiments were carried out with male weanling rats of the Wistar strain. Animals were fed ad libitum for 1 mo on either a balanced diet consisting of (in cal) 55% starch, 20% casein and 25% sunflowerseed oil or a fat-free diet containing 73.4% starch and 26.6% defatted casein. The fatty acid content of the balanced diet was 3.8 g % and the relative percentages of the fatty acids were 12.0 palmitic, 0.4 palmitoleic, 3.0 stearic, 34.1 oleic and 50.5 linoleic. Both diets were supplemented with minerals and a mixture of vitamins (11). Water was given ad libitum. After a month, the rats on the fat-free diet were divided into different groups. One group was maintained on the same diet; in the other groups the diet was supplemented with 1.5 g % of either ethyl γ-linolenate, ethyl linoelaidate, methyl linoleate or columbinic acid. Groups of rats were killed 24 and 48 hr after the supplementation of the diet. Six animals per group were used in these experiments. The rats were killed by decapitation. Livers were excised rapidly and immediately placed in ice-cold homogenizing medium (12). After the homogenization, microsomes were separated by differential centrifugation at 100,000 × g as described previously (12) and immediately used for the desaturation assays and for measuring the fatty acid composition.

Analytical procedure. Desaturation of palmitic acid by liver microsomes was measured estimating the percentage of conversion of [1-^{14}C]palmitic acid to palmitoleic acid. Five nmol of labeled acid and 95 nmol of unlabeled acid were incubated with 5 mg of microsomal protein in a metabolic shaker at 37 C for 10 min in a total volume of 1.5 ml of 0.15 M KCl-0.25 M sucrose solution. The medium contained 4 μmol ATP, 0.1 μmol CoA, 1.25 μmol NADH, 5 μmol $MgCl_2$, 2.4 μmol n-acetylcysteine, 62.5 μmol NaF, 0.5 μmol nicotinamide and 62.5 μmol phosphate buffer (pH 7). After 10 min the reaction was stopped by addition of 2 ml of 10% KOH in ethanol. The fatty acids were recovered by saponification of the incubation mixture (45 min at 85 C), acidification and extraction with petroleum ether (bp 30–40 C). The fatty acids were esterified with methanolic 3M HCl (3 hr at 68 C) and the distribution of the radioactivity between substrate and product was measured by gas liquid

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radiochromatography in an apparatus equipped with a Packard proportional counter. Percentage of conversion was calculated from the distribution of radioactivity between substrate and product measured directly on the radiochromatogram.

Lipids of liver microsomes were extracted with chloroform/methanol (2:1, v/v) by the procedure of Folch et al. (13). The fatty acids of the lipids were converted to methyl esters by the procedure described above and analyzed on a Hewlett-Packard, Model 5840-A gas liquid chromatograph equipped with a flame ionization detector. The column was packed with 10% SP2330 coated on Chromosorb WAW (100–200 mesh), Supelco Inc. (Bellefonte, Pennsylvania). The oven temperature was programmed from 140 to 220 C at 3 C/min to separate methyl esters ranging from 12 to 22:6ω3. Retention time and peak areas were determined electronically using a Hewlett-Packard reporting integrator. Identification of methyl esters was made by comparison with known standards.

Results were calculated as mean ± one SEM; statistical analyses were made by conventional t-test. Microsomal protein content was determined by the biuret method of Gornall et al. (14).

An additional experiment was done to find any competitive effect on the Δ9 desaturation activity when different concentrations of linoleic and columbinic acids were added to a microsomal system, separated from the liver of rats fed a balanced diet. The microsomal fraction of the livers of three rats was obtained as described above. Desaturation of palmitic to palmitoleic acid was measured using 5, 10 and 20 nmol of palmitic acid (5 nmol of labeled acid and different amounts of unlabeled acid) with increasing amounts of either linoleic or columbinic acid (10, 20 and 40 nmol). Five mg of microsomal protein were incubated in a metabolic shaker at 37 C for 10 min in the conditions previously described. The percentage of conversion was studied by gas liquid chromatography as already detailed.

RESULTS

Fatty acid composition. It is well known that the absence or presence of fat in the diet, as well as its fatty acid composition, changes the fatty acid composition of animal tissues. In this respect, it is important not only to know the changes produced after a long period of diet modification, which gives a picture of the stationary aspect of the adaptation, but also the effect produced in a short period of time, which informs about the dynamics of the adaptation. Correspondingly, it has been shown that liver microsomes are especially sensitive to these changes and quickly respond to them (7,15–17). For this reason, to study the effect of unsaturated acids of Δ9,12 structure on monoenoic biosynthesis, animals were first deprived of fat in the diet for a month and then refed with the unsaturated acids for only 24 or 48 hr. Histograms of Figures 1 and 2 show the percentage of the principal fatty acids of the microsomes under different dietary conditions.

The variations of saturated and monoenoic fatty acids in liver microsomal lipids are shown in Figure 1. This figure indicates that the fat-free diet, as expected, increased palmitic, palmitoleic and oleic acids, compared to the animals fed a balanced diet. The addition of the

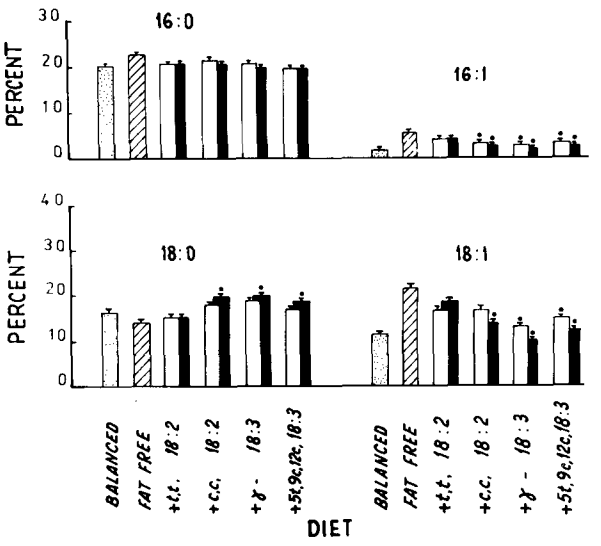

FIG. 1. Effect of different dietary fatty acids, supplemented to rats maintained on a fat-free diet, on the relative percentages of palmitic, palmitoleic, stearic and oleic acids of liver microsomes. Balanced diet (dotted bar), fat-free diet (lined bar), or fat-free diet supplemented with the different acids for 24 hr (open bar) or 48 hr (solid bar). Results are the mean of six animals. Vertical lines represent 1 SEM, *P < 0.01 compared to fat-free diet.

all-*trans* linoelaidic acid to the fat-deficient diet did not evoke differences in the relative percentage of those acids compared to the rats maintained on a fat-free diet, after either 24 or 48 hr of treatment. The supplementation of the diet with linoleate and γ-linolenate (both containing the 9-*cis*,12-*cis*-dienoic structure) returned the values of palmitoleic and oleic acids to controls, an effect that was more evident in the presence of γ-linolenate. The relative percentage of stearic acid also increased significantly after 48 hr of linoleate and γ-linolenate treatment. Columbinic acid evoked changes in saturated and monoenoic acids similar to those produced by linoleate or γ-linolenate administration.

The effects of the diets on the polyunsaturated fatty acid of microsomal lipids are illustrated in Figure 2. The fat-free diet produced the corresponding decrease of linoleic and arachidonic acids and an increase of eicosa-8,11,14-trienoic acid typical of this kind of diet. The addition of linoelaidic acid had virtually no effect on the polyunsaturated fatty acid pattern of the animals on the fat-free diet in spite of being incorporated into the microsomes. The percentage was 6.5 ± 0.4 after 24 hr and 7.7 ± 0.5 after 48 hr.

The addition of members of the ω6 fatty acid family tended to restore normal fatty acid pattern: the ω9 series was diminished and the ω6 series was increased. In spite of the common behavior, specific effects were easily recognized for each acid (Fig. 2) since linoleate restored both linoleic and arachidonic acid levels, whereas γ-linolenate restored arachidonic and docosapentaenoic acids but decreased the already low linoleic acid level. Besides, after the treatment with γ-linolenate the percentage of this acid found in the microsomes was only about 1%. Sprecher (19) also described a depression of eicosa-5,8,11-trienoic acid and an increase of arachidonic acid level in rat liver when γ-linolenic acid was the sole fat source in the diet.

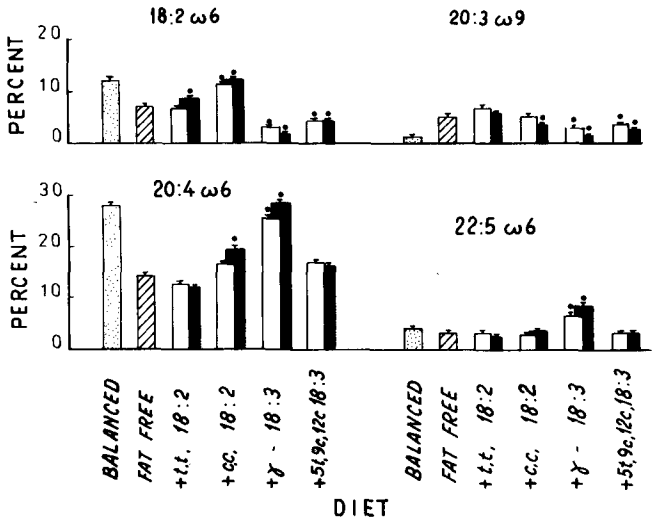

FIG. 2. Effect of different dietary fatty acids, supplemented to rats maintained on a fat-free diet, on the relative percentages of linoleic (18:2ω6), eicosa-5,8,11-trienoic (20:3ω9), arachidonic (20:4ω6) and docosa-4,7,10,13,16-pentaenoic (22:5ω6) acids of liver microsomes. Balanced diet (dotted bar), fat-free diet (lined bar), or fat-free diet supplemented with the different acids for 24 hr (open bar) or 48 hr (solid bar). Results are the mean of six animals. Vertical lines represent 1 SEM. *P < 0.01 compared to fat-free diet.

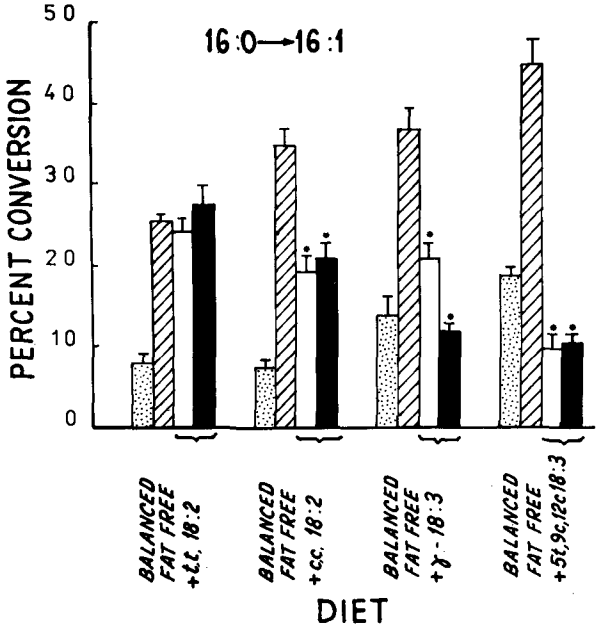

FIG. 3. Effect of different dietary fatty acids, supplemented to rats maintained on a fat-free diet, on the percentage of conversion of palmitic to palmitoleic acid of rat liver microsomes in vitro. Balanced diet (dotted bar), fat-free diet (lined bar), or fat-free diet supplemented with linoelaidic (t,t-18:2), linoleic (c,c-18:2), γ-linolenic (γ-18:3) and columbinic (5t,9c,12c-18:3) acids for 24 hr (open bar) or 48 hr (solid bar). Results are the mean of six animals. Vertical lines represent 1 SEM, *P < 0.001 compared to fat-free diet.

When columbinic acid was administered in the diet it was incorporated into microsomal lipids (9.9 ± 0.6 and 10.4 ± 0.6 after 24 or 48 hr of treatment, respectively) and was elongated to acids of 20 and 22 carbons in an amount

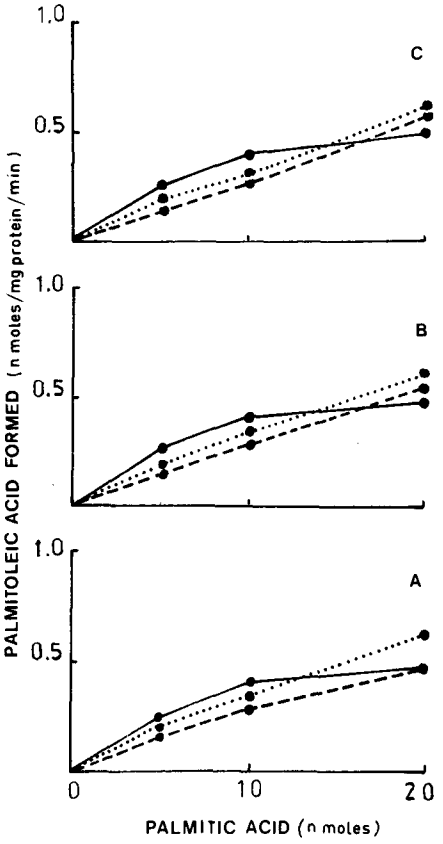

FIG. 4. Effect of exogenous linoleic and columbinic acids on Δ9 desaturation activity of liver microsomes of rats fed a balanced diet. Increasing amounts of palmitic acid were incubated in the absence (•——•) and presence of linoleic (.....) or columbinic (•— — —•) acid. A: 10 nmol; B: 20 nmol; C: 40 nmol. Results are the mean of the liver microsomal pool of three rats analyzed in duplicate.

of 1-3% (Fig. 2). Similar conversions were also observed in isolated HTC cells (18).

Columbinic acid addition decreased the microsomal concentration of linoleic and 20:3ω9 acids but increased arachidonic acid.

Δ9 Desaturation activity. The desaturation of palmitic acid in liver microsomes of rats fed for one month either a balanced diet, a fat-free diet or a fat-free diet supplemented during the last 24 or 48 hr with different acids of octadeca-9,12-dienoic structure is shown in Figure 3. The fat-free diet produced a significant increase in Δ9 desaturation activity compared to animals fed a balanced diet. The supplementation of the fat-free diet with linoelaidate for either 24 or 48 hr produced no changes in the conversion of palmitic to palmitoleic acid, as has been demonstrated already by others (20). The addition of linoleate or γ-linolenate to the fat-free diet produced a significant decrease in this enzymatic activity, an effect that was more remarkable when the rats were fed γ-linolenate for 48 hr. In this case, the values were similar to those obtained from the animals fed a balanced diet.

The additions of columbinic acid also produced a decrease in Δ9 desaturation activity that reached the control values 24 hr after the diet administration. However, the curves shown in Figure 4 demonstrate that the incubation of a variable concentration of either linoleic or columbinic acid and labeled palmitic acid with rat liver

microsomes produced no significant changes in the conversion of palmitic to palmitoleic acid in any of the different concentrations of the substrate analyzed.

DISCUSSION

The data presented in this study are in agreement with earlier reports showing that fatty acid desaturation in rat liver microsomes is regulated by dietary alterations. Now it is generally accepted that in mammalian systems, a fat-free diet enhances the activity of Δ9 desaturase (4,5) and the same results are shown in the present experiment (Fig. 3). This effect was reflected in the microsomal fatty acid composition and indicated by the increase in the relative percentage of palmitoleic and oleic acids (Fig. 1). The addition of essential fatty acids to the fat-free diet has been shown to inhibit the Δ9 desaturase (4,6,7) and is reflected in the present experiment by the decrease of the conversion of palmitic to palmitoleic acid. However, the specific effect produced by different Δ9,12 structures on the deactivation of the Δ9 desaturase has not been fully investigated. Using a model experiment based on dietary changes, we have tried to elucidate the effect produced by four typical acids that have in common the 18-carbon chain length and the Δ9,12 double bonds. However, whereas in linoelaidic acid the double bonds are all *trans*, linoleic, γ-linolenic and columbinic acids possess an all-*cis* Δ9,12 structure. Gamma-linolenic acid, having an extra *cis* double bond in Δ6, is biosynthesized from linoleic acid and is a precursor of arachidonic acid whereas the fourth acid tested, columbinic, has an extra *trans* double bond in Δ5 in addition to the *cis,cis* Δ9,12. This extra double bond in Δ5 position prevents the desaturation of the acid by the Δ6 desaturase (21).

Figures 1 and 3 show that linoleate or γ-linolenate decreased the Δ9 desaturase activity enhanced by the fat-free diet, and at the same time decreased oleic and palmitoleic acid percentages in the microsomes. Both acids were converted by the animal into higher homologs and predominantly to arachidonic acid. Therefore, the inhibitory effects could be due to a negative induction or competition on the Δ9 desaturase enzyme exerted by the tested acids or by their products of conversion, since it was already shown that the addition of arachidonic acid to a fat-free diet also produced a decrease in microsomal Δ9 desaturase activity (7). A *cis,cis* structure was essential for this effect, since t,t,18:2ω6 acid that is not desaturated to higher homologs (22,23) is an ineffective agent in the modulation of Δ9 desaturase activity (Fig. 3).

To elucidate if the effect of ω6 *cis* fatty acids on the Δ9 desaturase depended specifically on the Δ9,12 structure or on their transformation into arachidonic acid, the effect of columbinic acid was assayed. Columbinic acid is incorporated into all lipid classes of different tissues (21,24) but it is not a substrate for the Δ6 desaturase (21). In addition, it impaired Δ9 desaturation (Fig. 3) and decreased the amount of palmitoleic and oleic acids in the membrane (Fig. 1). Columbinic acid differs from linoleic acid only by the existence of the extra *trans* double bond in Δ5 position, but this *trans* double bond which prevents the desaturation of columbinic acid brings forth an extended conformation to this part of the molecule, which therefore is not very different from the antiperiplanar structure of the corresponding single -C-C- bond of linoleic acid. In consequence, columbinic and linoleic acids have a very similar spatial structure. The results of the present work show in consequence that the *cis,cis* Δ9,12 double bond structure of the unsaturated acid is inhibitory per se of Δ9 desaturase, and this effect is maintained even after the addition of a *trans* double bond in Δ5 or a *cis* double bond in Δ6 position (γ-linolenic acid). These results are in agreement with a prediction suggested by Abraham et al. (24) after the analysis of the total fatty acid composition of liver and mammary glands of lactating mice fed on linoleate, γ-linolenate and columbinate.

Besides, results of Figure 4 referring to the Δ9 desaturation activity found by incubating liver microsomes of rat fed a balanced diet in the presence of linoleic or columbinic acid would indicate that the inhibitory effect previously observed would not be a result of a direct competitive reaction.

Columbinic acid also decreased markedly the amount of 20:3ω9 acid already incorporated in liver microsomes, a finding that was previously reported (21,24).

Figure 2 also shows that columbinic acid decreased linoleic acid and increased the level of arachidonic acid upon that observed in essential fatty acid-deficient microsomes. These results can be explained considering that columbinic acid is incorporated into membrane lipids, replacing linoleic acid in the phospholipids of the bilayer. Then the linoleic acid released from the phospholipids moieties would provide an additional source of substrate for arachidonic acid biosynthesis. However, this effect is transient and will function meanwhile and until linoleic acid in the phospholipid depot is not exhausted. Therefore, the production of eicosanoid acids derived from ω6 acids would be maintained during this extra period but it would be decreased after the exhaustion of arachidonic acid sources.

The inhibition produced by columbinic acid on oleic and 20:3ω9 biosynthesis has further consequences in the composition and structure of the lipid bilayer of membranes. Moreover, since the biosynthesis of these ω9 fatty acids was decreased although the animals were in an EFA-deficient status, columbinic-treated animals presented a low 20:3ω9/20:4ω6 ratio. Therefore, this ratio cannot be used in these animals as an index of EFA deficiency.

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Fatty Acids in Crinoidea and Ophiuroidea: Occurrence of All-*cis*-6,9,12,15,18,21-tetracosahexaenoic Acid

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The fatty acid compositions of lipids from two species of Crinoidea and two species of Ophiuroidea have been investigated with open-tubular gas chromatography. About 5–10% of tetracosahexaenoic acid was found in total fatty acids from all the samples, and the structure was determined as all-*cis*-6,9,12,15,18,21-tetracosahexaenoic acid [24:6(n-3)] by ^{13}C-NMR of the methyl esters and mass spectrometric analyses of the methyl esters, the pyrrolidides and the ozonolysis products. The 24:6(n-3) was concentrated in the polar lipids rather than neutral lipids. The n-3 hexaenoic structure suggested chain elongation of 22:6(n-3) as the source.

The 5-olefinic acids (5-18:1, 5-20:1, 5,11- and 5,13-20:2) were low in Crinoidea (0.2–1.3%) but were present in higher levels (2.5–5.2%) in Ophiuroidea. Polyunsaturated acids found other than 24:6(n-3) were 20:4(n-6), 20:5(n-3) and 22:6(n-3) as major components and 16:3(n-3), 18:2(n-6), 18:3(n-6), 18:3(n-3), 18:4(n-3), 20:2(n-9), 20:2(n-6), 20:3(n-6), 20:3(n-3), 21:5(n-3) and 22:5(n-3) as minor components in all the samples.
***Lipids 21,* 430–433 (1986).**

Marine invertebrates usually contain a great number of unsaturated fatty acid isomers as their lipid components. To separate the isomers for determination of the components, open-tubular gas chromatography (GC) is a very effective tool. In echinodermata, fatty acids from some species of Echinoidea (1,2) and Asteroidea (3) have been investigated by open-tubular GC, and the 5-olefinic fatty acids such as 5-18:1, 5-20:1, 5,11- and 5,13-20:2 were found as their characteristic components. In this study, open-tubular GC was used for the investigation of fatty acids from Crinoidea and Ophiuroidea lipids, and 24:6 fatty acid was found as an unusual component with minor amounts of the 5-olefinic acids. The structure of 24:6 fatty acid was confirmed as all-*cis*-6,9,12,15,18,21-24:6 by ^{13}C-NMR and mass spectrometric analyses. Occurrence of 24:6(n-3) in the lipids of some marine fish has been reported (4,5), but the contents of 24:6(n-3) were low levels, below 2%. In this study, high levels of 24:6(n-3) (5–10%) in Crinoidea and Ophiuroidea lipids were elucidated.

MATERIALS AND METHODS

Materials. Two specimens of sea lilies, *Tropiometra afra macrodiscus* and *Comanthus japonica,* were caught at the coast of Miura Peninsula in Kanagawa prefecture on April 9, 1985. Two specimens of brittle stars, *Ophioplocus japonicus* and *Asteronyx loveni,* were caught at the coast of Usujiri near Hakodate on June 8, 1979, and at 200 m depth in Okhotsk Sea on June 25, 1979, respectively. The species of animals studied are listed in Table 1. They were kept frozen at −20 C for a few months until used.

Preparation of fatty acid methyl esters. Total lipids were extracted by the method of Bligh and Dyer (6). Fractionation of total lipids (TL) into neutral and polar lipids (NL, PL) was carried out by column chromatography using silicic acid (Kiesel Gel 60; Merck, Darmstadt, Federal Republic of Germany) with chloroform and methanol as the developing solvents. Lipids were saponified by refluxing with 1 M KOH-EtOH for 1 hr, and the unsaponifiables were extracted with ether. Following acidification with dilute HCl, fatty acids were recovered by ether extraction and converted to methyl esters by heating in a sealed tube at 100 C for 10 min with 7% BF_3-MeOH. Methyl esters were purified by thin layer chromatography (TLC) with Kiesel Gel 60 G plate of 0.5 mm thickness by developing with n-hexane/ether (85:15, v/v).

Open-tubular GC of methyl esters. Open-tubular GC of methyl esters was done with a Shimadzu GC 6AM instrument (Shimadzu Seisakusho Co., Kyoto, Japan) equipped with a dual FID detector and a glass capillary WCOT column (50 m × 0.28 mm id) coated with SP 2300 (Supelco Inc., Bellefonte, Pennsylvania). The carrier gas was H_2. The column temperature was 190 C, and the injector and detector were 230 C. Peak area percentages were obtained with a Shimadzu integrator C-R2AX.

TABLE 1

Samples and Their Contents of Lipids

Sample no.	Class	Genus and species	Lipid %[a]		Date	Place
			Total	Neutral		
1	Crinoidea	*Comanthus japonica*	0.98	52	April 1985	Misaki
2		*Tropiometra afra macrodiscus*	0.69	24	April 1985	Misaki
3	Ophiuroidea	*Asteronyx loveni*	1.63	—	June 1979	Okhotsk Sea
4		*Ophioplocus japonicus*	0.77	—	June 1979	Usujiri

[a]Wt %: Total lipid of a wet sample and neutral lipid to total lipid.

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Structural analyses of 24:6(n-3). Methyl esters were fractionated according to their degree of unsaturation on a silver nitrate impregnated layer of silica gel G (1) by developing with ethyl acetate/n-hexane (1:9, v/v).

The pentaenoates and hexaenoates were concentrated by argentation column chromatography using 20% $AgNO_3$-silica gel as the packing, and ether/n-hexane as the solvent for eluting. The percentages of ether in the solvents were progressively increased from 10 to 100%.

Methyl esters were fractionated according to their carbon number on Whatman KC18F reversed phase (RP) TLC plate by developing with acetonitrile.

A JEOL FX-90Q spectrometer (Nippon Denshi Co., Tokyo, Japan) in the Fourier transform mode at 22.5 MHz for ^{13}C was used to obtain the spectrum of methyl ester in $CDCl_3$.

Pyrrolidides of fatty acids were prepared by heating at 100 C for 30 min on a microscale (7) 10 mg of methyl esters in freshly distilled pyrrolidine, 1 ml, and acetic acid, 0.1 ml.

Reductive ozonolysis of 1–5 mg of methyl ester samples was carried out in methylene chloride at −70 C using the procedure of Kleiman et al. (8). The ozonides were reduced by addition of a few crystals of triphenylphosphine. Aldehydic products were analyzed by GC using 1.5 m × 3 mm id glass column packed with 5% Silar 10C on Gas Chrom Q (100–120 mesh). The column temperature was programmed from 60 to 280 C at 2 C/min. The detector and injector temperatures were maintained at 300 C. The carrier gas was nitrogen at 30 ml/min.

GC-mass spectrometry (MS) analyses of the methyl esters, the pyrrolidides and the ozonolysis products were carried out with a JEOL D-300 GC-MS system equipped with a glass column (1 m × 3 mm id) packed with 3% OV-1 on 100–120 mesh Gas Chrom Q for the methyl esters, 1.5% OV-17 on 100–120 mesh Gas Chrom Q for the pyrrolidides, and a glass column (2 m × 3 mm id) packed with 2.5% FFAP on 100–120 mesh Gas Chrom Q for the ozonolysis products using He as a carrier gas. All spectra were obtained at 23 eV ionizing electron energy, and a source temperature of 185 C for the methyl esters and 190 C for the pyrrolidides and the ozonolysis products.

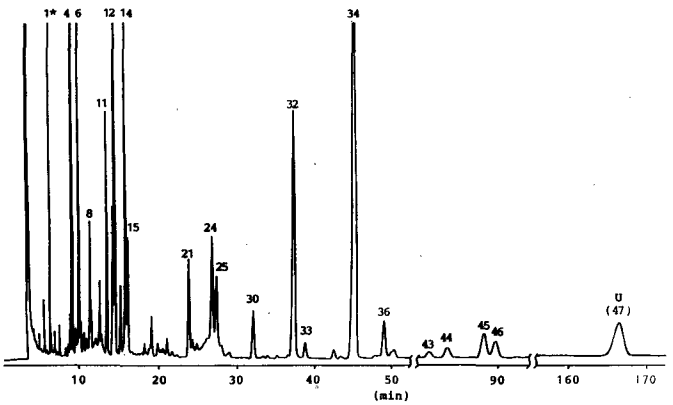

FIG. 1. Gas chromatogram of fatty acid methyl esters of total lipids from *Comanthus japonica*. *See Table 4.

TABLE 2

Comparison of Mass Spectra of 6-Oxo-hexanoate from Ozonolysis with a Reference Specimen

m/e	Intensity %[a] From 24:6(n-3)	Intensity %[a] From 18:1(n-12) (reference)	Relation with M[b]
126	0.3	0.5	M-18
115	1.4	1.3	M-29
113	37.0	33.5	M-31
112	21.2	19.9	M-31-1
101	46.0	42.9	M-43
95	10.8	14.6	M-31-18
87	100.0	100.0	M-57
85	11.7	13.9	M-59
84	26.8	29.6	M-59-1
74	51.1	49.9	M-70
73	6.6	7.8	M-71
71	3.4	7.7	M-73
70	12.1	15.8	M-74
69	6.8	9.1	M-74-1
67	34.2	40.9	M-59-18
59	52.3	54.2	M-85
57	15.1	21.9	M-87
56	8.0	11.9	M-59-29
44	5.8	10.5	M-100
43	25.7	24.9	M-101
42	1.9	2.5	M-71-31
41	8.2	8.9	M-74-29

[a]Percentages to the intensity of the parent ion m/e 87.
[b]See text for the assignment. M: m/e of molecular ion 144.

TABLE 3

^{13}C Chemical Shifts of Methyl Ester of 24:6(n-3)

Location of C atom	Found	Calcd[a]
1	174.01	173.83*
2	34.02	34.05
3	24.65	24.65
4	29.15	29.30
5	26.92	26.90
6	129.69	129.60*
7	128.45	128.38*
8	25.68	25.75
9	128.07	128.14*
10	128.61	128.46*
11	25.68	25.75
12	128.23	128.30
13	128.23	128.30
14	25.68	25.75
15	128.23	128.30
16	128.23	128.30
17	25.68	25.75
18	128.23	128.30
19	128.23	128.30
20	25.68	25.75
21	127.09	127.15**
22	132.02	131.97**
23	20.59	20.64**
24	14.25	14.30**
OCH_3	51.41	51.31*

[a]Calculated shifts for each carbon are obtained with the method reported by Bus et al. (10,11), except those for carbons which are taken from the data of *18:3(n-6) (12) and **22:6(n-3).

RESULTS AND DISCUSSION

In this study, a peak of an unknown component U appeared after the peak of 22:6(n-3) in GC on SP 2300 open-tubular column as shown in Figure 1. The fatty acids from the lipids of Crinoidea and Ophiuroidea contained about 5–10% of this unknown fatty acid.

The structural analyses of the unknown fatty acid were undertaken using the fatty acid methyl esters from *A. loveni*. The unknown fatty acid methyl ester was concentrated in the pentaenoate and hexaenoate fraction by $AgNO_3$-TLC, and then separated from 20:5(n-3) and

TABLE 4

Fatty Acid Compositions of the Lipids from Crinoidea and Ophiuroidea (Wt %)

Peak no.	Fatty acid	RRT[b]	ECL[c]	Sample no.[a] 1 TL[d]	1 NL[e]	1 PL[f]	2 TL	2 NL	2 PL	3 TL	4 TL
1	14:0	0.260	14.00	4.75	8.38	0.91	1.24	3.91	0.30	3.72	7.98
2	*iso*-15:0	0.310	14.51	0.18	0.28	0.10	0.10	0.26	0.03	0.57	0.57
3	15:0	0.363	15.00	0.25	0.35	0.22	0.33	0.56	0.26	0.28	0.56
4	16:0	0.514	16.00	13.36	17.14	8.74	8.86	14.81	6.36	5.99	8.92
5	16:1(n-11)	0.560	16.26	0.13	0.15	0.09	0.25	0.40	0.13	0.45	0.48
6	16:1(n-7)	0.589	16.41	3.64	5.75	1.18	1.53	4.95	0.32	2.56	3.93
7	*iso*-17:0	0.607	16.50	0.43	0.43	0.28	0.41	0.70	0.32	0.42	0.40
8	17:0	0.711	17.00	1.86	1.85	2.02	3.35	3.64	3.28	0.86	1.06
9	*iso*-18:0	0.843	17.49	0.52	0.87	0.37	0.83	0.55	1.11	0.27	0.26
10	16:3(n-3)	0.847	17.51	—	—	—	0.83	—	1.27	—	—
11	*anteiso*-18:0	0.902	17.69	3.43	1.02	6.95	0.33	0.18	0.37	0.07	—
12	18:0	1.000	18.00	8.70	9.62	8.64	10.00	12.42	9.96	4.22	4.57
13	18:1 5	1.057	18.17	1.08	1.72	0.40	0.05	0.19	0.03	1.86	1.74
14	18:1(n-9)	1.118	18.34	5.54	7.59	3.23	2.21	4.37	1.51	9.97	8.11
15	18:1(n-7)	1.141	18.40	1.79	2.00	1.47	0.88	1.94	0.43	6.73	3.01
16	18:1(n-5)	1.181	18.51	0.07	0.21	0.01	0.03	0.16	0.02	0.89	0.44
17	18:2(n-6)	1.329	18.87	0.18	0.54	0.14	0.19	0.47	0.08	0.54	0.77
18	18:3(n-6)	1.480	19.21	0.26	0.49	0.12	0.16	0.55	0.05	0.13	0.25
19	19:1(n-9)	1.533	19.32	0.47	0.21	0.74	0.31	0.11	0.42	—	—
20	18:3(n-3)	1.641	19.52	0.12	0.19	0.04	0.19	0.50	0.19	0.23	1.54
21	18:4(n-3)	1.829	19.86	2.25	3.24	0.80	1.46	5.21	0.24	0.36	0.91
22	20:0	1.914	20.00	0.22	0.20	0.24	0.31	0.41	0.28	0.07	0.25
23	20:1 5	2.029	20.18	0.06	0.07	0.03	0.03	0.08	0.02	0.10	1.20
24	20:1(n-11)	2.089	20.27	2.85	3.06	2.61	1.83	2.23	1.74	16.17	8.08
25	20:1(n-9)	2.136	20.34	1.64	1.42	1.64	0.63	0.80	0.46	3.21	0.90
26	20:1(n-7)	2.192	20.42	0.14	0.14	0.13	0.21	0.30	0.15	0.74	0.41
27	20:2 5,11	2.275	20.53	0.16	0.21	0.11	0.16	0.23	0.14	0.49	1.67
28	20:2 5,13	2.309	20.58	—	—	—	—	0.02	—	0.04	0.63
29	20:2(n-9)	2.395	20.69	—	0.02	—	0.01	0.04	—	—	1.34
30	20:2(n-6)	2.551	20.89	1.50	1.53	1.47	2.38	2.68	1.91	0.57	0.64
31	20:3(n-6)	2.822	21.20	0.08	0.06	0.09	0.09	0.14	0.05	0.06	0.64
32	20:4(n-6)	3.013	21.40	8.67	4.22	15.19	17.31	4.58	20.69	4.14	2.33
33	20:3(n-3)	3.144	21.53	0.52	0.58	0.41	0.81	1.15	0.63	0.23	—
34	20:5(n-3)	3.732	22.06	21.97	13.26	29.04	25.38	14.45	28.98	13.39	15.42
35	22:1(n-11)	3.971	22.26	0.24	0.24	0.12	0.44	0.49	0.43	5.66	3.04
36	22:1(n-9)	4.084	22.34	1.98	1.91	2.04	2.33	2.33	2.33	1.15	1.07
37	22:1(n-7)	4.197	22.43	0.94	0.64	1.24	0.55	0.57	0.53	—	0.20
38	22:2 7,13	4.431	22.59	0.12	0.12	0.13	0.14	0.21	0.06	—	0.18
39	22:2 7,15	4.515	22.65	0.05	0.07	0.03	0.10	0.13	0.04	—	0.21
40	23:0	5.066	23.00	—	0.17	—	0.36	0.23	0.58	—	—
41	21:5(n-3)	5.276	23.13	0.46	0.58	0.33	0.27	0.56	0.15	0.37	0.24
42	23:1(n-9)	5.611	23.32	0.18	0.19	0.17	0.48	0.35	0.51	0.23	0.36
43	24:0	6.984	24.00	0.54	0.54	0.55	0.61	0.60	1.05	—	—
44	22:5(n-3)	7.194	24.09	0.84	0.97	0.60	0.60	1.15	0.43	0.50	0.51
45	22:6(n-3)	7.614	24.26	1.90	2.04	1.33	1.48	2.92	1.21	4.45	5.63
46	24:1(n-9)	7.748	24.32	1.32	1.65	1.20	2.02	1.78	2.10	—	—
47	24:6(n-3)	14.806	26.28	4.59	4.06	4.82	7.92	5.67	8.84	8.30	9.53

[a]See Table 1.
[b]Relative retention time.
[c]Equivalent chain length.
[d]Total lipids.
[e]Neutral lipids.
[f]Polar lipids.

22:6(n-3) by RP-TLC. These results and the retention data of GC suggested that the structure of the unknown fatty acid was 24:6.

A molecular ion peak at m/e 370.28693 was obtained from the mass spectrum of the methyl ester of the unknown fatty acid under the high resolution condition with a D-300 instrument. It showed that the molecular formula of the methyl ester of the unknown fatty acid was $C_{25}H_{38}O_2$ (calculated mol wt 370.28718) and then the structure of the unknown fatty acid was confirmed as 24:6.

The position of the double bond closest to the ester group of 24:6 was determined by reductive ozonolysis followed by GC of the products. The peak component was identified on the basis of the agreement of the retention time with those of the reference specimen; ozonolysis products from the methyl ester of 6-18:1 (Tokyo Kasei Kogyo Co., Tokyo, Japan; purity ca. 96%) containing methyl 6-oxo-hexanoate. The ozonolysis product of 24:6 showed only one peak on GC, and the retention time of the peak agreed with that of methyl 6-oxo-hexanoate. The mass spectrum of methyl 6-oxo-hexanoate from the ozonolysis products of 24:6 was taken by the GC-MS instrument. The mass number of the fragment ion peaks and their intensities are shown in comparison with those of the reference specimen in Table 2. In the assignment of the ion peaks in Table 2, the mass numbers of the fragment ions were attributed to the loss of the following fragments: 18 (H_2O), 29 ($H\text{-}C{=}O^+$), 31 (H_3CO^+), 43 ($HOCCH_2^+$), 44 ($H\text{-}COH{=}CH_2^+$), 57 ($HOCCH_2CH_2^+$), 59 (H_3COOC^+), 73 ($H_3COOCCH_2^+$) and 74 ($H_3CO\text{-}COH{=}CH_2^+$). The mass spectrum of the peak of the ozonolysis products of 24:6 agreed with that of methyl 6-oxo-hexanoate. The agreement supported that the double bond position closest to the ester group of 24:6 existed between C_6 and C_7.

The mass spectrum of the pyrrolidide from the methyl ester of 24:6 showed the following irregular intervals of m/e 12 between the maxima in the fragment ion peak for each carbon atom: C_5 (m/e 154, intensity 5.5% to the parent ion peak)-C_6 (m/e 166, 2.8%), C_8 (m/e 194, 4.3%)-C_9 (m/e 206, 1.6%), C_{11} (m/e 234, 2.8%)-C_{12} (m/e 246, 3.8%), C_{14} (m/e 274, 1.6%)-C_{15} (m/e 286, 3.0%), C_{17} (m/e 314, 1.1%)-C_{18} (m/e 326, 1.8%) and C_{21} (m/e 354, 0.7%)-C_{22} (m/e 366, 1.0%). A molecular ion peak appeared at m/e 409, 2.4%. These fragments showed that the structure of the unknown fatty acid was 24:6(n-3).

Infrared spectra of the methyl ester of 24:6(n-3) showed no absorbance near 971 cm^{-1}. The fact confirmed the all-*cis* configuration of 24:6(n-3) (9).

The ^{13}C-NMR of 24:6(n-3) showed the peaks listed in Table 3. The ^{13}C-NMR spectrum for 24:6(n-3) was in fair agreement with those calculated by the set of additive substituent parameters (10–12). These results offer further corroborating evidence for the structure of 24:6(n-3).

The compositions of the fatty acids from Crinoidea and Ophiuroidea are shown in Table 4. The fatty acids contained a noticeable content of 24:6(n-3) (5–10%). It is noteworthy that in all the samples listed in Table 4, 24:6(n-3) is higher than 4%, since the occurrence of 24:6(n-3) has not been reported as an appreciable fatty acid component of animals. It has been reported as a minor component of certain marine animals such as Baltic herring *Clupea harengus* (4) and Baltic salmon *Salmo salar* (5) at levels below 2%.

The n-3 hexaenoic structure suggested the formation of 24:6(n-3) from 22:6(n-3) by chain elongation. Another possible origin of 24:6(n-3) is the diet. The fatty acid composition of a sea cucumber, *Stichopus japonicus,* has been elucidated by GC analysis in another study (Kaneniwa, M., Itabashi, Y., Endo, S., and Takagi, T., submitted for publication). The sea cucumber was caught in the same area where *O. japonicus* was caught, and their diets are detritus, similar to *O. japonicus.* However, 24:6(n-3) has not been detected in fatty acids from sea cucumber lipids. It suggested that 24:6(n-3) in Crinoidea and Ophiuroidea did not originate from the diets.

The compositions of the fatty acids from NL and PL of Crinoidea are shown in Table 4. The 24:6(n-3) was concentrated in PL rather than NL analogously to 20:4(n-6) and 20:5(n-3). It showed that 24:6(n-3) was important for function and structure rather than for storage.

Appreciable amounts of 5-olefinic fatty acids such as 5-18:1, 5-20:1, 5,11- and 5,13-20:2 have been found in fatty acids from sea urchin lipids (9.4–21.4% of the total) (1,2). In addition, 7,13- and 7,15-22:2 fatty acids were found and were presumed to be formed by C_2 elongation of 5,11- and 5,13-20:2. These fatty acids were also found in Crinoidea and Ophiuroidea lipids. The 5-olefinic acids were low (0.2–1.3%) in Crinoidea but higher in Ophiuroidea (2.5–5.2%). The 7-olefinic acids were very low in these species (0–0.4%).

The major polyunsaturated acids of Crinoidea and Ophiuroidea, other than 24:6(n-3), were 20:4(n-6), 20:5(n-3) and 22:6(n-3). The other polyunsaturated acids in these two genera constituted less than 2.5% of the total.

The odd chain monoenoic fatty acid 23:1(n-9) was found in Crinoidea and Ophiuroidea lipids (0.2–0.5%). It has been found in the fatty acids from the lipids of some holothurians (1.3–5.5%) (Kaneniwa, M., Itabashi, Y., Endo, S., and Takagi, T., submitted for publication).

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Analysis of Exoskeleton Triglycerides of *Leptinotarsa decemlineata* Say

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The exoskeleton triglyceride fraction isolated from the Colorado beetle, *Leptinotarsa decemlineata* Say, was studied. Analysis of the hydrolysis products and direct capillary column gas chromatography showed that, apart from triglycerides, the fraction contained no diol lipids. The composition of the natural triacylglycerol mixture was assessed by gas chromatography and by field desorption and electron impact mass spectrometry. Triglycerides isolated from the Colorado beetle are typical glycerol triesters, with long chain (mainly oleic or palmitic) fatty acids. The structures of two major components (91%), i.e., trioleoyl glycerol and 1,2-dioleoyl-3-palmitoyl glycerol, were determined.

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The physiology, biology and biochemistry of the Colorado beetle, *Leptinotarsa decemlineata* Say, have been studied for many years. The present state of knowledge on this notorious pest of potato cultures has been reviewed (1). Previous studies were concerned with, among other things, quantitative changes in neutral lipids depending on the developmental stage of the Colorado beetle, and the influence of nutrition on metabolism and development (2). In our studies, we obtained a fraction of neutral lipids which consisted primarily of triglycerides. Earlier reports (1) pointed out that lipid content varies considerably at various stages of beetle development, reaching a peak prior to and at the onset of diapause. However, no precise literature data exist on the composition and content of individual components of the neutral lipid fractions, which may contain triglycerides and also neutral diol lipids (3,4). These compounds are common in plants and animals and occur as mixtures with triglycerides.

This paper reports studies on the chemical composition of the triglyceride fraction isolated from the Colorado beetle, *L. decemlineata* Say, prior to diapause. The content of individual components of the natural triglyceride mixture in the Colorado beetle can be quickly identified and assessed by gas chromatography and by field desorption (FD) and electron impact (EI) mass spectrometry.

MATERIALS AND METHODS

Extraction of lipids. The Colorado beetle, *L. decemlineata* Say, was collected from a field in northeastern Poland late in August 1978. Adult beetles (natural population of males and females) were placed directly into methylene chloride, 300 cm^3 CH_2Cl_2 for 1000 beetles. After 30 days, the solvent was distilled off under reduced pressure, yielding 5.64 g of primarily exoskeleton extract.

Isolation of the neutral lipid fraction. Neutral lipids were isolated from the extract by preparative TLC on plates (25 × 30 cm) which had been precoated (3 mm) with Merck Kieselgel 60 $PF_{254+366}$ and activated for 12 hr at 130 C; 400 mg of extract was applied to the plates and separated using a solvent system of hexane/acetone/chloroform (4:1:20, v/v/v). Zones were scraped from the plates and extracted with ethyl acetate. The zone with R_f 0.7–0.8, which corresponded to the triglyceride fraction according to infrared (IR) and nuclear magnetic resonance (NMR) spectra, was again subjected to chromatography, using cyclohexane/acetonitrile/chloroform (8:1:49, v/v/v), which yielded 210 mg of triglyceride which was homogenous as judged by thin layer chromatography (TLC).

Methanolysis of the triglyceride fraction and separation of products. A triglyceride fraction, 100 mg, was subjected to methanolysis according to the method of Baumann et al. (5). The products obtained were separated on Kieselgel 60 plates for preparative TLC (Merck, Darmstadt, Federal Republic of Germany; 20 × 20 cm, 2 mm thick), using the solvent system hexane/ether (85:15, v/v). The zone with R_f 0.5 (methyl esters of fatty acids) was extracted with diethyl ether, and the zone with R_f 0.0 (polyol) was extracted with methanol. The methyl esters of fatty acids (89 mg) were analyzed by gas chromatography (GC), and the polyols (10 mg) were studied by TLC, then microacetylated (6) and analyzed by GC.

TLC of polyols. The polyol fraction obtained by methanolysis of the triglyceride fraction was examined by TLC on Kieselgel 60 (Merck, 10 × 20 cm, 2 mm thick) using the solvent system chloroform/methanol (4:1, v/v). Fractions were detected with $AgNO_3$ ammonia solution, 5% $Na_2Cr_2O_7$ solution in H_2SO_4 and 1% $Pb(OAc)_4$ solution in anhydrous benzene. Ethanediol, glycerol, 1,2-propanediol, 1,3-propanediol, 1,3-butanediol and 1.6-hexanediol were used as standards.

Glass capillary column GC. Chromatographic analysis was performed on capillary columns connected to a modified Varian Aerograph 1400 with flame ionization (FID) detector. Samples were injected into an all-glass inlet system with a split ratio of 1:20. Argon was used as carrier gas. The temperature of the injector and the detector was 330 C.

The fatty acid methyl esters were analyzed on soda-glass capillary columns (20 m length, 0.28 mm id coated with Silar 10C liquid phase). The columns were pre-etched with 20% HCl at 150 C for 12 hr (7), prior to coating with barium carbonate (8), and were deactivated with Carbowax-20M.

The chromatographic analysis of polyol acetates was done on a glass column coated with Dexsil 300 liquid phase (40 m length, 0.26 mm id, 0.1 μm thickness of the liquid phase). Chromatographic analyses were performed with the temperature programmed between 45 C and 170 C at 4 C/min. Identification was verified by coinjection of glycerol, ethanediol, 1,2-propanediol, 1,3-propanediol, 1,3-butanediol and 1,6-hexanediol as acetates.

Direct chromatographic analysis of the triglyceride fraction was done on a 10 m Pyrex glass column of 0.23 mm id coated with SE-30 phase. The surface of the

capillary was prepared similarly as for a Silar 10C column. The column was coated by evaporating the 0.2% SE-30 phase solution in pentane from the capillary.

Catalytic hydrogenation. A mixture of triglyceride (40 mg) from the Colorado beetle was dissolved in 10 ml of anhydrous ethyl acetate and perhydrogenation was performed using Adams platinum catalyst with 0.23 mmol of H_2 consumed.

Mass spectrometry of triglycerides. Mass spectra were measured using a Varian MAT 711 spectrometer equipped with a combined EI/FI/FD ion source and a Spectrosystem 100 SS. The sample was injected directly into the source using a probe with controlled temperature; 70 eV ionization energy and 8 kV accelerating voltage were used. FD spectra were recorded using benzonitrile-activated emitters in a commercial Varian MAT apparatus. Heating current of the emitter, 15 mA; additional cathode voltage, 100 V.

RESULTS

Polyol fractions. The polyol fractions obtained by methanolysis of the triglyceride fraction were first examined by TLC. Apart from glycerol, no polyols were detected. TLC results confirmed by capillary GC of polyol acetates showed that glycerol was the only polyol constituent of the isolated triglyceride fraction.

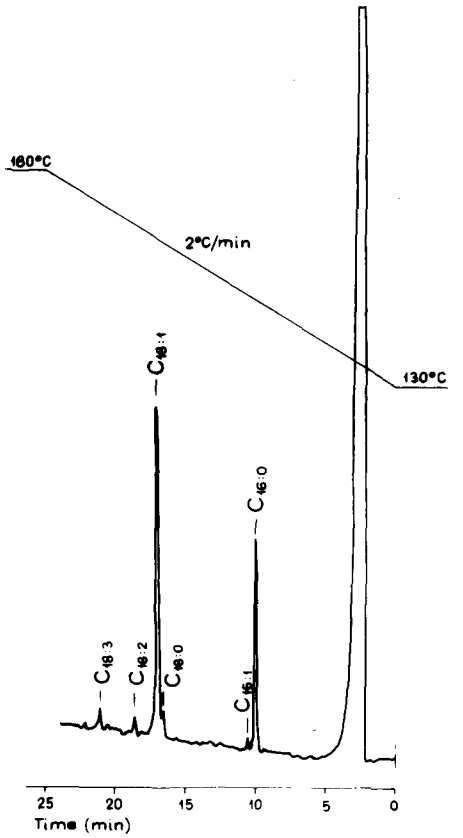

FIG. 1. GC analysis of fatty acid methyl esters on a Silar 10C capillary column programmed from 130 C at 2 C/min.

TABLE 1

Fatty Acid Composition of the Triglyceride Fraction of Colorado Beetle

Fatty acid	Carbon number	Double bonds	Content (%)
Palmitic	16	0	26.5
Palmitoleic	16	1	1.5
Stearic	18	0	3.5
Oleic	18	1	63.0
Linoleic	18	2	2.5
Linolenic	18	3	3.0
Unknown	20	2 or 3	trace

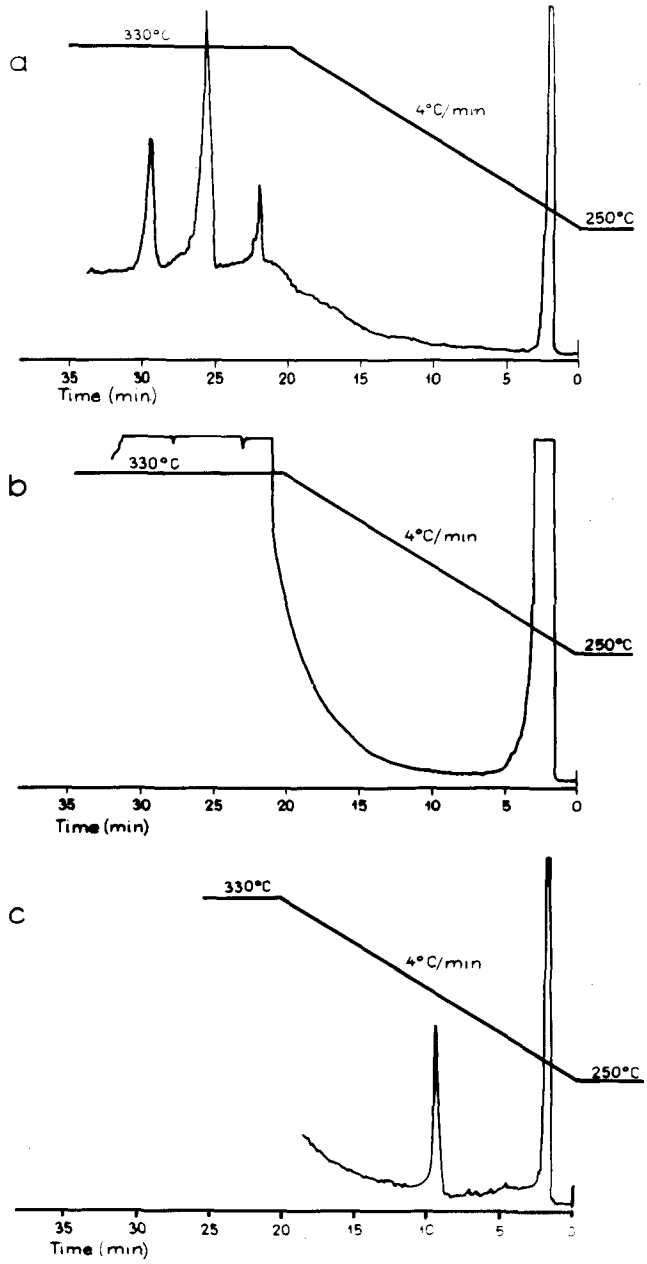

FIG. 2. GC analysis of triglyceride fractions on an SE-30 capillary column programmed from 250 C to 330 C at 4 C/min. (a) Colorado beetle triglyceride fraction 0.4 μl; (b) Colorado beetle triglyceride fraction 1.0 μl; (c) 1,3-propanediol dipalmitate 0.3 μl.

Fatty acids. The composition of fatty acids in the triglyceride fraction was established by capillary GC of their methyl esters (Fig. 1), by comparison with a standard mixture of fatty acid methyl esters. The results are listed in Table 1.

Composition of individual triglycerides in natural mixtures. The triglyceride fraction from the Colorado beetle and the synthetic 1,3-propanediol dipalmitate were analyzed by capillary GC. The triglyceride fraction of the beetle was rechecked for neutral diol lipid content (Fig. 2), but no diol esters were detected despite overloading the column with the sample analyzed (Fig. 2). Comparison of the above results with the chromatogram of the synthetic triglyceride standards has shown that the neutral lipid fraction isolated from the Colorado beetle contains three groups of triglycerides differing from one another by two methylene groups.

Molecular ions of the triglyceride (Fig. 3) are observed in the mass spectrum produced by the field desorption technique which makes the technique suitable for semiquantitative analysis of mixtures of high molecular weight compounds. The distribution of ion intensity in the spectrum corresponds to the approximate content of each triglyceride in the mixture (9). In the mass spectrum (Fig. 3), three ion groups appear which correspond to triglycerides with a total number of carbon atoms of 53, 55 and 57, respectively, and a trace ionization current which corresponds to a triglyceride group with 59 carbon atoms. Base ions in each group are ions with m/z 832, 858 and 884, for which the composition of fatty acids may be as follows: $M^+ = 832$, 16:0 + 16:0 + 18:1; $M^+ = 858$, 16:0 + 18:1 + 18:1; $M^+ = 884$, 18:1 + 18:1 + 18:1.

The above fatty acid composition is delineated from the mass of the M^+ ion and from the chromatographic fatty acids analysis (Table 1).

The relative intensities and the most probable fatty acid composition of the molecular ions are listed in Table 2. In accounting for the relative abundance of natural isotopes, only ^{13}C and ^{18}O contents were considered. Quantitative interpretation of the results is furthermore complicated by the fact that in the spectrum of triglycerides $(M + H)^+$ ions occur whose intensity, depending on instrumental conditions, may exceed the intensity of M^+ ions (9,10). The ionization cross-sections of triglycerides with various unsaturation degrees are not known, which makes the result difficult to assess quantitatively. According to Evans et al. (9), detection coefficients cannot be expected to be identical.

The EI mass spectrum of the triglyceride mixture is shown in Figure 4. The spectrum is characterized by low relative intensity of the molecular ions M^+. All fragmentation ions in the spectrum can be attributed to one of the following groups (11):

I) ions $(M-18)^+$
II) fragmentation ions formed through loss of the acyloxy group or elimination of a molecule of acid, $(M-RCOO)^+$ and $(M-RCOOH)^+$
III) ions containing a fatty acid fragment and a portion of glycerol, $(RCO+74)^+$ and $(RCO+128)^+$
IV) acyl ions, RCO^+

TABLE 2

Composition of Triglycerides from the Colorado Beetle

Triglyceride	M^+	Fatty acid composition	Relative intensity (%)	Corrected intensity (%)[a]
C_{53}	826	16:0 + 16:1 + 18:3	3.9	4.0
	828	16:0 + 16:0 + 18:3	4.9	4.5
	830	16:0 + 16:1 + 18:1	4.9	4.5
	832	16:0 + 16:0 + 18:1	13.2	13.0
C_{55}	852	16:0 + 18:2 + 18:3 16:1 + 18:1 + 18:3	12.0	12.5
	854	16:0 + 18:1 + 18:3 16:1 + 18:1 + 18:2	31.2	30.0
	856	16:0 + 18:1 + 18:2 16:1 + 18:1 + 18:1	28.0	23.5
	858	16:0 + 18:1 + 18:1	100.0	100.0
	860	16:0 + 18:0 + 18:1	27.1	10.0
	862	16:0 + 18:0 + 18:0	5.9	4.5
C_{57}	878	18:1 + 18:2 + 18:3	7.1	7.5
	880	18:1 + 18:1 + 18:3	7.1	6.0
	882	18:1 + 18:1 + 18:2	9.8	9.0
	884	18:1 + 18:1 + 18:1	61.0	62.0
	886	18:0 + 18:1 + 18:1	32.0	21.0

[a]Corrected intensity was computed by taking into account the intensity of the ion M+1.

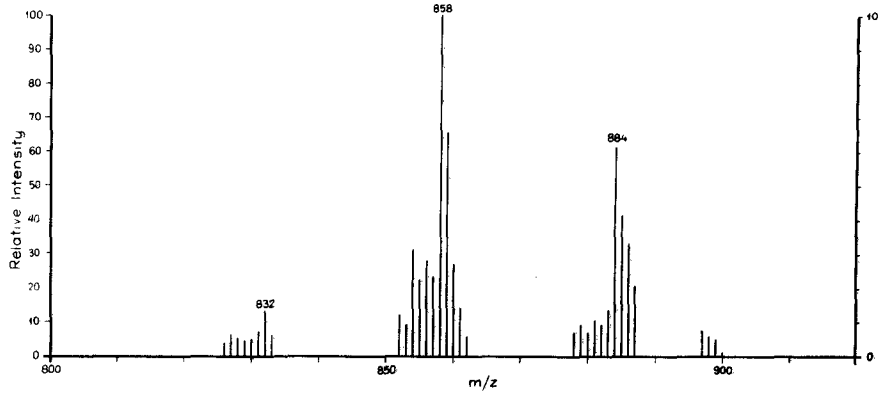

FIG. 3. Field desorption mass spectrum of total triglyceride fraction from Colorado beetle.

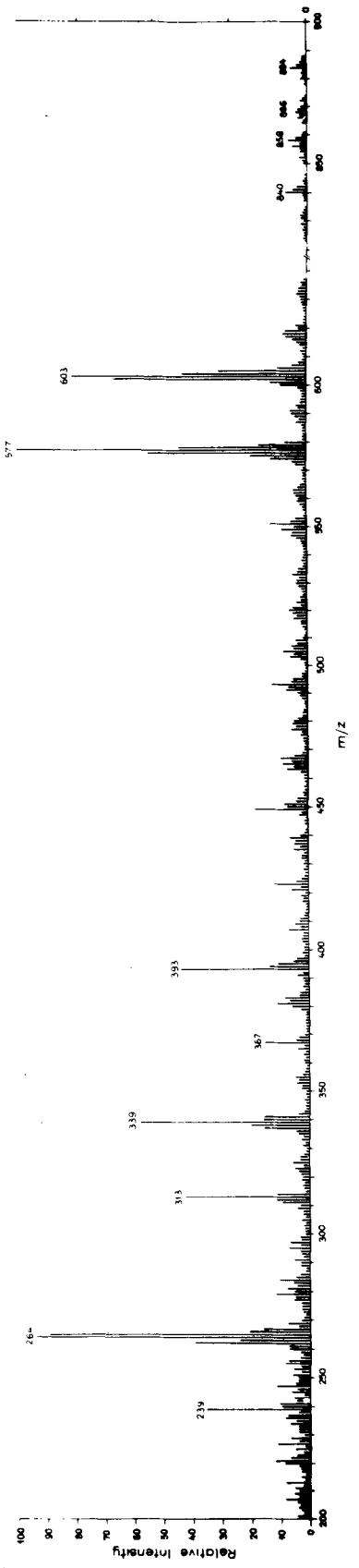

FIG. 4. Electron impact mass spectrum of total triglyceride fraction from Colorado beetle.

TABLE 3

Direct Inlet Electron Impact Mass Spectra of Triglycerides Before and After Hydrogenation[a]

Ion class	m/z	Fragmentation ion assignment	Relative intensity (%)
I	866	$(M_{884} - 18)^+$	3.3
	840	$(M_{858} - 18)^+$	7.3
II	603/609[a]	$(M_{884} - C_{17}H_{33}COO)^+$ or $(M_{858} - C_{125}H_{31}COO)^+$	81.0/98[a]
	602	$(M_{884} - C_{17}H_{33}COOH)^+$ or $(M_{858} - C_{15}H_{31}COOH)^+$	67.1
	577/581[a]	$(M_{858} - C_{17}H_{33}COO)^+$	100.0/100.0[a]
	576	$(M_{858} - C_{17}H_{33}COOH)^+$	54.4
III	393	$(C_{17}H_{33}CO + 128)^+$	44.3
	367	$(C_{15}H_{31}CO + 128)^+$	15.2
	339	$(C_{17}H_{33}CO + 74)^+$	58.2
	313	$(C_{15}H_{31}CO + 74)^+$	43.0
IV	265	$C_{17}H_{33}CO^+$	89.9
	264	$(C_{17}H_{33}CO - H)^+$	93.7
	239	$C_{15}H_{31}CO^+$	35.4

[a]Indicates relative intensity of ion triglycerides after hydrogenation.

TABLE 4

Comparison of Triglyceride Content (C_{53}, C_{55} and C_{57}) Determined by GC and FD-MS

Triglyceride	Composition determined by GC (%)	Composition determined by FD-MS (%)
C_{53}	9.5	8.5
C_{55}	69.0	58.0
C_{57}	21.5	33.5

Spectral interpretations are given in Table 3.

DISCUSSION

We used two approaches in our neutral lipid analyses: (a) direct analysis of neutral lipids by capillary GC, and (b) analysis of the polyol fraction obtained after hydrolysis. No diol except glycerol was found in the polyol fraction. According to the literature (4), diol derivatives are widespread in nature, even though there is a lack of information on their occurrence in the lipids of beetles. However, it is possible that some diol derivatives may occur at developmental stages other than prior to diapause.

Chromatographic analysis of fatty acid methyl esters has shown the prevalence of two acids (ca. 90%) in the mixture which simplified the investigations of the main triglyceride structures. Also, direct GC analysis (12) of the triglyceride mixture from the Colorado beetle revealed two predominant components.

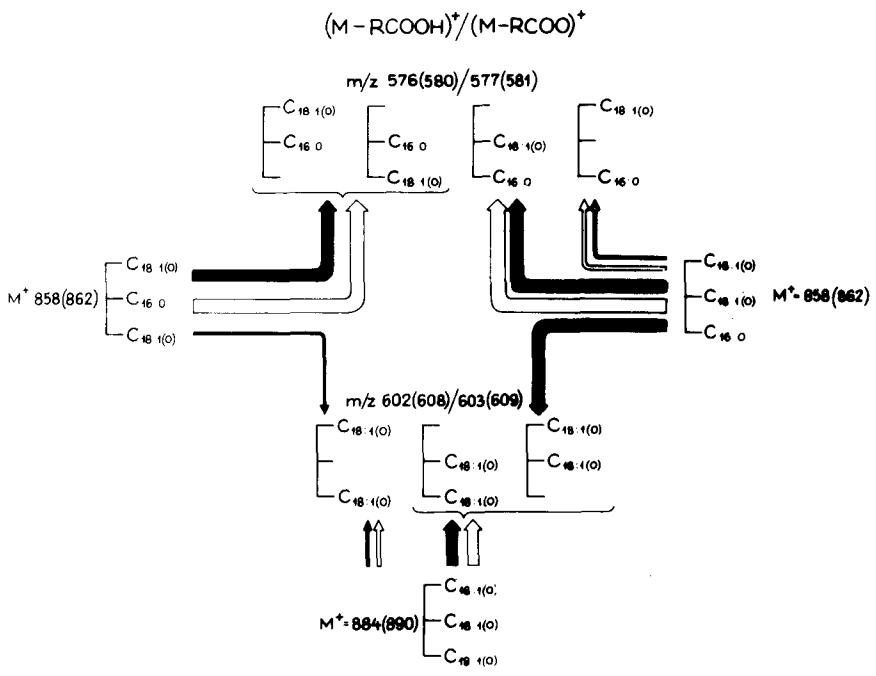

FIG. 5. The origin of specific ions (M-RCOOH)$^+$, (M-RCOO)$^+$ from the triglyceride mixture. Preferential fragmentations are depicted by wider arrows. Pathways labeled black and the figures in parentheses refer to hydrogenated triglycerides.

A semiquantitative comparison of the analytical results of the triglyceride mixture obtained by GC and FD mass spectrometry is listed in Table 4. Despite the totally different analytical techniques used, the results were almost the same. The two main components (Table 2), dioleoyl palmitoyl glycerol and trioleoyl glycerol, constitute about 90% of the mixture of natural triglycerides from the Colorado beetle, which is in agreement with the statistical distribution of fatty acids in the triglycerides.

Fragmentation ions in the EI spectrum of group II, i.e., (M-RCOO)$^+$ (Table 3), may be used to determine the position of individual fatty acids in the triglycerides. According to the literature (11), the fragmentation process leading to these ions prefers the elimination of terminal acyloxy groups and of unsaturated acids.

One of the major triglycerides in the sample investigated is 1,2,3-glycerol trioleate, according to its m/z value of the molecular ion (m/z 884). The triglyceride with m/z 858 should be composed of the fatty acids 18:1, 18:1, 16:0. Since preferential cleavage of the ester bonds (11) could not be used for the mass spectral analysis of a triglyceride mixture to determine the position of palmitic acid, we catalytically hydrogenated the triglyceride mixture. Saturated triglycerides were characterized by EI mass spectra with fragmentation preference only in regard to the position of fatty acids. The fragmentation trend and consequently the intensity changes in the fragmentation ions (M-RCOO)$^+$, (M-RCOOH)$^+$ of hydrogenated triglycerides for the two possible structures are presented in Figure 5. As shown in the figure, the intensities of the (M-RCOOH)$^+$/(M-RCOO)$^+$ ions with m/z 608/609 in the hydrogenation products for the sequence of 18:0, 18:0 and 16:0 acids must be considerably higher, as has in fact been observed (Table 3). The findings show that the main triglyceride in the Colorado beetle is most probably 2-dioleoyl-3-palmitoyl glycerol, in accordance with the natural preference of unsaturated acids for the position 2 of glycerol (13).

The lipid fractions of various insects are commonly composed of neutral glycerides and free fatty acids which constitute about 75% of the total amount of lipids (14). Insect triglycerides, whose greatest concentration is found in fatty bodies, epidermis and hemolymph (15), are typical glycerol esters with long chain fatty acids, mainly oleic, palmitoleic and linoleic. The content and composition of triglycerides in the Colorado beetle, as we determined, is in accordance with literature data (15). The beetles were studied before they entered the state of diapause, prior to hibernation, i.e., at a time when they store large lipid reserves, mainly triglycerides, which appear indispensable in metabolic processes (16,17). The triglyceride analyses presented here can be applied to the analysis of triglycerides from other sources.

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Effects of Estradiol and Environmental Temperature Changes on Rat Liver Δ6 Microsomal Desaturase Activity

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The regulation of Δ6 desaturase activity by environmental temperature changes was studied in the microsomal membranes from female and ovariectomized female rat liver. Female rats adapted at 30–32 C for 20–25 days and then shifted to 13–15 C for 5 days showed an increased Δ6 desaturase system. Ovariectomized rats adapted under the same conditions did not show significant changes in this enzyme. The fatty acid compositions of microsomal phosphatidylcholine showed a decrease in arachidonic acid in female rats at 30 C compared to females at 15 C and ovariectomized rats at both temperatures. These results suggest that a modification of ovaric sex hormone levels might be responsible for the different Δ6 desaturase activity in female rats acclimated at both temperatures. In this regard, serum estradiol radioimmunoassay yielded slight differences between the two groups of female rats, suggesting that estradiol could play a role in the regulation of the Δ6 desaturase. The administration of a pharmacological dose of 17-β estradiol to female and ovariectomized rats kept at 30 and 15 C decreased the Δ6 microsomal desaturase activity. These data suggest that estradiol levels are involved in the regulation of the Δ6 desaturase during cold adaptation.
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Many investigations have tried to elucidate the metabolic regulation of the Δ6 desaturase system in liver microsomes. It is well known that in rats, diet and hormonal factors modify the activity of this enzyme and have shown that the hyperglycemic hormones such as glucagon (1,2), epinephrine (2), thyroxine (3) and glucocorticoids (4) inhibit the Δ6 desaturase.

In addition, our previous studies (5) where female rats were adapted to warm temperature (30–32 C) for 20–25 days and then shifted to cooler temperature (13–15 C) for different periods of time showed that Δ6 desaturase activity increased after 24 hr of cold exposure. Male rats adapted under the same conditions did not show significant changes on this desaturase.

Endocrine response to cold exposure in homeotherms goes with changes in the plasma levels of catecholamines (6), prostaglandins (7), thyrotropin (8), corticosterone (8,9), prolactin (8), triiodothyronine (10) and thyroxine (8,10). Since only female rats showed different Δ6 desaturase activity upon temperature shift, it may be suggested that female sex hormones could be involved. In consequence, the investigation was undertaken to determine whether a modification of female sex hormone levels might be at least in part responsible for the different Δ6 desaturase activity in female rats acclimated at 30 C and 15 C. We have used two model systems: female and ovariectomized rats, and the administration of 17-β estradiol to both of them.

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MATERIALS AND METHODS

Chemicals. [1-^{14}C]Linoleic acid (54.7 mCi/mmol, 98.5% radiochemically pure, 1% *trans* isomer) was purchased from New England Nuclear (Boston, Massachusetts). 17-β Estradiol hemisuccinate was provided by Gador Laboratories (Argentina) and cofactors used for the enzymatic reactions were provided by Sigma Chemical Co. Inc. (St. Louis, Missouri).

Animal treatment. Female and bilaterally ovariectomized Wistar rats (45–50 days old) were used in all experiments. The last group was ovariectomized under ether anesthesia and allowed to recover for 10 days; at that time the acclimation treatment was initiated. Female rats were subjected to estrous cycle study by vaginal smear and those found under proestrous were chosen to perform the acclimation.

For the acclimation treatment, female and ovariectomized rats were kept for 20 days in a temperature-controlled room maintained at 30–32 C and were fed Purina rat chow and tap water ad libitum. After this time half of the animals from each group were maintained at this temperature and the other half were placed in a cold room (13–14 C) and allowed to cool down over a period of five days under the same diet conditions. A comparison of relative growth and food intake in females and ovariectomized rats was performed during the final five days. All animals were subject to a daily photoperiod of 12 hr light and 12 hr darkness, with midnight as the midpoint of the dark period.

When the effect of estradiol was studied, the rats kept at 30 C or 15 C (following the acclimation system previously described) were given two daily (every 12 hr) subcutaneous injections of estradiol hemisuccinate. The dose chosen was 0.75 mg/100 g body weight. Cold-exposed rats received the first administration three days after the temperature shift. The animals maintained at 30 C were injected at the same time as those exposed to cold temperature. All the animals were killed after two consecutive days of hormonal treatment.

Serum and microsome preparation. The rats placed at 30 C and 15 C with or without estradiol administration were decapitated and the serum from blood was stored frozen until assayed. Livers were rapidly removed and homogenized in a solution containing 0.25 M sucrose, 0.15 M KCl, 62 mM potassium phosphate buffer pH 7.4, 5 mM $MgCl_2$ and 1.4 mM N-acetyl-L-cysteine. The homogenate was centrifuged at 10,000 g for 20 min, the pellet was discarded and the supernatant was centrifuged again at 110,000 g for 60 min to obtain the microsomal pellet. These steps were carried out at 4 C. The microsomal fraction was suspended in a small volume of homogenizing solution. All subsequent enzymatic assays and composition analysis used these microsomal membrane fractions.

Enzymatic assays. Five nmol of labeled linoleic acid and 45 nmol of unlabeled acid were incubated with 3 mg of

the microsomal protein in constant shaking at 36 C for 10 min. In these conditions, the enzyme was saturated by the substrate. The incubation solution contained 0.25 M sucrose, 0.15 M KCl, 0.04 M phosphate buffer (pH 7.4), 1.41 mM N-acetyl cysteine, 0.04 M KF, 1.3 mM ATP, 0.06 mM CoA, 0.87 mM NADH and 5 mM $MgCl_2$ in a final volume of 1.6 ml.

The protein was determined by microbiuret procedure using crystalline bovine serum albumin as standard (11). The desaturation reaction was stopped by addition of 2 ml of 10% KOH in ethanol. Saponification and esterification of fatty acids were performed and the analyses were carried out by gas liquid radiochromatography in a Model 893 Packard apparatus equipped with a proportional counter using GP 10% SP-2330 on Chromosorb WAW (100–120 mesh) (12).

The relative radioactivity in linoleic and γ-linolenic acids was determined by measuring the area of the counter output peaks by triangulation in the radiochromatograms. A control assay without the addition of microsomes was done and no desaturation was observed.

Fatty acid analysis. Serum and microsome total lipids were extracted using the method of Folch et al. (13). Phosphatidylcholine was separated by a two-step thin layer chromatographic procedure (14).

Appropriate standard was visualized by exposure to iodine vapor. Unexposure samples were scrapped and fatty acid methyl esters prepared as previously described (15). Fatty acid esters were analyzed by gas liquid chromatography in a Hewlett-Packard model 5840-A Chromatograph equipped with the 5840-A GC terminal and using a 6-ft column filled with 10% SP-2330 on 100–200 Chromosorb WAW.

Radioimmunoassay (RIA). Serum estradiol levels were measured with haptene RIA using ^{125}I estradiol as a marker (16).

Statistics. Data are reported as mean ±1 S.E. The significance of differences in a treatment series was determined by the Student's t-test.

RESULTS AND DISCUSSION

A comparative study of the Δ6 desaturase activity of female and ovariectomized rat liver microsomes was made. The ovariectomized rats were studied to elucidate their response to temperature changes in the absence of ovaric hormones, taking into account that there still exists a small suprarrenal supply of progesterone and androstene 3,17-dione (estrogen precursor).

A first set of experiments (Table 1) supports earlier observations regarding the differences in Δ6 desaturase activity of female rats by temperature shift: cold exposure markedly increases the activity (5). Moreover, Table 1 shows that ovariectomized rats exposed to cold evidence no changes in the Δ6 desaturase activity. The comparison of relative growth/daily increase in body weight (g) between female and ovariectomized rats at 30 C and 15 C did not show statistical significance. The daily food intake (g) increased about 15% in female and ovariectomized rats at 15 C when compared to those at 30 C. Since the increased food intake in female and ovariectomized rats at 15 C was the same, it is concluded that this food intake increment is not related to the different Δ6 desaturase activity appearing in the already mentioned groups.

The total lipid fatty acid composition of microsomal membrane (not reported here) from female and ovariectomized rats at both temperatures did not show significant changes. However, when microsomal phosphatidylcholine was isolated, important differences in the fatty acid composition were found. The results are summarized in Table 2; there is an increase of palmitic and oleic acids and a decrease of arachidonic acid in female rats at 30 C. The different response of Δ6 desaturase system (Table 1) and the compositional fatty acid changes (Table 2) in female rats (not observed in ovariectomized rats) suggest that sex hormones would be involved in the modulation of this enzyme activity in female rats acclimated at both ambient temperatures. For this reason, we have assayed the effect of estradiol on the Δ6 desaturase activity on female as well as ovariectomized rats.

In order to determine the best doses of estradiol able to modify Δ6 desaturase activity from ovariectomized rats at 30 C, different doses of estradiol treatment were studied. Table 3 shows that treatment with estradiol at 0.185 and 0.37 mg/100 g body weight did not influence desaturase activity level. In contrast, treatment with estradiol at 0.75 mg/100 g body weight was effective in decreasing the enzyme activity. The same decrease in enzyme activity was observed with a dose higher than 0.75 mg. For this reason, this latter dose was used in the subsequent experiments.

In the next experiment, female and ovariectomized rats acclimated to 30 C and 15 C were used. Half of the rats of each group were injected with 17-β estradiol following the administration system described in Materials and Methods, and the other half received the hormone vehicle. The Δ6 desaturase activity of liver microsomes from every group can be seen in Table 4. Data demonstrate that female rats at 30 C having a higher plasma estrogen level than ovariectomized rats presented a smaller Δ6 desaturase activity. On the other hand, estradiol administration to female and ovariectomized rats at both temperatures decreased the microsomal enzyme activity. The present results show that under our experiment conditions, the significant decrease in Δ6 desaturase activity could be ascribed to the modulating action of the estradiol.

It should be pointed out that female rats placed at 15 C present higher desaturating levels (12.1% conversion)

TABLE 1

Effect of Environmental Temperature Changes on Δ6 Desaturase Activity from Female and Ovariectomized Rat Liver Microsomes

Group	Δ6 Desaturase activity (% conversion)	Student's t-test
Female, 30 C	9.1 ± 0.8	P < 0.001
Female, 15 C	14.3 ± 0.6	
Ovariectomized, 30 C	13.0 ± 0.7	N.S.
Ovariectomized, 15 C	12.5 ± 0.5	

Results are the mean of the percentage conversion of five animals ±1 S.E. Duplicate samples from individual animals were analyzed. N.S. = not significant.

TABLE 2

Changes in Fatty Acid Composition of Microsomal Phosphatidylcholine Fraction of Female and Ovariectomized Rats at 30 C and 15 C

Fatty acids (mol %)	Female		Ovariectomized	
	30 C	15 C	30 C	15 C
16:0	25.4 ± 0.5	19.3 ± 0.2	19.8 ± 0.6	18.7 ± 1.4
18:1	12.6 ± 0.4	9.7 ± 0.3	7.1 ± 0.3	7.2 ± 0.5
20:4ω6	11.9 ± 0.9	20.1 ± 0.6	24.7 ± 1.0	25.2 ± 0.8

Results are the mean of five samples ±1 S.E. Only fatty acids with significant differences were included.

TABLE 3

Effect of Different Doses of 17-β Estradiol on Δ6 Desaturase Activity from Ovariectomized Rat Liver Microsomes Kept for 20 Days at 30 C

	Dose of 17-β estradiol (mg/100 g body weight)[a]					
	None (control)	0.187	0.375	0.75	1.5	3.0
Δ6 Desaturase activity (% conversion)	11.4 ± 0.7	11.2 ± 0.4	11.3 ± 0.6	6.9 ± 0.5[b]	6.5 ± 0.3[b]	6.1 ± 0.4[b]

Each value is the mean ±1 S.E. of five rats.

[a]Animals received hormonal injections every 12 hr and were killed after two consecutive days of treatment. Control received the hormone vehicle.

[b]$p < 0.01$ by Student's t-test for paired comparison with control.

TABLE 4

Effect of 17-β Estradiol on Δ6 Desaturase Activity from Female and Ovariectomized Rats at 30 C and 15 C

Animals	Group 1 (30 C)	Group 2 (30 C + E)	Group 3 (15 C)	Group 4 (15 C + E)	$P < 0.01$
Δ6 Desaturase (% conversion)					
Female	8.2 ± 0.6	5.2 ± 0.3	12.1 ± 0.6	7.6 ± 0.3	1 vs 2 1 vs 3 3 vs 4
Ovariectomized	12.8 ± 0.3	7.1 ± 0.8	13.1 ± 0.6	7.7 ± 0.4	1 vs 2 3 vs 4

E = 17-β estradiol. Results are expressed as percentage conversion of four rats ±1 S.E.

TABLE 5

Serum Estradiol Levels of Female and Ovariectomized Rats at 30 C and 15 C With or Without 17-β Estradiol Treatment

Group	30 C	30 C + E	15 C	15 C + E
Female pg Estradiol/ml serum	28.1 ± 3.0 (6)	68.3 ± 13.0 (4) $P < 0.001$[a]	18.1 ± 2.7 (7) $P < 0.05$[a]	46.7 ± 7.0 (4) $P < 0.01$[a]
Ovariectomized pg Estradiol/ml serum	8.3 ± 2.7 (6)	80.0 ± 7.3 (6) $P < 0.001$[b]	9.2 ± 1.6 (7) NS[b]	82.7 ± 1.9 (6) $P < 0.001$[b]

E = 17-β estradiol. The number of observations are given in parentheses. Results are expressed as mean ±1 S.E.

[a]Refers to female at 30 C (Student's t-test).

[b]Refers to ovariectomized at 30 C (Student's t-test).

TABLE 6

Effect of 17-β Estradiol on Serum Fatty Acid Composition of Female and Ovariectomized Rats at 30 C and 15 C

Fatty acids (mol %)	Female				Ovariectomized			
	Group 1 (30 C)	Group 2 (30 C + E)	Group 3 (15 C)	Group 4 (15 C + E)	Group 1 (30 C)	Group 2 (30 C + E)	Group 3 (15 C)	Group 4 (15 C + E)
16:0	23.6 ± 0.3	22.4 ± 0.5	18.3 ± 0.4^{b}	21.5 ± 0.2^{c}	20.4 ± 0.5	23.2 ± 0.2$^{a'}$	18.6 ± 0.5	21.7 ± 0.4$^{c'}$
16:1	2.9 ± 0.2	4.0 ± 0.4	1.9 ± 0.03	2.6 ± 0.03^{c}	2.3 ± 0.06	3.0 ± 0.1	1.8 ± 0.08	2.4 ± 0.09
18:0	13.7 ± 0.3	9.9 ± 0.2$^{a'}$	13.8 ± 0.3	12.5 ± 0.7	12.4 ± 0.3	10.0 ± 0.6	13.8 ± 0.8	11.8 ± 0.5
18:1	15.8 ± 0.5	19.5 ± 0.6$^{a'}$	11.3 ± 0.4$^{b'}$	16.2 ± 0.5^{c}	10.2 ± 0.3	18.5 ± 0.4$^{a'}$	9.8 ± 0.5	16.4 ± 0.9^{c}
18:2ω6	25.9 ± 0.7	31.4 ± 0.6^{a}	26.1 ± 0.3	28.2 ± 1.4	27.8 ± 0.2	31.4 ± 0.8$^{a'}$	25.2 ± 1.0	29.0 ± 1.4
20:3ω6	0.8 ± 0.1	0.8 ± 0.1	1.0 ± 0.3	0.7 ± 0.03	0.7 ± 0.06	0.6 ± 0.05	0.9 ± 0.1	1.3 ± 0.07
20:4ω6	15.0 ± 0.6	10.0 ± 0.4^{a}	24.1 ± 0.4^{b}	14.6 ± 0.8^{c}	23.7 ± 1.0	9.4 ± 0.6^{a}	25.2 ± 1.0	15.4 ± 1.0^{c}
22:5ω3	0.7 ± 0.09	0.6 ± 0.03	1.1 ± 0.2	1.2 ± 0.3	0.6 ± 0.04	1.2 ± 0.6	1.4 ± 0.3	0.6 ± 0.06
22:6ω3	1.6 ± 0.02	1.4 ± 0.2	2.4 ± 0.4	2.4 ± 0.6	1.9 ± 0.1	2.7 ± 1.2	3.0 ± 0.3	1.4 ± 0.2

E = 17-β estradiol. Results are the mean values ±1 S.E. based on five samples. Letters above the data indicate significant differences, $P < 0.001$: a, group 1 vs group 2; b, group 1 vs group 3; c, group 3 vs group 4. a', b' and c' indicate $P < 0.01$. Minor components were not considered.

compared to those kept at 30 C (8.2% conversion) and similar to ovariectomized rats at both temperatures (12.1 or 13.1% conversion). A possible explanation for these results would be that estrogen plasma levels of female rats at 15 C were lower than those at 30 C.

Table 5 shows the serum concentration of estradiol determined by RIA in the female and ovariectomized animals; it demonstrates that female rats at 15 C had lower estradiol levels than the ones at 30 C. In the light of these results the Δ6 desaturase inhibition could be attributed to changes of estradiol physiological levels produced by the temperature shifting.

Given the fact that estrogen increases the plasma levels of triacylglycerols (17), we decided to study the fatty acid composition of serum on the four groups of female and ovariectomized rats. The results in Table 6 demonstrate an increase of oleate (18:1) and a decrease of arachidonate (20:4ω6) in serum from female rats placed at 30 C with respect to female at 15 C and ovariectomized rats at both temperatures. The arachidonate decrease would arise from estradiol inhibitory effect on Δ6 desaturase, since in the synthesis of this fatty acid from linoleate (18:2ω6) the Δ6 desaturation is the rate-limiting step (18). The oleate increase suggests a high Δ9 desaturase activity, or that the decreased activity on Δ6 desaturase favored the generation of ω9 derivates at the expense of ω6 compounds (19).

We can conclude that the estradiol injection evokes an inhibitory effect on the Δ6 desaturase activity in female as well as in ovariectomized rats. According to this, the increased Δ6 desaturase activity in ovariectomized compared to female rats would be due to the low estradiol concentration. In the same way, the fact that female rats exhibit a lower desaturase activity at 15 C compared to those at 30 C would suggest a higher estrogenic effect. This hypothesis seems to be confirmed by the serum estradiol concentration determined by RIA.

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A Liquid Crystalline Phase in Human Intestinal Contents During Fat Digestion

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A viscous, gel-like phase was found in ultracentrifuged human upper intestinal contents collected during rapid fat lipolysis. This "gel" phase was layered in the ultracentrifuge tube between the micellar and oil phase. The gel contained lipids typical of the micellar and not the oil phase. The concentration of these lipids was higher and the ratio of total bile salts to lipids was lower in the gel than in the micellar phase. The gel, unlike the micellar phase, was birefringent between crossed polarizers. These data demonstrate that lipids in this gel phase can form liquid crystals in the gut during fat digestion.
Lipids 21, 444–446 (1986).

Triglyceride digestion in the upper intestinal lumen occurs through the integrated action of pancreatic lipase and colipase and of bile, resulting in the formation of monoglyceride and fatty acid (1). During fat digestion, gall bladder contraction adds bile salts to duodenal contents which incorporate the insoluble products of lipase hydrolysis into mixed micelles (2). Bile also contributes phospholipids, principally phosphatidylcholine, and cholesterol into the lumen where phosphatidylcholine may be further hydrolyzed to lysophosphatidylcholine by phospholipase A_2 (3). During digestion and absorption, these lipids of different chemical composition exist in several physical states. The classic studies of Hofmann and Borgstrom (4) showed that ultracentrifugation separated two major physical phases of intestinal contents, an isotropic clear aqueous (micellar) phase and a floating oil phase. In addition, small amounts of lipid can be precipitated as a pellet during centrifugation (5).

The present report describes observations made in the early 1970s (6) on the physical and chemical composition of lipids from duodenal contents in man, collected as a part of studies of fat and carbohydrate absorption. After ultracentrifugation of specimens collected during rapid fat digestion, a viscous or gelatinous phase was layered below the oil. Physical observation and chemical composition studies of this gel phase are consistent with the interpretation that lipids can form a liquid crystalline phase in the intestinal lumen. Since these observations, a viscous isotropic and a liquid crystalline phase have been observed under the microscope when triglyceride digestion was simulated in vitro (7) and similar findings in human intestinal contents have been presented in preliminary form (8,9).

EXPERIMENTAL METHODS

During studies of intestinal carbohydrate and fat absorption rates in 35 normal volunteers and patients with fat malabsorption (10), a gel phase was observed in specimens of duodenal content in four normals and two patients with sprue. These six subjects had received one of two test formulas. Two volunteers received 250 ml of a commercial formula (Ensure, Ross Laboratories, Columbus, Ohio), containing 34.9 g of fat per thousand kcal which was taken within 5 min. Two patients with sprue and two volunteers received corn oil (6 g), milk protein (4.5 g) and glucose and sucrose (16 g per dl plus polyethylene glycol 0.5% in water), which was perfused into the upper duodenum (15 cm cephaled from a collection portal) at a rate of 2.2 ml/min. Intestinal contents were collected by gravity, immediately heated to 70 C for 10 min to inactivate pancreatic lipase (11) and an aliquot (5 ml) was ultracentrifuged at 100,000 × g in an SW 50.1 rotor (Beckman ultracentrifuge, L50) for 4 hr at 34–37 C. When a gel phase was present, the top of the centrifuge tube contained a floating oil phase (ca. 0.2–0.3 ml), a narrow sharp protein interface that separated an opaque gel phase (ca. 0.8–1 ml) from the micellar phase below. The oil and gel phases were removed by tube slicing. Duplicate samples of the subnatant micellar phase were collected by gentle syringe aspiration of the middle of the punctured ultracentrifuge tube.

The oil, gel and micellar phases were extracted with 3 vol of ethanol/heptane mixture (1:1:1, v/v/v) (12). The total lipid content was measured gravimetrically and fatty acid concentration determined by titration with 0.05 M tetramethyl ammonium hydroxide to a phenolphthalein end point. Additional aliquots were extracted with chloroform/methanol (2:1, v/v) and the lipid phosphorus (13) and cholesterol (14) concentrations were measured. Micellar and gel neutral lipids and phospholipids were separated by thin layer chromatography (15) and quantitated after charring by densitometry (Densicord recording densitometer, Photovolt Corp., New York, New York). Total bile salt concentrations were measured by the hydroxysteroid method (16). Polarizing microscopy was used to examine specimens of the gel and micellar phases obtained from the same centrifuge tube. These samples were placed in thin-walled quartz capillaries (1 mm in diameter) for polarizing microscopy and x-ray diffraction. X-ray diffraction patterns of the gel showed a high background in the small angle region which precluded identification of liquid crystal patterns.

RESULTS

The present observations on an unusual phase in ultracentrifuged intestinal contents were made in duodenal and jejunal specimens collected by syphonage during studies of intestinal fat absorption (6). Gel phases were seen in duodenal and not in jejunal samples, suggesting that they occurred only during very rapid triglyceride hydrolysis. The pH of duodenal specimens varied between 6.6 and

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8.0. The gel phase was usually dark green and opaque, separated from the oil above by a sharp interface. Separation of the gel from the translucent micellar phase below was not always sharp, and the gel tended to diffuse if the centrifuge tube was disturbed.

The gel appeared birefringent between crossed polarizers (Fig. 1a). The presence of striations of irregular dimensions in the capillary is unusual and suggests that the liquid crystals occur in domains of about the same size as the striations and that these domains are not aligned parallel to each other. In an attempt to effect parallel orientation of the liquid crystals, the gel was flowed back and forth in the capillary tube and then allowed to settle. This shearing force left a central line of almost uniform extinction throughout most of the tube. When partial evaporation was allowed to occur prior to sealing the capillary, the highly hydrated liquid crystals reformed into focal conics (Fig. 1b). Birefringence was not observed in the micellar phase from these specimens nor from specimens that did not have a gel-like appearance. Total lipid, fatty acid and phospholipid concentrations in the gel were significantly greater than in the micellar phase; bile salts also were slightly but significantly more concentrated (Table 1). More importantly, ratios of total lipid to bile salt in the gel were higher than in the micellar phase. The molar ratios of monoglyceride and fatty acid to bile salt also were significantly greater in the gel than in micellar phases (Table 2). Phospholipid to bile salt and cholesterol to bile salt ratios were correspondingly greater in the gel than in the micelles.

Addition of 0.5 ml of a 100 mM sodium taurocholate solution to 0.25 ml aliquots of the opaque gel caused immediate formation of a clear isotropic phase. The gel phase did not change when phosphatidylcholine or lysophosphatidylcholine was added nor after incubation for 30 min with trypsin.

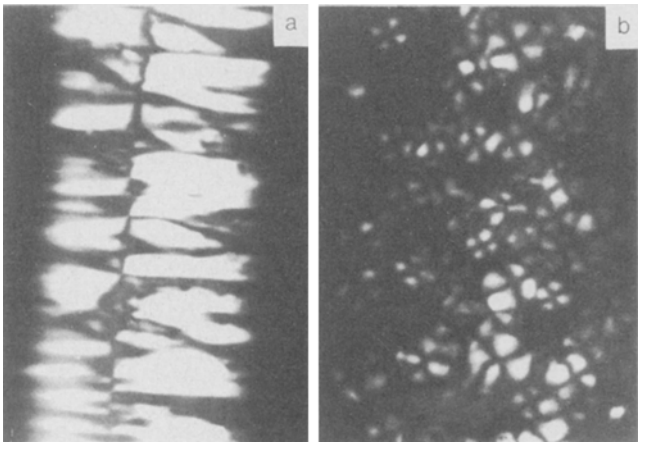

FIG. 1. Appearance of the gel phase between crossed polarizers. a: Initial appearance of fresh gel specimens. Note the irregular striations which suggest that liquid crystals occur in small nonparallel domains. b: Appearance after partial evaporation. Typical recrystallization into focal conics. Specimens examined in quartz capillary tubes, 1.0 mm diameter. Magnification × 20.

TABLE 1

Chemical Composition of Gel and Micellar Phases

	Total lipid (mg/ml)	Fatty acid (μmol/ml)	Bile salt (μmol/ml)
Gel	16.5 ± 4.5	25.7 ± 9.1	8.8 ± .9
Micellar phase	6.0 ± .4	8.5 ± .7	6.0 ± .7
Difference (p)	<.05	<.05	<.01

Specimens of gel and micellar phases prepared from duodenal fluid as described in Methods. Mean ± SEM from six samples analyzed in duplicate. Differences calculated by Student's t-test.

TABLE 2

Concentration Ratios of Total Bile Salts to Lipid Classes in Gels and Micellar Phases from Centrifuged Intestinal Contents

	Ratio				
	BS	MG	FA	PL	C
Gel	1	2.2 ± .3	2.9 ± .3	.18 ± .04	.063
Micellar phase	1	1.1 ± .1	1.4 ± .2	.07 ± .02	.025
Difference (p)		<.01	<.025	NS	<.001

Mean ± SEM of four samples analyzed in duplicate. BS, bile salt; MG, monoglyceride; PL, phospholipid; C, cholesterol; FA, fatty acid; NS, not significant. Based on molar concentrations.

DISCUSSION

The present studies reveal that upper intestinal contents, collected during rapid hydrolysis of triglycerides, can contain a liquid crystalline phase which was identified by the presence of birefringence between crossed polarizers. The micellar phases collected from the same ultracentrifuged intestinal contents as the gels showed no characteristics of liquid crystals. In the gel, the concentration of all lipid classes was much higher than in the micellar phase. Furthermore, the molar ratios of fatty acid, monoglyceride and cholesterol to bile salt were much higher in the gel than in the micellar phases of these specimens and the concentrations of lipids were close to micellar saturation (10). Ultracentrifugation was unlikely to have induced the appearance of liquid crystals (17) since similar treatment of intestinal contents from the remaining 29 subjects did not demonstrate such a phase.

During the 20 years following the initial description of two major phases, an oil emulsion and a clear isotropic micellar phase, in centrifuged postprandial intestinal contents (4), several authors have suggested that luminal lipids exist in other physical forms. For example, small precipitates were described at the bottom of centrifuged tubes (5) representing nonpolar lipids like cholesterol which had precipitated from mixed micellar phases after monoglyceride absorption (18) or calcium soaps of fatty acids (7). An aqueous bile salt-fatty acid gradient also was described (19) and a slightly turbid aqueous filtrate noted (20). Unpublished data described in a recent comprehensive review (21) implied that a turbid solution containing a liquid crystalline phase saturated with bile salts could be found in postprandial intestinal contents. These suggestions from in vivo studies are supported by the data of Patton and Carey, who simulated digestion on a

microscope slide in vitro (7) and described rapid formation of a birefringent liquid crystalline shell on the edge of oil droplets followed by the development of a viscous isotropic phase, and by Lindstrom et al. (22), who studied the formation of liquid crystalline phases of mixtures of fatty acids, monoglycerides and triglycerides in aqueous systems in the test tube.

Monolayer penetration data (23) and consideration of phase equilibria suggest that liquid crystalline phases could form in intestinal contents during fat digestion. Such a phase would contain a higher concentration of bile salts, fatty acids and monoglycerides than the micellar phase. In intestinal contents, a liquid crystalline phase would exist only transiently since it would equilibrate rapidly into smaller aggregates of micellar size upon addition of bile salts. The concentrations of neutral lipids and cholesterol were much higher in the gel than in the micellar phase in our studies (Table 2) and taurocholate instantly cleared the gel, whereas addition of phospholipids and trypsin digestion of protein were without effect. Since the interaction of the many lipids in the gel with water is complex, the precise chemical composition of the liquid crystals is uncertain.

Lipid absorption is believed to occur principally from molecularly dispersed species in the unstirred layer adjacent to mucosal cells. The micellar phase increases the availability of lipid molecules to the unstirred water layer. Whether insoluble amphiphiles such as cholesterol are absorbed solely by molecular dispersion from a micellar phase is not entirely clear. The higher concentration of lipids in an intermediate liquid crystalline phase would increase their rate of transfer to the mucosal membrane for cellular uptake. The quantitative role of liquid crystalline phases in overall fat absorption remains to be clarified.

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Metabolism and Incorporation into Glycerolipids of Exogenous 18:3(n-3) and 18:3(n-6) by MDCK Cells

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The extent to which exogenous 18:3(n-3) and 18:3(n-6) were desaturated and elongated and the degree to which they and their derivatives altered the unsaturation index of cell glycerolipids were compared using clone 4 MDCK cells grown in lipid- and serum-free medium. Despite differences in the degree of unsaturation of the individual polyunsaturated fatty acids produced from 18:3(n-3) or 18:3(n-6), the unsaturation index of phospholipids increased similarly from 0.7 in control cells grown in serum- and lipid-free medium to ca. 1.6 in those supplemented with fatty acid. The added fatty acids had little effect on cell growth. The conversion of 18:3(n-6) to 20:3(n-6) and 20:4(n-6) was more rapid than that of 18:3(n-3) to 20:4(n-3) and 20:5(n-3). No significant quantities of 20:3(n-3) or 18:4(n-3) were noted. When both 18:3 isomers were supplied simultaneously, marked differences in the amounts of some species of n-3 and n-6 polyunsaturated fatty acids were observed. The presence of 18:3(n-6) and/or its derivatives suppressed levels of 20:4(n-3) and 20:5(n-3), perhaps through inhibition of the Δ6 and Δ5 desaturases responsible for their synthesis from 18:3(n-3). Similarly 18:3(n-3), and/or its longer more unsaturated derivatives, diminished the formation of 20:4(n-6) from 18:3(n-6). No marked effect on the products derived from elongation alone were observed.
Lipids 21, 447–453 (1986).

Recent data suggest that changes in membrane phospholipid composition accompany the development of polarity by epithelial cells in culture (1,2). There is also speculation that components of the lipid bilayer may play a role, directly or indirectly, in the function of tight junctions between such cells (3,4). One means of exploring further the relationship between lipids and these phenomena is to perturb the membrane lipid composition and measure its effect on the functions of interest. This strategy has been successfully employed to demonstrate that many membrane-bound enzymes and transport systems are affected by alterations in the properties of the lipid bilayer (5,6). Frequently changes in the degree of acyl group unsaturation have been used for this purpose. It is important to note, however, that such modifications often involve changes not only in the physical properties of the membrane systems under study, but also in the availability of n-6 fatty acids which act as precursors to biologically active eicosanoids (7,8). Inhibitors of phospholipases or cyclooxygenase are usually employed to dissociate prostaglandin dependent events from those related to alterations in membrane physical properties (9,10). Because these inhibitors can affect enzymes other than cyclooxygenase (11), it is undesirable during long term fatty acid supplementation of intact cells to include them in the medium. In such instances, the n-3 and n-6 isomers of 18:3 could be particularly useful. They and the products of their elongation and desaturation both have very low melting points (5,12) and may, therefore, affect membrane functions which depend on the physical state of the membrane. On the other hand, while some members of the n-6 family are important precursors of the major prostaglandins, those of the n-3 family are not (8,13,14).

The present study is the first of several concerned with the role of plasma membrane lipids in the function of clone 4 MDCK cells, an established epithelial cell line. Clone 4 MDCK cells were chosen for this study because they can be cultured in a chemically defined, serum-free medium, supplemented with, among other things, linoleic acid to form high resistance junctions in monolayer (Schneeberger, E. E., Lynch, R. D., and Rabito, C. A., unpublished observations). The objective was to adapt these cells to a fatty acid-free medium and to define the conditions necessary to induce significant and similar changes in the phospholipid unsaturation index when the cells were supplemented with 18:3(n-3) or 18:3(n-6).

MATERIALS AND METHODS

Cell culture. Monolayer cultures of MDCK cells (clone 4), provided as a gift by Julia Lever (University of Texas Medical School, Houston, Texas), were maintained in 25- or 75-cm^2 tissue culture flasks (Falcon Plastics, Becton Dickinson, Oxnard, California). The medium consisted of a 50:50 mixture of Dulbecco's Modified Eagle Medium and Ham's F-12 Nutrient mixture (Gibco, Grand Island, New York), and 10% newborn calf serum (Hyclone Laboratories, Logan, Utah), 1.45 mM Hepes and 1.45 mM $NaHCO_3$; this medium was designated S-12. For experiments requiring serum- and lipid-free conditions, the medium was modified as follows: Ham's F-12 was replaced with Ham's F-10, which lacks 18:2(n-6) but is otherwise similar. The medium was supplemented with 1.45 mM Hepes, 1.45 mM $NaHCO_3$, 25 ng/ml prostaglandin E_1, 5 μg/ml insulin, 5 μg/ml transferrin, 50 nM hydrocortisone, 5 pM triiodo-L-thyronine and 1.75 ng/ml selenous acid (15). This medium is referred to as T-10. All solutions for cell culture were prepared with glass-distilled, deionized water. Cultures were grown at 37 C in a humidified incubator in a 5% CO_2 atmosphere. Stock cultures were periodically monitored for Mycoplasma contamination (16).

Cultures were split using 0.05% trypsin (Worthington, Diagnostic Systems Inc., Freehold, New Jersey) in Ca^{++}-free, Mg^{++}-free Earles balanced salt solution (EBSS). The action of trypsin was inhibited by the addition of a neutralizing amount of soybean trypsin inhibitor. After centrifugation at 560 × g the cells were resuspended in fresh medium to yield a split ratio of 1:10 for cell propagation and 1:5 when preparing for fatty acid supplementation. Medium was changed in stock cultures every 2–3 days. Subconfluent cultures were allowed to adapt to

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serum-free medium for four days prior to initiating experiments in which they were to be incubated with fatty acid- and albumin-supplemented serum-free medium. All experiments were with cells between the 180th and 240th passage. Cell growth rates were monitored by measuring fluorometrically the μg of DNA present per culture (17).

Fatty acid supplementation. The contents of freshly opened ampuls of 18:3 (Supelco Inc., Bellefonte, Pennsylvania) were diluted immediately with ice cold, N_2 saturated heptane. After the heptane solution was washed with a 3:1 (v/v) mixture of methanol/water to remove polar products of lipid peroxidation (18), it was dried under a stream of N_2 in a tared vessel and quickly dissolved in sufficient 0.08 N NaOH to yield a final sodium salt concentration of 50–60 μmol/ml (6,19). The fatty acid salt solution was stored in liquid N_2 until needed.

Fatty acid/albumin complexes (molar ratio of fatty acid to albumin, 6.8:1) were prepared in serum-free medium by dissolving the required amount of fatty acid-free albumin (Miles Laboratories, Elkhart, Indiana) in the medium, heating the resulting solution rapidly to 56 C and then, while stirring vigorously, adding the fatty acid salt prepared as described above (6,20). The solution was quickly cooled to room temperature in an ice bath, and filter-sterilized through a 0.2 μm filter (Schleicher and Schull Inc., Keene, New Hampshire). This solution was either used immediately or stored at −24 C overnight. Incubation of the cells in 25-cm^2 tissue culture flasks with 4 ml of the various media, containing either albumin alone or albumin/fatty acid complex, was initiated in cultures at 75–85% confluence. Concentrations of fatty acids used varied from 62.5 nmol/ml to 500 nmol/ml; total incubation time, unless noted otherwise, was 48 hr.

Lipid extraction. Incubations were terminated by rinsing the monolayers twice, each time with 5 ml of ice-cold EBSS. Two ml of EBSS were immediately added, the flask was tightly capped and the cells were disrupted by rapidly and repeatedly passing the exterior of the growth surface of the flask over the tip of an inverted ultrasound probe (approximate surface of 2 cm^2) powered by a Sonifier Cell Disrupter model W185 (Heat Systems Ultrasonics Inc., Plainfield, New York) with the output control set at 50% of maximum. The use of ice-cold balanced salt solutions, prechilled monolayers and a rapid motion of the flask on the probe tip resulted in complete cell disruption within 5–10 sec with little heat generation. The resulting sonicate was transferred to a glass conical centrifuge tube containing 2.0 ml of ice-cold butanol (21,22), with 0.1% butylated hydroxytoluene as an antioxidant, and 50 μg each of triheptadecanoin and diheptadecanoyl phosphatidylcholine as internal standards for quantitation of the cell triglyceride and phospholipid fractions, respectively. The experimental flask was rinsed with another 2.0 ml of EBSS which was added to the sonicate.

After vigorous vortex mixing, the centrifuge tubes were placed in a water bath at 55–60 C for 15 min, after which they were centrifuged in the cold at 1000 × g for 5 min. The upper (butanol) phase was transferred to a clean tube, and the lower phase was extracted a second time with 2.0 ml of butanol. After centrifugation, the butanol extracts were combined and washed with an equal amount of water saturated with butanol.

Lipid analyses. Lipid extracts were dried under N_2 and redissolved in 4–5 drops of $CHCl_3/CH_3OH$ (1:1, v/v). The entire extract was applied to a silica gel H-coated (0.3 mm thick) thin layer chromatography plate. Plates were prepared as previously described (23) except that the slurry contained 0.07% ammonium sulfate. Plates were activated for 30 min at 100 C immediately prior to use.

Separation of the triglyceride and phospholipid fractions was accomplished by developing the chromatogram in a solvent mixture of hexane/diethyl ether/glacial acetic acid (60:39:1, v/v) (6). The chromatogram was then air-dried in a fume hood for 5 min and sprayed with an aqueous solution of 0.1% 8-anilino-1-napthalenesulfonic acid (Supelco). Individual lipid classes were visualized under a UV lamp, and the appropriate zones were collected in test tubes for methylation with a mixture of benzene/CH_3OH (60:40, v/v) containing 5% (w/v) NaOH (24). Transmethylation was terminated after 5 min by the addition of an equal volume of 8% sulfuric acid in CH_3OH, and the methyl esters were extracted into heptane. After concentration under N_2, the fatty acid methyl esters were separated by gas chromatography on a 6′-long glass column (1/8″ i.d.) packed with 10% SP-2330 on 100/120 Chromasorb (Supelco). The column was mounted in a Varian series 2400 gas chromatograph equipped with a flame ionization detector; nitrogen was the carrier gas. Injector and detector temperatures were 235 C, and the column oven temperature was 198 C. The amount of each fatty acid present was calculated as a percentage of the total acyl groups in the sample using an Hewlett Packard 3380A integrator. With the exceptions of 18:4(n-3) and 20:4(n-3), fatty acid identifications were based on a comparison of sample fatty acid retention times with those of standards (Supelco). The tentative identification of 18:4(n-3) and 20:4(n-3) was based on a comparison of relative retention times from the literature (25,26) with those of the methyl esters in this study. Absolute amounts of triglyceride and phospholipid per culture were determined by comparing the areas of the 17:0 peak, derived from the added internal standards, with areas of the sample peaks.

Unsaturation indices were calculated by multiplying the percentage of each fatty acid present in the fraction by the number of double bonds it contained. The products were then summed and divided by 100.

RESULTS

Cell growth. During the first 24 hr, growth of MDCK cells in serum- and lipid-free medium was less than that of cells in medium supplemented with serum. From that point onward, however, they multiplied at approximately the same rate as those in serum-supplemented medium (Fig. 1). A small increase in cell growth was noted during the first days when either albumin alone or albumin complexed with either 18:1 or 18:3(n-6) was added to the culture medium. Continued cell growth in the presence of exogenous fatty acid at a rate equal to or greater than in its absence indicates that, at the concentration employed, these fatty acids were not cytotoxic. Moreover, whether cultures received fatty acid or not, the cells were greater than 90% viable as determined by trypan blue dye exclusion. Cytoplasmic lipid droplets, which were evident to a limited extent in cells from all cultures, were more numerous in those from cultures supplemented with fatty acid.

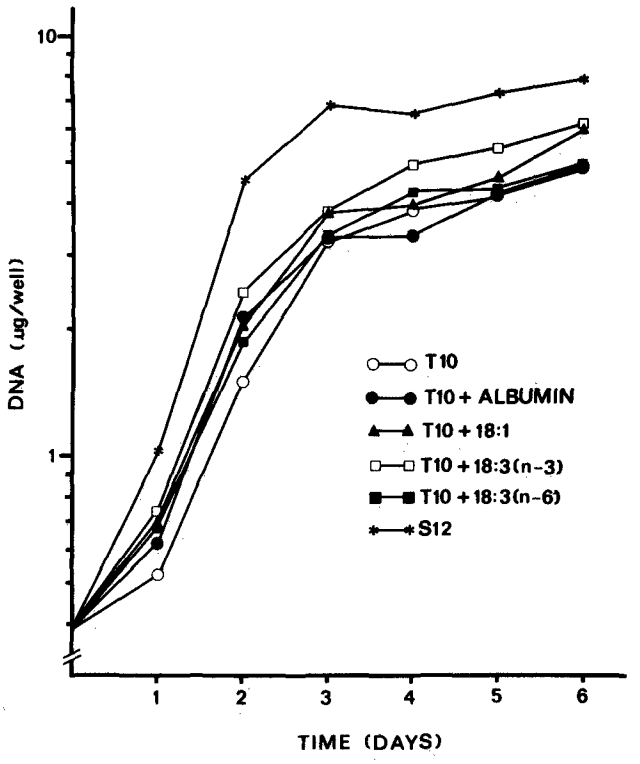

FIG. 1. Growth of clone 4 MDCK cells in media supplemented with either 18:1, 18:3(n-3) or 18:3(n-6) complexed with albumin. Cells, adapted to serum- and lipid-free medium for 4 days, were transferred to multiwell plates and allowed to attach overnight yielding monolayers ca. 20% confluent. One set of triplicate wells was analyzed for its DNA content, while medium on the remainder was replaced with 1.0 ml of serum-free medium (○); medium supplemented with serum (*); albumin alone (●); or medium with albumin complexed to 0.25 µmol/ml of 18:1 (▲), 18:3(n-3) (□), or 18:3(n-6) (■). At the indicated times the µg of DNA per well was determined. All data points represent the mean of three wells. Standard deviations in all cases were <10% of the mean.

TABLE 1

Phospholipid Fatty Acid Composition of Control, 18:3(n-3) and 18:3(n-6)-supplemented Clone 4 MDCK Cells (µg %)[a]

Fatty acid	Serum, supplemented	Serum, lipid-free	Exogenous fatty acid 18:3(n-3)	Exogenous fatty acid 18:3(n-6)
16:0	15.7 ± 1.3	10.9 ± 1.1	8.6 ± 0.9	9.9 ± 0.6
16:1	6.0 ± 0.5	5.7 ± 0.6	1.4 ± 0.4	1.3 ± 0.2
18:0	17.2 ± 1.1	15.9 ± 1.0	26.4 ± 0.7	22.5 ± 0.4
18:1(n-9)	37.7 ± 0.7	53.8 ± 0.5	27.3 ± 0.9	27.6 ± 1.0
18:2(n-6)	7.0 ± 0.3	3.7 ± 0.2	1.0 ± 0.1	1.2 ± 0.3
18:3(n-6)	—	trace	trace	3.4 ± 0.3
18:3(n-3)	—	—	12.5 ± 1.0	trace
20:1(n-9)	0.8 ± 0.2	1.3 ± 0.1	trace	trace
20:2(n-9)	2.5 ± 0.4	—	—	—
?[b]	1.8 ± 0.2	6.2 ± 0.3	0.5 ± 0.1	0.2 ± 0.1
20:3(n-6)	6.3 ± 0.3	0.9 ± 0.1	0.5 ± 0.1	19.7 ± 1.3
20:4(n-6)	4.6 ± 0.2	0.6 ± 0.1	trace	13.5 ± 1.2
20:4(n-3)	—	—	10.4 ± 0.6	—
20:5(n-3)	0.2 ± 0.1	trace	10.8 ± 0.3	0.2 ± 0.1
22:6(n-3)	0.2 ± 0.1	trace	trace	trace

Cells adapted to T-10 medium for 4 days were incubated for 48 hr with either albumin alone or albumin complexed with 0.25 µmol/ml of the appropriate fatty acids. Lipids were then extracted and separated and the acyl group composition of the phospholipids was determined.

[a]Data are the mean ±1 S.D. of three determinations.

[b]This fatty acid was tentatively identified as 22:0 or 20:3(n-9).

Phospholipid acyl group composition. Marked changes in phospholipid acyl group composition were observed after MDCK cells were maintained for 4 days in serum- and lipid-free medium (Table 1). Less than 5% of the fatty acids in the phospholipid fraction of MDCK cells maintained for 4 days in serum- and lipid-free medium contained two or more double bonds. Quantitatively, 18:1 accounted for approximately half of all fatty acids in the phospholipids (Table 1). The addition of an albumin/18:1 complex to the medium resulted in only minor changes in phospholipid acyl group composition (data not shown), which were similar to those reported by others (27).

When an albumin complex containing either 18:3(n-3) or 18:3(n-6) was used, however, the percentage of polyunsaturated fatty acid in the phospholipid fraction increased approximately sevenfold over 48 hr. The n-3 polyunsaturated fatty acids identified in the phospholipid fraction isolated from cultures incubated with 18:3(n-3) were 18:3(n-3), 20:4(n-3) and 20:5(n-3). No quantitatively significant amounts of 18:4(n-3), 22:5(n-3) or 22:6(n-3) were detected. After incubation with 18:3(n-6), increases were observed in the levels of 18:3(n-6), 20:3(n-6) and 20:4(n-6). The decrease in 18:1, which accompanied the increase in polyunsaturated fatty acid content, is consistent with observations in other cell lines and likely reflects inhibition by the polyunsaturated fatty acids of a Δ9 desaturase system (28,29). Despite large changes in acyl group composition, the total amount of phospholipid fatty acid per culture was similar whether fatty acid was added to the medium or not. This lack of an effect by exogenous lipid on the amount of phospholipid per culture has been noted in other cell lines (6,30).

Exogenous fatty acid concentration and phospholipid unsaturation index. Regardless of the isomer used and differences in the polyunsaturated fatty acids derived therefrom, the unsaturation index of the phospholipid was elevated to a similar extent as the concentration of exogenous 18:3 was increased (Fig. 2). There was no

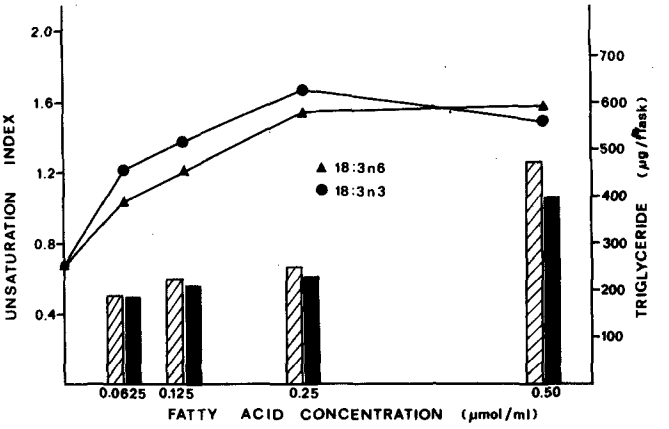

FIG. 2. Concentration-dependent effects of exogenous 18:3(n-3) and 18:3(n-6) on phospholipid unsaturation index and triglyceride content of clone 4 MDCK cells. Cells were adapted to T-10 medium for 4 days in 25-cm² tissue culture flasks. On day 5, when cells were ca. 75% confluent, medium was replaced with 4 ml of T-10 containing either 18:3(n-3) or 18:3(n-6) at the concentration indicated. After 48 hr, the monolayer was washed and the unsaturation index of the phospholipid and the triglyceride content of the culture determined. Lines represent the unsaturation index; bars represent the triglyceride content of the culture. Hatched bars are 18:3(n-3); solid bars are 18:3(n-6).

significant rise in the unsaturation index at initial concentrations of exogenous fatty acid greater than 0.25 μmol/ml. As in other systems (6,31), however, the size of the triglyceride pool increased as the amount of fatty acid supplied exceeded demands for phospholipid synthesis.

Time course for utilization of exogenous 18:3 by MDCK cells. Analysis of the medium taken from several cultures after 72 hr of incubation with 0.25 μmol/ml of either isomer showed that between 80 and 90% of the exogenous fatty acid originally added had been taken up. Only traces, if any, of the longer, more unsaturated fatty acids derived from the precursor 18:3, originally supplied, were detected in the medium. The total amount of the principal n-3 fatty acids in esterified form (triglyceride and phospholipid) was at a maximum within 12 hr, while that for n-6 fatty acids was not achieved until 48 hr (Figs. 3a and 3b). After 72 hr, the total amount of n-3 or n-6 fatty acid recovered in cell glycerolipid was less by 10 and 30%, respectively, than the amount taken up by the cells, suggesting that some of the added fatty acid had been either oxidized or converted to non-fatty acid derivatives.

The relatively rapid accumulation of 20:3(n-6) and the low levels of its immediate precursor, 18:3(n-6), in the total glycerolipid fraction of cells incubated with the latter, suggest the existence of a very active elongase (Fig. 3a). On the other hand, levels of esterified 20:4(n-6) produced by desaturation of 20:3(n-6) increased at a rate less than half that for the formation of 20:3(n-6). By 48–72 hr of incubation, however, the levels of each of the n-6 fatty acids were changing only slightly.

The utilization of 18:3(n-3) by MDCK cells is qualitatively and quantitatively different from that of 18:3(n-6) (Fig. 3b). Cell levels of 18:3(n-3), although declining during the first 48 hr, were maintained at 3–4 times those of its isomer in the preceding experiment (Fig. 3a). Concomitantly, the products derived from 18:3(n-3) accumulated more slowly than did those from 18:3(n-6), and were still increasing at 72 hr. Neither 20:3(n-3), a product resulting from elongation of the exogenous fatty acid, nor 18:4(n-3), derived from the direct desaturation of 18:3(n-3), were detected in significant amounts at any time. Although the amount of 20:4(n-3) present in the lipids of cells incubated with 18:3(n-3) was less than the 20:3(n-6) formed during incubations with 18:3(n-6), the rates at which the products of their desaturation, 20:5(n-3) and 20:4(n-6), respectively, accumulated were similar.

At all times, the fraction of 18:3(n-3) and 18:3(n-6) in phospholipid was less than that in triglyceride (Figs. 3a and 3b). As indicated in these figures, the numbers in parentheses above the bars represent the fraction of that particular fatty acid esterified in phospholipids. A similar distribution of 20:4(n-3) and 20:3(n-6) between phospho-

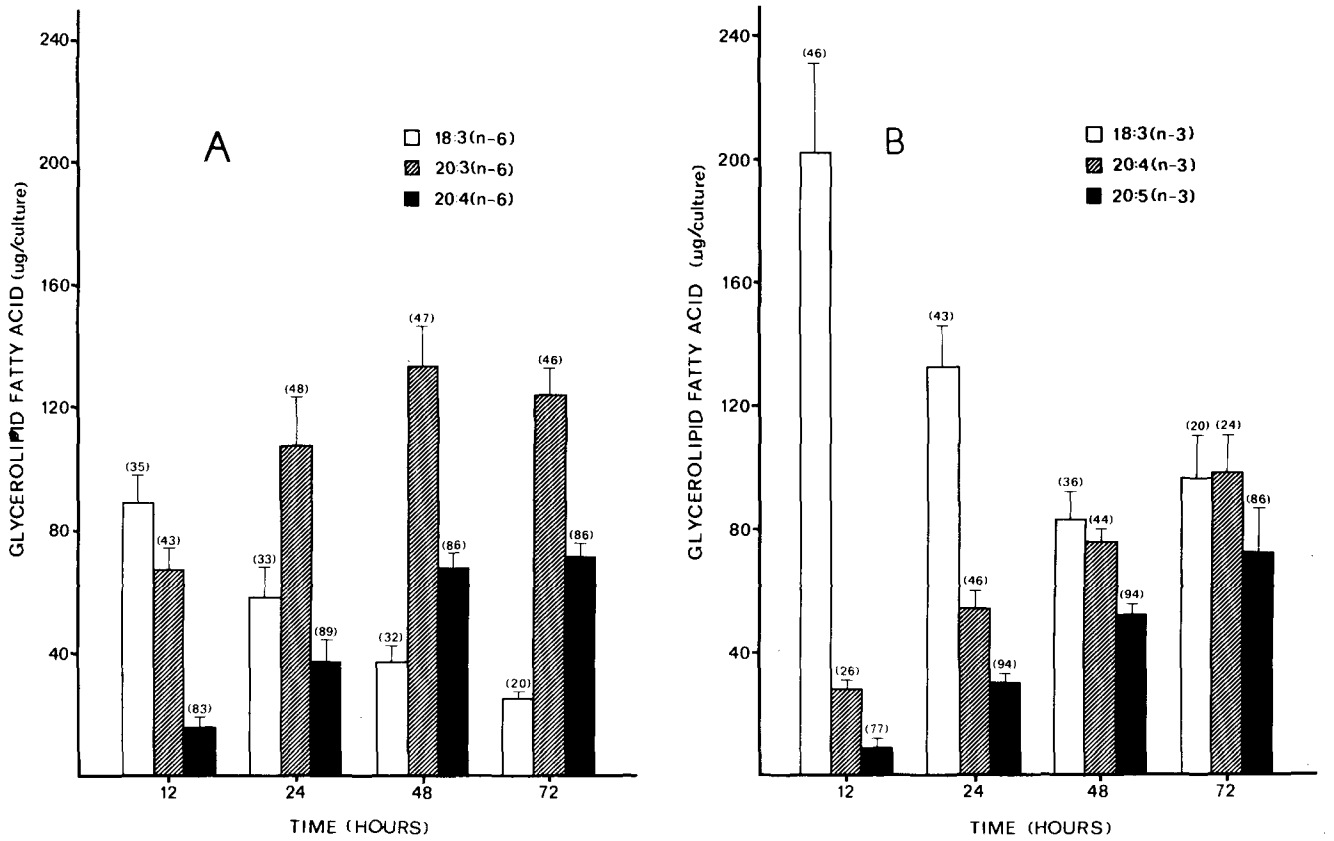

FIG. 3. Time course for the accumulation of n-3 and n-6 fatty acids in glycerolipids of MDCK cells incubated with 18:3(n-3) or 18:3(n-6). Replicate cultures of MDCK cells, ca. 75% confluent, were incubated in 25-cm² tissue culture flasks with 0.25 μmol/ml of 18:3(n-3) or 18:3(n-6) for the times indicated. Lipids were extracted and the total amounts of fatty acids of the n-3 and n-6 families in the glycerolipid fraction were determined. A: Cells incubated with 18:3(n-6); B: cells incubated with 18:3(n-3). Each bar represents the mean of data from three experiments; vertical lines represent ±1 S.D. Numbers in parentheses are the percentage of that fatty acid in the phospholipid fraction. For baseline glycerolipid fatty acid composition, see data for serum-free cells in Table 1.

lipid and triglyceride was also noted. More striking was the preferential esterification of 77% of the 20:4(n-6) and 94% of the total 20:5(n-3) in the phospholipid fraction.

Substrate mixing experiments. Although the n-3 fatty acids likely occupy the same esterification sites in the various phospholipid classes as members of the n-6 family, it is conceivable that fatty acids of each family may to some extent enter exclusive phospholipid pools. If the latter were true, providing cells with a mixture of both isomers might result in a value for the unsaturation index greater than the 1.6 obtained when only one was added. With all combinations of 18:3(n-3) and 18:3(n-6) tested, the unsaturation index did not differ significantly from values obtained with each fatty acid added separately. There were, however, marked changes in the relative amounts of the various fatty acids in the n-6 and n-3 families in the total cell lipids (Figs. 4 and 5). When a fixed concentration of 18:3(n-6) was tested for its effect on the utilization of varying initial amounts of 18:3(n-3), a marked decline in both 20:4(n-3) and 20:5(n-3) levels was noted (Fig. 4). Conversely, the presence of 18:3(n-3), or perhaps one or more of its derivatives, decreased the levels of 20:4(n-6) (Fig. 5). Note that at all combinations of concentrations of 18:3(n-3) and 18:3(n-6) employed, the amount of 20:5(n-3) was the same or slightly greater than 20:4(n-6). This occurred even though the amount of 20:3(n-6), the precursor for the latter, was nearly twice that of 20:4(n-3), the immediate precursor for 20:5(n-3). These data suggest that this Δ5 desaturase system shares with others (12) a preference for n-3 substrates.

DISCUSSION

With the possible exception of 18:3(n-3), any positive growth effects noted when fatty acid/albumin complexes were added to the cultures of MDCK cells were accounted for by the presence of albumin alone. The addition of relatively high concentrations of exogenous fatty acids to MDCK cells in serum-free medium did not diminish cell growth rates. In fact, in the presence of 18:3(n-3), the culture growth rate was slightly enhanced. Furthermore, even after trypsinization, greater than 90% of the cells excluded trypan blue.

Except for those fatty acids oxidized or converted to other non-fatty acid products (e.g., prostaglandins), nearly all cell-associated fatty acids were present in esterified form in either the phospholipid or triglyceride fraction. In fact, no measurable unesterified fatty acid was detected after separation of the lipids by thin layer chromatography. In addition, there were little, if any, of the longer, more unsaturated n-3 or n-6 derivatives identified in the medium. For these reasons, discussion of the total amount of the exogenously derived fatty acids in the culture is limited to those in the glycerolipid fractions of the cell. Any differences, therefore, between fatty acids with respect to their amounts and distribution between phospholipid and triglyceride must arise from differences in the quantities and selectivities of enzymes involved in fatty acid activation, elongation, desaturation, retroconversion, acylation, deacylation and oxidation.

One of the chief differences between cells in serum-supplemented medium and those adapted to serum- and lipid-free conditions was a rise in 18:1 levels as the n-3 and n-6 polyunsaturated fatty acids fell. An increase in a fatty acid with a retention time of 22:0 or 20:3(n-9) was also observed. Although the ambiguity remains unresolved, it should be noted that the increase in the amount of this fatty acid in cells grown in serum- and lipid-free conditions, and its decrease when n-3 or n-6 fatty acids were supplied, is consistent with its being 20:3(n-9). The latter is commonly observed in lipids from essential fatty acid-deficient animals. Depending on the relative amounts of 22:0 or 20:3 (n-9) present in these serum- and lipid-free cells, the value for their unsaturation indices could vary from 0.7 to 0.9.

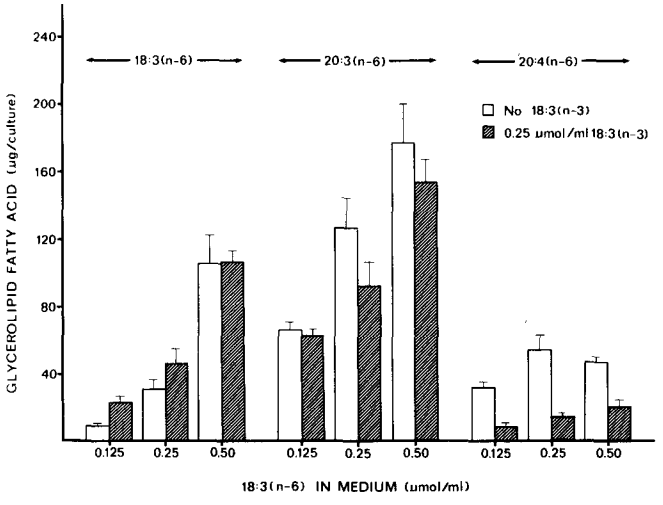

FIG. 4. Effects of fixed amounts of 18:3(n-6) on the metabolism of increasing amounts of 18:3(n-3). Cells in 25-cm² flasks after adaptation to serum- and lipid-free medium for four days were ca. 75% confluent. They were incubated for 48 hr with increasing concentrations of 18:3(n-3) in the absence (open bars) or presence (hatched bars) of 0.25 μmol/ml of 18:3(n-6). Lipids were extracted and the total amount of each of the n-3 fatty acids in the glycerolipid fraction was determined by gas chromatography. Each bar represents the mean from three experimental flasks. Vertical lines represent ±1 S.D.

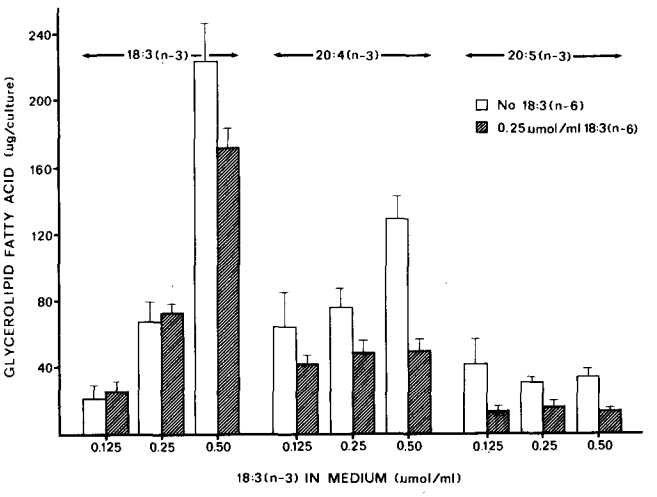

FIG. 5. Effects of fixed amounts of 18:3(n-3) on the metabolism of increasing amounts of 18:3(n-6) by clone 4 MDCK cells in culture. Protocol was similar to that described for Fig. 4 except that the concentration of 18:3(n-6) was increased in the absence or presence of 0.25 μmol/ml (18:3(n-3), and the fatty acids quantitated were of the n-6 series.

Despite qualitative and quantitative differences in polyunsaturated fatty acid composition, the increase in the cell phospholipid unsaturation index after incubation with either 18:3(n-3) or 18:3(n-6) was similar in magnitude and in its response to changes in the concentration of exogeneous fatty acid. With either isomer, polyunsaturated fatty acids constituted a maximum of ca. 40% of the total fatty acids in the phospholipid fraction even when there was a large supply of those fatty acids in the triglyceride pool. This suggests that, as in many other systems (6,33), the specificity of the phospholipid acylating enzymes for the sn-1 and sn-2 positions of glycerol limit the polyunsaturated fatty acid content of phospholipid to values less than 50%.

The conversion of 18:3(n-3) and 18:3(n-6) to 20:5(n-3) and 20:4(n-6), respectively, may proceed through pathways which have some enzymes in common (31,34). The sequence of reactions by which 18:3(n-3) and 18:3(n-6) were converted to 20:5(n-3) and 20:4(n-6), respectively, however, and the rate at which intermediates accumulated differed markedly. In MDCK cells provided with exogenous 18:3(n-6), the rate-limiting Δ6 desaturase, normally involved in controlling synthesis of 20:4(n-6) from 18:2(n-6) (35), was bypassed. Instead, this fatty acid was directly elongated to yield 20:3(n-6), which accumulated at a rate greater than that observed for 20:4(n-6), suggesting that the Δ5 desaturase responsible for the formation of the latter from 20:3(n-6) is a second point for regulating the steady state levels of arachidonate and other n-6 fatty acids. It is of interest that even with elevated levels of 20:3(n-6) in cell glycerolipids the amount of 20:4(n-6) formed via Δ5 desaturase was only slightly in excess of that utilized for phospholipid synthesis.

In contrast to its n-6 isomer, the transformation of 18:3(n-3) to 20:5(n-3) involves both a Δ6 and a Δ5 desaturase (13). If the elongation reaction required between these two desaturation steps is rapid (35), then, as in the formation of 18:3(n-6) from 18:2(n-6), it is the Δ6 desaturase which limits the rate of formation of 20:4(n-3). Flux through this step may be further reduced by inhibition of the Δ6 desaturase by 18:3(n-3) (36,37) or another of the n-3 series of fatty acids. To account for its presence in negligible quantities in cell glycerolipids, 18:4(n-3) must be immediately elongated to 20:4(n-3). That the rate of accumulation of 20:4(n-3) was greater than that of 20:5(n-3) is consistent with a role for the Δ5 desaturase in regulating the synthesis of the end products of both the n-3 and n-6 pathways.

The absence of quantitatively significant amounts of 20:3(n-3) in MDCK glycerolipids suggests either that the system responsible for elongation of 18:3(n-6) or 18:4(n-3) did not recognize 18:3(n-3) or that any 20:3(n-3) formed underwent retroconversion. Although 22:5(n-3) and 22:6(n-3) have been reported in several tissues (13,14,38, 39), neither accumulated in measurable amounts in lipids of MDCK cells incubated with 18:3(n-3). It is conceivable that, as in some other systems (26,40–42), these fatty acids were synthesized and either retained or released into the medium in quantities too small for detection by gas chromatography alone.

No additive effects on phospholipid unsaturation index were apparent when mixtures of 18:3(n-3) and 18:3(n-6) were provided to cultures. This is consistent with there being equal access to all phospholipids by members of either family of polyunsaturates. Interpretation of data from substrate mixing experiments, however, was confounded by large changes in the relative amounts of various members of both the n-3 and n-6 families of polyunsaturated fatty acids. These changes, in turn, resulted from interactions between these polyunsaturates and the desaturases and elongases required for their formation and/or utilization. Although the conclusion is speculative, the fall in 20:4(n-3) levels observed in the presence of 18:3(n-6), for example, may reflect inhibition by the latter or other n-6 fatty acids (as well as the n-3 fatty acids) of Δ6 desaturase activity (36,37,43). The net effect is a decrease in the supply of precursor for 20:5(n-3) production. On the other hand, 18:3(n-6), which is not a substrate for Δ6 desaturase, was elongated to 20:3(n-6) in amounts proportional to the supply of 18:3(n-6), and was affected little, if at all, by 18:3(n-3) or other n-3 fatty acids derived from it. As a consequence of these various interactions, the ratio of 20:3(n-6) to 20:4(n-3) was elevated greatly in cells incubated with a mixture of both isomers.

This fact notwithstanding, the amount of 20:5(n-3) produced was either equal to or greater than the 20:4(n-6) in cell lipids. This suggests that in these cells the enzyme(s) responsible for determining steady state levels of the latter two fatty acids is selective for the accumulation of 20:5(n-3) relative to 20:4(n-6). Similar selectivities have been described in other systems (12). Further work is necessary to assess the role of acyltransferase selectivities in determining the distribution of the various n-3 and n-6 fatty acids among the individual phospholipid classes.

The data in the present study show that despite qualitative and quantitative differences in the metabolism of 18:3(n-3) and 18:3(n-6), remarkably similar changes in the unsaturation index of MDCK cell phospholipid were achieved when cultures were supplemented with either 18:3(n-3), 18:3(n-6) or both. When these two isomers were added to the medium of a human lung fibroblast cell line (6), the change in unsaturation index was similar, but higher than reported for the epithelial line employed in the present study. This was due to the production of more highly polyunsaturated fatty acids by the fibroblasts. Further work is necessary to determine whether the reported similarities in phospholipid unsaturation indices obtained when cells are incubated with either 18:3(n-3) or 18:3(n-6) are due to regulatory mechanisms or are merely coincidental.

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Effect of Nutritional Status on the Fatty Acid Composition of Rat Liver and Cultured Hepatocytes

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The lipid concentration and fatty acid composition of the whole liver and of cultured hepatocytes isolated from the livers of rats fed ad libitum (fed), fasted for 24 hr (fasted), or fasted for 48 hr and then refed a fat-free, high carbohydrate diet for 48 hr (refed) was studied. Hepatocytes were maintained as monolayer cultures in serum-free, lipid-free media and their fatty acid composition was analyzed at 3, 24, 48, 72 and 96 hr. The livers of fed animals, as well as their hepatocytes, contained less total lipid than those from animals on either of the other dietary regimes. Livers of fasted animals had three times the amount of lipid found in the livers of fed animals, and the livers of refed animals contained five times the amount of lipid as the livers of fed animals (all based on mg lipid/g wet weight of liver). The fatty acid composition of hepatocytes after 3 hr of culturing was very similar to that of fresh liver when compared in each of the dietary regimes. However, while the fatty acid compositions of livers and hepatocytes from fed and fasted animals were similar, the pattern in liver of refed animals was quite distinct from that of the fed animals. In the fed and fasted animals palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1[n-9]), linoleic acid (18:2[n-6]) and arachidonic acid (20:4[n-6]) were the major fatty acids of the liver; in refed animals 16:0, palmitoleic acid (16:1[n-7]), 18:0, 18:1(n-9) and *cis*-vaccenic acid (the n-7 isomer of oleic acid) were the major fatty acids. During maintenance in culture the 18:1(n-9) content of the hepatocytes increased in cells from livers of animals on all three dietary regimes. The polyunsaturated fatty acid content was similar in fresh livers and isolated hepatocytes in all samples when compared on the basis of μg fatty acid/mg of hepatocyte or liver protein. It was also found that the polyunsaturated fatty acid content of hepatocytes was remarkedly stable with time of culture when the cells were incubated in serum-free, lipid-free medium. Thus, isolated hepatocytes maintained in serum-free medium appear to be a possible system for the evaluation of the effects of prior nutritional status on fatty acid metabolism in the whole animal, not subject to hormonal and other somatic influences which often complicate the interpretation of such nutritional studies.
Lipids 21, 454–459 (1986).

The liver is the primary organ for the metabolism of dietary fatty acids (1–3). It also is well established that dietary fatty acids repress endogeneous fatty acid synthesis in the liver, while fat-free, high carbohydrate diets stimulate fatty acid synthesis (4–7). Short term fasting in rats increases liver lipid content (8,9) while decreasing total liver mass and liver glycogen. The mechanisms of repression, induction and fat mobilization have not yet been completely elucidated (3,4,7).

Hepatocytes can be isolated from rat liver and maintained in serum-free media for several days (10). After a recuperative period of variable length depending on treatment and culture media, the cells recover many of their characteristic enzyme activities and cellular functions (11). In this work, the effects of either feeding ad libitum, fasting for 24 hr, or fasting for 48 hr and then refeeding a high carbohydrate, fat-free diet (referred to as fed, fasted and refed animals, respectively) on the fatty acid composition of fresh whole liver and isolated hepatocytes maintained in serum- and lipid-free medium for 96 hr was studied. The objective of this research was to determine the effect of prior nutritional status of the animals on the fatty acid composition of the hepatocytes in culture. It was also of interest to observe how the polyunsaturated fatty acids are affected in apparently healthy cells maintained in medium devoid of these essential nutrients. It was suspected that they must be conserved under these conditions.

MATERIALS AND METHODS

Materials and animals. Male Sprague-Dawley rats (100–150 g) were obtained from Bantin and Kingman (Fremont, California). They were fed laboratory chow obtained from Ralston-Purina (Richmond, Indiana). Waymouth's MB 752/1 and Swim's S-77 media were purchased from GIBCO (Santa Clara, California). Dexamethasone sodium phosphate and crystalline pig insulin were gifts of K. Bohra (Organon, West Orange, New Jersey) and W. W. Bromer (Eli Lilly and Co., Indianapolis, Indiana), respectively. Triiodothyronine and BSA Fr V (essentially fatty acid-free) were bought from Sigma Chemical Co. (St. Louis, Missouri). Collagenase type II was obtained from Worthington Biochemicals (Freehold, New Jersey). Purified fatty acid methyl ester reference standard mixtures were purchased from Nu-Chek-Prep (Elysian, Minnesota and Supelco Inc. (Bellefonte, Pennsylvania). Organic solvents were obtained from Burdick and Jackson (Muskegon, Michigan).

The animals were placed on one of three dietary regimes: 1, fed lab chow ad libitum; 2, fasted for 24 hr; 3, fasted for 48 hr, then refed a high carbohydrate diet for 48 hr. The high carbohydrate diet contained 5% glycerol, 30% egg albumin and 65% sucrose. The animals were maintained in individual cages with free access to water. The ambient temperature of the animal quarters was 25 C. The room was lighted between 6 a.m. and 6 p.m. Animals were anesthesized with nembutal between 8 and 9 a.m.; the livers were either removed immediately and extracted or perfused for isolation of hepatocytes as described below. Animals whose livers were perfused weighed 300–400 g at the time of perfusion. Animals whose livers were extracted directly weighed ca. 300 g.

Hepatocyte isolation and culture. Hepatocytes were isolated by the method of Berry and Friend (12) as modified by Bonney et al. (13) with the following additional changes. Livers in situ were perfused at 37 C with 300 ml of calcium-free Swim's S-77 medium containing BSA (1 mg/ml) at a flow rate of 25 ml/min. After

perfusing for 10 min with the calcium-free medium, calcium (3 mM) and collagenase (0.5 mg/ml) were included in the perfusion medium. The collagenase-containing medium was recirculated through the liver while the calcium-free medium was not. The liver cells were dispersed after 7-8 min perfusion with the collagenase-containing medium. Liver cells were collected in calcium-free Swim's S-77 medium, treated with DNase (10μg/ml) and then cooled, after which trypsin inhibitor was added to the cell suspension as previously reported by Kelley and Potter (14). Hepatocytes were isolated by filtration and centrifugation as previously described (15). Hepatocytes were suspended (2 million/ml) in Waymouth's MB 752/1 medium supplemented with insulin (2×10^{-7} M), dexamethasone (10^{-6} M), triiodothyronine (10^{-6} M), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin (20 μg/ml) and defatted bovine serum albumin, fraction V (0.5 mg/ml). The cell suspension was inoculated (6 ml/dish) in 100-mm collagen-coated culture dishes, maintained at 37 C in an atmosphere of 95% air and 5% CO_2. Medium was initially changed at 3 hr after plating and then every 24 hr. The Waymouth's medium was aspirated, cells were washed four times with Hank's Hepes BSS and then cells from each culture dish were scraped into 5 ml of H_2O containing hydroquinone (20 μg/ml).

Lipid extraction. The lipid extractions were performed as described previously (16,17) with some modifications. Briefly, the samples, either washed hepatocyte suspension or homogenized fresh liver, were first lyophilized to remove water. The freeze-dried samples were extracted with $CHCl_3$/MeOH (2:1, v/v). The extracts then were filtered to remove insoluble protein, inorganic salts and carbohydrates.

The extraction solvents were removed by evaporation with dry N_2 at 40 C. The total lipid extracts were further purified by redissolving in dry chloroform, refiltering, evaporating and drying in a vacuum dessicator. Then they were weighed and either prepared for transmethylation or stored at low temperature (−20 C) until analyzed further.

Transmethylation. The total lipid extracts were transmethylated directly without prior separation of the various lipid classes. The samples were placed in 15 × 150 mm screw cap culture tubes fitted with reflux condensers (18). Five ml of 7% methanolic-HCl was added to each tube which was then heated to between 85 and 90 C for 2 hr. The tubes were cooled to room temperature and 10 ml of redistilled H_2O was added to each tube. The fatty acid methyl esters (FAME) then were extracted by three washings of the aqueous phase with 3 ml of hexane. The hexane washes were combined and dried over $NaHCO_3/Na_2SO_4$ (4:1, w/w) for 1 hr and evaporated to dryness at 40 C. The FAME then were transferred to vials which had been previously tared. The weights of FAME were determined and the samples diluted to an appropriate concentration in hexane and prepared for analysis by gas liquid chromatography (GLC).

GLC. The fatty acid methyl ester samples were analyzed on fused silica, wall-coated capillary column, 0.025 mm I.D. × 30 m long. The columns, coated with SP-2340, were obtained from Supelco Inc. Samples were run on either a Hewlett-Packard 5880A gas chromatograph with an attached Hewlett-Packard automated data station or a Perkin-Elmer Sigma 2000 gas chromatograph coupled to a Perkin-Elmer 7500 computer with a Chrom 3 data analysis program.

The column conditions were as follows: The carrier gas was helium at 14.5 psi inlet pressure. The samples were injected in 1 μl of hexane at a split ratio of 50:1. The FAME were separated on the column using a temperature program from 150 to 190 C. The first ramp was 2 C/min for 10 min to 170 C, with a hold at that temperature for 5 min. The second ramp was 5 C/min for 4 min to 190 C, with a hold at that temperature for 15 min. The FAME were tentatively identified by comparison of their retention times (RT) to RT of reference standards obtained from either Nu-Chek-Prep or Supelco Inc. The GLC detector was a hydrogen flame ionization detector.

The quantitative accuracy of the GLC procedures was evaluated by using either purified single FAME or reference mixtures, and the reference FAME or mixtures were selected to cover the range of fatty acid methyl esters present in the samples. Quantitative reference standards (either single FAME standards or mixtures) were prepared in the concentration ranges expected for the FAME mixtures in the natural samples. Accuracy of the quantitation was estimated to be within 5% for major components in the samples (those present in amounts greater than 10% of total FAME in the sample) and within 10% for components present in less than 10% of total FAME in the sample.

It should be noted that the cholesterol extracted into the hexane washes of the transmethylation mixture was not separated from the FAME prior to injection of the samples in the chromatograph. However, free cholesterol does not elute as a discrete peak from a SP-2340 column operated under the conditions described here. Hence, there is no interference in FAME analysis either quantitatively or qualitatively.

The percentage compositions of FAME in the rat hepatocyte samples were calculated as wt % transformed from the area percentages in the chromatogram. The transformation was accomplished by using a response factor for each individual FAME calculated from purified reference standards and the calibration procedures provided by the instrument manufacturers. Generally, the flame ionization detector is quite linear in response over the range of FAME found in the samples with respect to both the mass of compound and its RT, particularly for the sample sizes used with capillary columns (19,20).

Only the major fatty acids are listed individually in Tables 2 and 3 as the capillary GLC analytical system used to collect this data yielded between 40 and 80 discrete peaks depending on the load of sample placed on the column. Usually individual minor peaks contributed less than 0.1% to the total area of the chromatogram, and many were unidentified. In a typical analysis, 20 identified components comprised 97-98% of the total fatty acid present, while 20 to 40 unidentified components accounted for the remaining 2-3%. Thus, to simply the presentation of the data, the 10 fatty acids identified in Tables 2 and 3 are the only compounds for which individual data are presented. The residue is collectively presented as "sum of trace components" and varies from ca. 5-9% of the total fatty acids present. Both identified and unidentified components are grouped together in this category.

The data in Figures 1, 2 and 3 are presented in terms of μg fatty acids/mg cellular protein because this allows the amount of each fatty acid to be compared in absolute terms rather than the normalized values given by a wt % calculation. Proteins were analyzed by the method of Lowry et al. (21).

RESULTS

Table 1 gives data for the content of lipids and total fatty acids in the livers of rats in the three nutritional states studied in this work. After fasting for 24 hr the liver had lost 1/3 (5–6 g) of its wet weight. Livers from rats fasted for 48 hr and then refed a fat-free diet for 48 hr regained 2 to 3 g of wet weight. However, both the fasted and refed animals had a larger percentage of their liver weight as lipid than the fed animals when results were compared as percentage of wet weight or μg lipid/mg of liver protein. Under our experimental conditions, mg protein/g wet weight of liver did not change significantly among the three dietary groups of rats. These values were 149.8 ± 14.4, 164.3 ± 8.0 and 150.8 ± 20.5 for the fed, fasted and refed rats, respectively.

Fatty acids made up ca. 32% of the total liver lipid in fed animals and 24% in the fasted animals, while in the refed animals fatty acids were over 45% of the total lipid fraction. The phospholipids comprised ca. 14, 46 and 61% of the total lipid in the liver of refed, fed and starved animals, respectively.

Table 2 gives the fatty acid composition of fresh whole liver lipids from rats on the three dietary regimes. In the fed animals the five fatty acids, palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1[n-9]), linoleic acid (18:2[n-6]) and arachidonic acid (20:4[n-6]), made up ca. 80% of total fatty acids, while docosahexaenoic acid (22:6[n-3]) accounted for almost 8% of the total fatty acids.

TABLE 1

Effect of Dietary Status on Lipid Content of Rat Liver

Dietary status of animals	Weight of fresh liver (g)	Wt % lipid[a]	% Phospholipid of total lipid[b]	μg Fatty acid / mg Protein[c]	Wt % fatty acid of total lipid[d]
Ad lib fed	15.97 ± 1.95	3.67 ± 0.70	45.8 ± 8.0	65.85 ± 21.28	31.7 ± 2.7
24-hr fasted	9.69 ± 0.71	4.69 ± 1.15	60.5 ± 9.3	163.37 ± 69.57	24.2 ± 6.6
48-hr fasted, 48-hr refed fat-free diet	12.13 ± 2.53	5.59 ± 0.51	13.5 ± 4.5	245.77 ± 47.58	45.4 ± 3.6

Data shown are averages ± S.D. (n = 3 for each treatment).

[a]Both starved and refed group significantly different than fed, $P < 0.01$.

[b]Calculated using μg phosphorus time 25. Both fed and starved group significantly different than refed, $P < 0.05$.

[c]Calculated from GLC mass areas and Lowry Protein determination. Refed group significantly different than fed, $P < 0.05$.

[d]Calculated from GLC mass areas and initial gravimetric wt of total lipid extract. All three groups significantly different from each other, $P < 0.01$.

TABLE 2

Fatty Acid Composition of Whole Rat Liver as Affected by Dietary Status

	Ad libitum-fed		24-Hr fasted		48-Hr fasted, 48-hr refed fat-free diet	
Fatty acid	Wt %	μg FA/mg protein	Wt %	μg FA/mg protein	Wt %	μg FA/mg protein
14:0	0.34 ± 0.17	0.22 ± 0.11	0.26 ± 0.03	0.42 ± 0.05	1.25 ± 0.39*	3.07 ± 0.96*
16:0	21.06 ± 0.75*	13.87 ± 0.49*	16.30 ± 0.79*	26.63 ± 1.29	32.53 ± 3.07*	79.95 ± 7.55*
16:1(n-9)	0.33 ± 0.09*	0.22 ± 0.06*	0.18 ± 0.04*	0.29 ± 0.07*	0.75 ± 0.09*	0.61 ± 0.22*
16:1(n-7)	1.08 ± 0.17*	0.71 ± 0.11*	0.60 ± 0.05*	0.98 ± 0.08*	15.39 ± 1.85*	37.82 ± 4.55*
18:0	14.89 ± 1.99*	9.81 ± 1.31*	21.03 ± 0.31*	34.36 ± 0.51*	3.51 ± 0.52*	8.63 ± 1.28*
18:1(n-9)	11.76 ± 1.88*	7.74 ± 1.29*	6.00 ± 0.25*	9.80 ± 0.41*	28.30 ± 1.03*	69.55 ± 2.53*
18:1(n-7)	2.77 ± 0.13*	1.82 ± 0.09*	1.76 ± 0.10*	2.88 ± 0.16*	6.70 ± 0.68*	16.47 ± 1.67*
18:2(n-6)	16.80 ± 0.26*	11.86 ± 0.17*	15.02 ± 0.64*	24.54 ± 1.05*	2.39 ± 1.56*	5.87 ± 3.83*
20:4(n-6)	14.22 ± 0.36*	9.36 ± 0.24*	21.44 ± 0.50*	35.03 ± 0.82*	2.89 ± 1.17*	7.10 ± 2.88*
22:6(n-3)	7.68 ± 1.06	5.86 ± 0.70	8.42 ± 1.28	13.76 ± 2.09	1.31 ± 0.52*	3.22 ± 1.28*
Sum of trace components	9.00 ± 0.36	5.73 ± 0.24	8.68 ± 0.61	14.18 ± 1.00	4.57 ± 0.84*	11.23 ± 2.06

Fatty acid designated by chain length, number of double bonds and the position of the first double bond from the methylene end of the molecule. Data shown are the average ± S.D. (n = 3 for each treatment).

*Concentration of fatty acids in the fed and starved groups indicated by asterisks are significantly different at $P < 0.05$, and both are significantly different from the corresponding values in the refed group by at least $P < 0.01$.

The fatty acid pattern was markedly different in livers of the refed animal compared to that in livers of fed or fasted rats. The percentage of palmitic, palmitoleic acid (16:1[n-7]) and oleic acid was elevated in liver of refed rats and accounted for over 75% of the total fatty acids present, but stearic acid and the polyunsaturated fatty acids were noticeably decreased and accounted for ca. 10% of total fatty acids collectively.

Table 3 gives the distribution of fatty acid in hepatocytes which were isolated from the livers of rats on the three different dietary regimes and maintained in the described culture medium for 3 hr. The fatty acid patterns of the isolated hepatocytes were quite similar to that found in the corresponding fresh intact livers; the cells from fed and fasted animals had a similar fatty acid composition which was distinctly different from the composition found in the refed animal.

While the experiments reported here were not designed to measure secretion of fatty acids into the medium, in a few instances all medium was collected and pooled from each change of medium and analyzed for total fatty acid content. Significant amounts of fatty acids could not be detected in medium used to culture cells from fasted or refed animals (detection limits about 5% of the cellular fatty acid content). Medium collected for 96 hr from cultured cells from fed animals had a total fatty acid content at the level of 10% of the cellular fatty acid content. The major fatty acids present in the medium were palmitic, palmitoleic, stearic and oleic. Thus, cellular fatty acid content or composition would not be markedly altered by secretion of fatty acids into the culture medium in these series of experiments.

Figure 1 shows the concentration of six major fatty acids for hepatocytes isolated from the liver of a fed rat and analyzed at five different times in culture. It was only when the data were analyzed in terms of μg fatty acid/mg cellular protein that the constancy of the concentration of polyunsaturated fatty acids in the cell with time in the medium became readily apparent. Conversely, the saturated and monounsaturated fatty acid concentration in the cells was altered significantly with time in culture. As with the whole liver, we could detect no significant changes in the average protein content per hepatocyte during the time course of this experiment; thus, the μg fatty acid/mg cellular protein is an accurate reflection of amount of fatty acid per cell in these experiments.

Figure 2 gives time-dependent compositional information on the identical fatty acids from the hepatocytes isolated from livers of fasted animals and maintained in culture for 96 hr. The changes parallel those observed in the cells from fed animals (Fig. 1) with only minor variations. Figure 3 shows the data for these fatty acids in hepatocytes isolated from the liver of refed animals and maintained in culture for 96 hr. In these cells the pattern of the changes in the cellular fatty acid concentration with time in culture was strikingly different from those of either the fed or fasted animals. The concentration of palmitic acid decreased significantly while the concentration of stearic and oleic acids increased and the concentration of polyunsaturated fatty acids appeared relatively unaltered.

DISCUSSION

The liver weights and lipid content of the livers reported here are consistent with literature values (8,22,23) for normal animals of the age and size used in this work. A 24-hr fast decreased the total liver wet weight by one-third, presumably by the loss of liver glycogen (24,25), while refeeding a fat-free diet high in carbohydrate for 48 hr restored some of the wet weight of the liver by replacing some of the liver glycogen and increasing the triglyceride content of the liver (6–8). It is interesting, however, that in the liver of fasted animals, the total lipid as well as the phospholipid content was higher than the corresponding values in the liver of fed animals, even when corrected for the decrease in the weight of the liver in the starved animals.

Even though de novo fatty acid synthesis is repressed in fasted animals (4,5), phospholipid synthesis or turnover

TABLE 3

Fatty Acid Composition of 3-Hr Cultured Hepatocytes as Affected by Prior Nutritional Status of Intact Animal

	Ad libitum-fed		24-Hr fasted		48-Hr fasted, 48-hr refed fat-free diet	
Fatty acid	Wt %	μg FA/mg protein	Wt %	μg FA/mg protein	Wt %	μg FA/mg protein
14:0	0.69 ± 0.10*	0.67 ± 0.09*	0.42 ± 0.13*	0.64 ± 0.20*	1.57 ± 0.05	5.62 ± 0.18*
16:0	21.30 ± 0.37*	20.87 ± 0.36*	17.58 ± 0.78*	26.90 ± 1.19*	28.28 ± 0.41	101.27 ± 1.47*
16:1(n-9)	0.41 ± 0.07*	0.40 ± 0.07*	0.23 ± 0.07*	0.35 ± 0.11*	0.65 ± 0.05	2.33 ± 0.18*
16:1(n-7)	1.69 ± 0.09*	1.66 ± 0.09*	0.65 ± 0.13*	0.99 ± 0.20*	13.02 ± 0.21	46.62 ± 0.75*
18:0	12.99 ± 0.22*	12.73 ± 0.22*	20.60 ± 1.54*	31.52 ± 2.36*	4.29 ± 0.33	15.36 ± 1.18*
18:1(n-9)	11.76 ± 0.23*	11.52 ± 0.22*	6.11 ± 0.46*	9.35 ± 0.70*	30.18 ± 1.19	108.07 ± 4.26*
18:1(n-7)	4.45 ± 0.05*	4.36 ± 0.05*	2.26 ± 0.17*	3.46 ± 0.26*	7.37 ± 0.13	26.39 ± 0.47*
18:2(n-6)	18.12 ± 0.21*	17.76 ± 0.21*	14.76 ± 0.50*	22.58 ± 0.77*	4.34 ± 0.17	15.54 ± 0.61*
20:4(n-6)	14.24 ± 0.27*	13.96 ± 0.26*	22.04 ± 1.22*	33.72 ± 1.87*	4.77 ± 0.44	17.08 ± 1.58*
22:6(n-3)	4.92 ± 0.28*	4.82 ± 0.27*	7.00 ± 0.34*	10.71 ± 0.52*	1.62 ± 0.16	5.80 ± 0.57*
Sum of trace components	9.42 ± 0.55	8.25 ± 0.54	7.72 ± 1.23*	11.81 ± 1.88	3.92 ± 1.11	14.04 ± 3.97

Data shown are the average ± S.D. (n = 4 for each treatment).

*Concentration of fatty acids in the fed and starved groups indicated by asterisks are significantly different at $P < 0.05$, and both are significantly different from the corresponding values in the refed group by at least $P < 0.01$.

presumably continues using fatty acids mobilized from peripheral tissues and adipose stores (8,26,27). The livers of animals refed a fat-free diet exhibit very active synthesis of fatty acids (6,7) which are rapidly incorporated into triglycerides (28–30). This concept is consistent with data in Table 1 which indicates that the wt % of fatty acids of the total lipid in the liver was highest in refed animals and lowest in the fasted animal. Apparently fasted animals rapidly hydrolyze any liver triglyceride for energy or convert it to phospholipid (27,28).

The data reported here on the fatty acid pattern of whole rat liver is consistent with that reported previously (31–35). There was little difference between the overall pattern found in the livers of fed and fasted animals. Fatty acid synthesis is inhibited in livers of the fasted animals and existing triglyceride fatty acids are converted to carbohydrate or phospholipids (28). However, chain elongation and some desaturation may still be occurring in the liver in both fed and fasted animals (36,37).

The liver from refed rats showed a very different fatty acid pattern relative to the wt % distribution of the major fatty acids. Palmitate, palmitoleate, oleate and *cis*-vaccenic acids (the n-7 isomer of oleic acid) were the predominant species present. The increase in palmitoleate was particularly striking as it rose from about 1% in the livers of fed animals to over 15% in the livers of refed animals. Almost equally dramatic was the decrease in the polyunsaturated fatty acids, linoleate, arachidonate and docosahexaenoate, which all decreased by a factor of 5 or more. This marked change in fatty acid pattern in the whole liver between fed and refed animals has been observed by others (38–41).

These differences between the refed and the fed or fasted animals were probably caused by the rapid synthesis of saturated and monounsaturated fatty acids in the liver of the refed animals (3,28,40,41), which were receiving a fat-free, high carbohydrate diet. As no polyunsaturated fatty acids were present in the diet of these animals, saturated and monounsaturated fatty acids increased in their liver due to de novo synthesis, while the amount of the polyunsaturated fatty acids was constant in terms of μg fatty acid/mg protein and decreased in percentage of total fatty acids.

Several metabolic processes in hepatocytes are initially disturbed by their isolation and maintenance in culture; however, most of these functions recover by 24 hr (11,42). The fatty acid composition of the cells at 3 hr after plating (Table 3) resembled the fatty acid composition of the corresponding whole liver (Table 2). Various changes in the cellular fatty acid composition occurred during 24–96 hr in culture. These changes indicate that there was vigorous metabolic activity related to fatty acid lipogenesis in cultured hepatocytes. This supposition is supported by

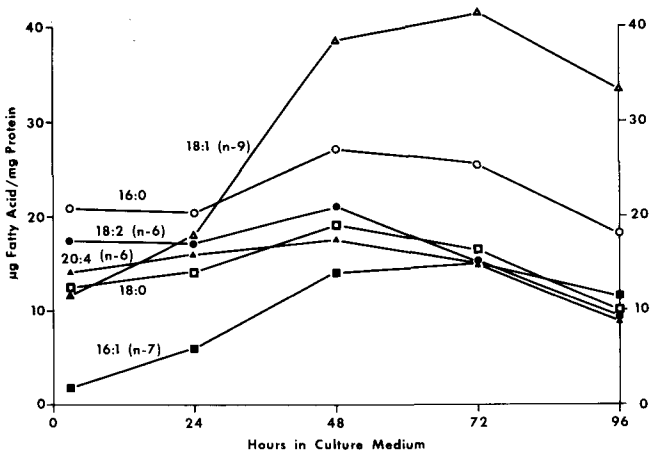

FIG. 1. Individual fatty acid content in μg fatty acid per mg cellular protein for six major fatty acids of rat hepatocytes isolated from livers of ad libitum-fed rats. The cells were maintained in a lipid-free, serum-free, high carbohydrate medium for 96 hr. Each point represents an average value from four experiments. Standard deviations are not shown to avoid clutter on figure, but they were generally less than 10% of the mean value. Fatty acids are designated by chain length, number of double bonds, and the position of first double bond from methylene end of the chain. In comparing the 3-hr and 96-hr points, all values for the fatty acids are significantly different ($P < 0.01$) except for 18:0, which is not.

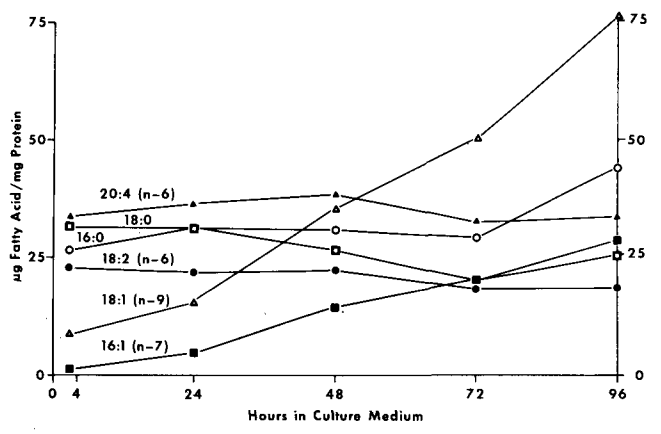

FIG. 2. Individual fatty acid content in μg fatty acid per mg cellular protein for six major fatty acids of rat hepatocytes isolated from livers of rats fasted for 24 hr. In comparing the 3-hr and 96-hr points, values for 16:0, 16:1, 18:0 and 18:1 are significantly different ($P < 0.05$); 18:2 and 20:4 values are not. Refer to the legend for Fig. 1 for additional details and explanation of shorthand designations of fatty acids.

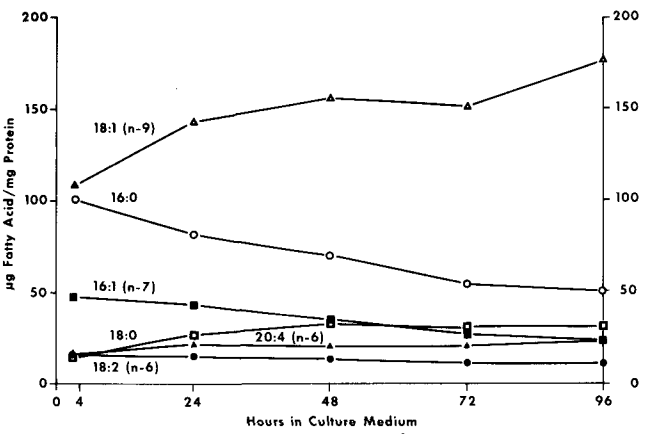

FIG. 3. Individual fatty acid content in μg fatty acid per mg cellular protein for six major fatty acids of rat hepatocytes isolated from livers of rats starved for 48 hr, then refed for 48 hr a high carbohydrate, fat-free diet. In comparing the 3-hr and 96-hr points, values for the fatty acid content of the cells are significantly different for 16:0, 16:1 and 18:1 ($P < 0.001$) but not for 18:0, 18:2 and 20:4. Refer to the legend for Fig. 1 for additional details and explanation of shorthand designations of fatty acids.

our data for the activity of several lipogenic enzymes in these cells (43).

To our knowledge there are no previous reports on the fatty acid composition of primary cultures of hepatocytes maintained for 96 hr in serum-free medium, nor are there any reports on the fatty acid composition of hepatocytes cultured from rats fed high carbohydrate, fat-free diets prior to isolation of the cells. Previous reports (32,40,42, 44,45) on the fatty acid composition of cultured hepatocytes were mainly for hepatoma cell lines rather than primary hepatocyte cultures; however, our data obtained with primary cultures are consistent with the earlier reported values for these cultures.

When hepatocytes were maintained in culture, de novo synthesis of fatty acids occurred primarily in the cells from fed and fasted animals (Kelley et al., in preparation). Where there was de novo synthesis of fatty acids, the content of palmitic acid in the cells remained constant (fasted animals) or increased (fed animals). Conversely, in the cells from the refed animals, the palmitic acid content decreased continuously with time in culture, presumably because it was being elongated to stearic acid which in turn was desaturated to oleic acid (36,37). A similar hypothesis can be constructed to explain the changes in the other saturated and monounsaturated fatty acids for which data are presented in Figures 1, 2 and 3. It is particularly interesting to note the relation between palmitic, palmitoleic and *cis*-vaccenic acids. These fatty acids appear to have a metabolic pathway somewhat independent of oleic acid. Indeed, in many reports, the *cis*-vaccenic acid content of mammalian tissue is ignored, assumed to be trivial or lumped with oleic acid; these are all unwarranted assumptions, in our opinion, as it appears *cis*-vaccenic is a major fatty acid at least in rat liver.

Our results appear to indicate that active synthesis of saturated and monounsaturated fatty acids occurred during maintenance of primary cultures of rat hepatocytes for 96 hr while polyunsaturated fatty acids were conserved during this time. This observation does not rule out subtle changes in the polyunsaturated fatty acid metabolism, such as conversion of linoleate to arachidonate at a level below that detectable with the techniques used in this work. Thus, maintenance of hepatocytes in serum-free, lipid-free medium offers an opportunity to study fatty acid metabolism in a system unperturbed by both hormonal agents and extraneous lipids associated with heterologous serum.

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Glycerolipid Biosynthesis in Rat Adipose Tissue. Influence of Age and Cell Size on Substrate Utilization[1]

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The conversion of [^{14}C]-labeled compounds such as acetate, glucose, pyruvate and palmitate into CO_2, glyceride-glycerol, glyceride fatty acids and total lipids was monitored in the average and matching adipocyte (with respect to size) preparations from young (6–9 wk) and old (age 56–60 wk) male Sprague-Dawley rats. The average cell size populations from young and old rats were 46 ± 3 and 83 ± 11 μm in diameter, respectively. The incorporation of [^{14}C]acetate, pyruvate and glucose into fatty acids was significantly reduced in the adipocytes from older rats, irrespective of their sizes. The production of CO_2 and glyceride-glycerol did not change significantly as a function of either cell size or animal age. Palmitate incorporation into lipids was similar in the average cell population derived from old and young rats, but it was considerably lower in the smaller adipocytes (46–50 μm diameter) from old animals. Irrespective of the cell size, triacylglycerol formation from *sn*-glycerol-3-phosphate was also significantly diminished in the adipocytes from older animals compared to younger ones as evidenced by decreases in activities of several enzymes, including *sn*-glycerol-3-phosphate acyltransferase, Mg^{2+}-dependent phosphatidate phosphohydrolase and diacylglycerol acyltransferase. However, triacylglycerol formation from monoacylglycerol did not change as a function of either cell size or age. These measurements of the metabolic and enzymic activities provide evidence that the synthesis of fatty acids from various precursors and triacylglycerol formation from *sn*-glycerol-3-phosphate are significantly reduced in adipocytes from older animals and that such changes occur independently of adipocyte size.
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Several metabolic functions of adipose tissue are subject to change with adipocyte size. These functions include glucose incorporation into lipid (1), lipoprotein lipase activity (2), lipolysis (3) and esterification of exogenously added fatty acids into lipid (1,4).

To study adipose metabolism as a function of cell size, two approaches are commonly used. In the first approach, various metabolic parameters of smaller adipocytes from young animals are measured and compared with larger adipocytes from older animals (1,5). In the second approach, adipocytes from the same donor are separated into different sizes before measuring various metabolic parameters (6–12). The second approach is preferable because, the source of adipocytes being the same, such measurements clearly reflect the changes as a function of cell size rather than the secondary changes caused by the differences in the hormonal, dietary and age-related conditions of the donors. By using the second approach, and by isolating adipocytes from age-matched lean and obese animals, studies from this and other laboratories (7–15) demonstrate that large adipocytes are relatively more active in glyceride-glycerol and fatty acid synthesis compared to smaller adipocytes. In contrast, recent studies by Francendese and DiGirolamo (16) and May (17) show that, in comparison with smaller adipocytes, the capacity of fatty acid synthesis from various precursors (glucose, pyruvate, acetate and lactate) is impaired in the larger adipocytes without any appreciable effect on the capacity to synthesize glyceride-glycerol. In these studies, however, smaller adipocytes were obtained from young rats (~6–8 wk old), whereas larger adipocytes were derived from older animals (8–12 mo old). Therefore, it is not presently clear whether these conflicting reports on the capacity of larger adipocytes to synthesize lipids relate to differences in the cell size as a result of different approaches used or simply reflect the age-related differences in adipose lipid metabolism.

Therefore, in the present investigation, we have used both of these approaches to investigate adipose lipid metabolism as a function of adipocyte size and age.

MATERIALS AND METHODS

Materials. sn-[U-^{14}C]Glycerol-3-phosphate (sp. radioactivity 20 mCi/mmol) and D-[U-^{14}C]glucose (sp. radioactivity 8 mCi/mmol) were purchased from ICN Chemicals and Radioisotope Division, (Irvine, California). [1,2-^{14}C]-Acetate (sp. radioactivity 96.8 mCi/mmol) and palmitoyl CoA [palmitoyl-1-^{14}C] (sp. radioactivity 58.5 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, Massachusetts). [1-^{14}C]Palmitic acid (sp. radioactivity 45.55 mCi/mmol) was obtained from Research Products International Corp. (Mt. Prospect, Illinois). Collagenase (218 units/mg of protein; lot 4194 44 N6783) was obtained from Worthington Corp. (Freehold, New Jersey). 2-Monoolein and 2-monooleyl ether were purchased from Serdary Research Laboratories Inc. (Port Huron, Michigan). Phosphatidate (Na salt) from egg lecithin and palmitoyl CoA were purchased from Sigma Chemical (St. Louis, Missouri). Most other chemicals were of analytical reagent grade quality and were purchased from the sources reported previously (18).

Male Sprague-Dawley rats were from our animal colony. Rats were placed in an air-conditioned animal facility maintained on a 12-hr dark (18-06 hr)/12-hr light (06-18 hr) schedule. Animals were fed laboratory chow (Ralston Purina Laboratory, St. Louis, Missouri). Rats were killed by decapitation between 8 and 10 a.m. Young rats were 6–9 wk old and the older animals were about 56–60 wk old.

Isolation of adipocytes and determination of cell size and number. Epididymal fat pads from 3–4 young rats and one old rat were used for each experiment to isolate adipocytes by the method of Rodbell (19) with slight modification (11). The resultant adipocytes were used for metabolic and enzymatic studies and for determination

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of adipocyte size and number. For cell size determination, isolated adipocytes were fixed in OsO_4 for 24–48 hr at 37 C (20). After processing (11,21), the cell diameter of the osmium-fixed cells was determined using a micrometer eyepiece (11).

To obtain adipocytes of different sizes from the same donor, adipocytes derived from young and old animals were filtered through 52 μm nylon screen (Telko Inc., Elmsford, New York) as described previously (11).

The rates of substrate utilization and enzymatic assays were expressed in relation to adipocyte number which was determined using 0.2 mm hemocytometer (11).

Metabolic studies. For metabolic studies, various adipocyte preparations (0.2–0.22 × 10^6 adipocytes/vial) were incubated (in duplicate or triplicate) in 20 ml plastic scintillation vials in the presence of 2 ml of Krebs-Ringer bicarbonate buffer containing 4% BSA. The incubation mixture contained either [^{14}C]acetate (0.13 μCi), [^{14}C]glucose (0.5 μCi), [^{14}C]pyruvate (0.2 μCi) or [^{14}C]palmitate (0.25 μCi). In addition to radioactive substrates, each incubation mixture contained nonradioactive substrate, such as acetate, glucose or pyruvate at 6 mM (final concentration), respectively. The final concentration of palmitate was 3 mM. Previous studies from various laboratories (10,16,17,22,23) demonstrate that the incorporation of pyruvate, acetate and palmitate into lipid is further stimulated in the presence of glucose. Therefore, in the vials that contained acetate, palmitate and pyruvate, 6 mM glucose was included. This concentration of various substrates was similar to that used earlier by Francendese and DiGirolamo (16) in their studies. The contents in the vials were incubated in a shaking water bath at 37 C with O_2/CO_2 (19:1) as the gas phase. Under these conditions, incorporation of radioactive substrates into lipids was linear with time up to 2 hr.

In the standard assay, the incubation was continued for 1.5 hr. At the end of incubation, CO_2 was released by injecting 0.25 ml of 0.5 M-H_2SO_4 and collected as described by Rodbell (19). Adipocyte lipids along with the medium were extracted with chloroform/methanol (2:1, v/v) and purified as described by Folch et al. (24). One portion (100 μl) of the lipid sample was counted for radioactivity to measure incorporation of label into total lipids. Another portion was subjected to saponification with 0.5 M-NaOH at 90 C for 1 hr. The samples were acidified by the addition of 0.5 ml of 12 M HCl and diluted with 1.5 ml of water. Fatty acids were extracted with 2 × 4 ml of petroleum ether (bp 30–60 C). The ether extracts were combined, dried under N_2 and processed to determine incorporation into fatty acids. The radioactivity in the glyceride-glycerol fraction was estimated by the difference between the total lipid radioactivity minus the radioactivity present in the fatty acid fraction.

To study [^{14}C]palmitate incorporation into lipids, the dry lipid samples obtained by the Folch procedure (24) were resuspended in 5 ml heptane. The heptane suspensions were washed 3× with 5 ml of 0.05 M sodium bicarbonate dissolved in ethanol/water (1:1, v/v) to remove unincorporated [^{14}C]palmitate (10). The heptane extracts were dried and mixed in 10 ml of scintillation fluid and counted.

Preparation of adipocyte homogenates and enzyme assays. Adipocytes isolated from various sources were homogenized in 2–3 ml of cold Medium A (0.25 M Sucrose, 1 mM Tris, pH 7.5, 1 mM EDTA and 1 mM DTT) with a Teckmar-tissuemizer (10N shaft; Teckmar Co., Cincinnati, Ohio) at speed 5 for 30 sec at 4 C. The resultant suspension was centrifuged at 2500 rev/min for 15 min in a refrigerated centrifuge (International Equipment Co., model B20-A) to prepare cell-free homogenates as described previously (11). After protein determination (25), adipocyte homogenates were used for various enzyme assays.

sn-Glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15), phosphatidate phosphohydrolase (PPH; EC 3.1.3.4) and diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) were assayed as described previously (15,18,27).

The acylation of *sn*-glycerol-3-phosphate was studied in the presence of palmitate, ATP, CoA and Mg^{2+} (18). In a final volume of 0.75 ml, the reaction mixture contained 24 mM Tris/HCl buffer, pH 7.5, 50 mM KCl, 0.84 mM *sn*-glycerol-3-phosphate, 0.1 μCi *sn*-[1,3-^{14}C]glycerol-3-phosphate, 0.7 mM dithiothreitol, 1.05 mM ammonium palmitate, 3 mM ATP, 3.6 mM $MgCl_2$, 0.01 mM CoA and 1.25 mg of fatty acid-poor albumin. The reaction was started with 0.1–0.3 ml of enzyme containing 100–140 μg of homogenate protein. Incubation was under air at 37 C in a shaking water bath. The reaction was linear with time for 20 min when fat-free homogenate was used as the enzyme source. Incorporation of [^{14}C]glycerol-3-phosphate into total lipids (phospholipids [phosphatidate and lysophosphatidate] diacylglycerol and triacylglycerol) was taken as a measure of enzyme activity.

Mg^{2+}-dependent phosphatidate phosphohydrolase was assayed in the presence of aqueous-dispersed substrate as previously described (27) and the release of P_i was taken as a measure of phosphatidate phosphohydrolase activity. In a final volume of 1 ml, the reaction mixture contained 60 mM Tris/maleate buffer, pH 7.5, 1 mM DDT, 0.5 mM phosphatidate, 5 mM $MgCl_2$ and 0.4 mg of bovine serum albumin. The reaction was started by the addition of 100–140 μg of homogenate protein and was linear with time up to 30 min. After incubation at 37 C for 20 min, the reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid, and the protein-free filtrate was collected by centrifugation. A portion of this filtrate (1 ml) was used to measure P_i content. The difference in the phosphate content between experimental reaction and the reaction stopped at zero time was taken as a measure of phosphatidate phosphohydrolase activity.

1,2-Diacyl-*sn*-glycerol acyltransferase was measured in the presence of 1,2-diacyl-*sn*-glycerol dispersed in 0.1% Tween 20 (w/v) (15). The standard assay was in a final volume of 1 ml, containing 50 mM Tris/HCl, pH 7.5, 1.2 mM 1,2-diacyl-*sn*-glycerol, 0.25 mM dithiothreitol, 8.0 mM $MgCl_2$, 0.08 mM palmitoyl-CoA (containing 0.1 μCi of [^{14}C]palmitoyl-CoA) and 1.25 mg of fatty acid-poor albumin. The contents were incubated at 37 C. The reaction was started by adding 0.1 ml–0.3 ml of homogenate (100–140 μg of protein). The reaction was linear with time up to 20 min. The formation of [^{14}C]triacylglycerol was taken as a measure of the enzyme activity.

Monoacylglycerol acyltransferase (MGAT) was assayed in the presence of 2-monooleyl ether dispersed in 0.1% Tween 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 $MgCl_2$, 0.5 mM monooleyl ether, 0.75 mM

dithiothreitol, 65 μM [^{14}C]palmitoyl CoA (0.1 μCi) and 1.25 mg of bovine serum albumin. The reaction was started by the addition of adipocyte homogenate protein (100–140 μg). Incubation was carried out at 37 C and was linear with time for 8 min. The formation of [^{14}C]di- and triacylalkylglycerol was taken as a measure of enzyme activity. In the absence of monooleyl ether, very little (less than 5% of the total) incorporation of [^{14}C]palmitoyl CoA into diacylalkylglycerol and triacylalkylglycerol occurred. The proportion of di- and triacylalkylglycerol formed from monoalkylglycerol was 1:1. The enzyme reaction stopped at zero time was subtracted as blank.

Radiochemical analysis of lipids. The radioactive lipids formed during the reaction were extracted as described by van den Bosch and Vagelos (28) and dried under N_2. The dry lipids were dissolved in 0.5 ml of benzene. Samples were applied in a volume of 0.1 ml and separated on thin layer plates coated with Silica Gel G (E. Merck, Darmstadt, Federal Republic of Germany). The lipid classes were identified by appropriate standards in an adjacent lane.

The separation of phosphatidate was performed with the solvent system chloroform/methanol/3M-NH_3 (65:38:8, v/v/v) and chloroform/methanol/acetic acid/water (50:25:8:4, v/v/v/v) (29,30). Neutral lipids were separated with hexane/diethyl ether/acetic (73:25:2, v/v/v) (31). The lipids were located by exposure of the plates to I_2. After sublimation of I_2 at room temperature, appropriate areas from the plates were scraped directly into scintillation vials containing 10 ml of Liquifluor in toluene. The radioactivity was measured in a Beckman LS 1800 scintillation counter.

RESULTS AND DISCUSSION

Influence of age and cell size on utilization of various precursors. Table 1 shows the utilization of acetate, glucose, pyruvate and palmitate in different preparations of adipocytes derived from young and old animals. Acetate was mainly incorporated into CO_2 and fatty acid fractions, with no significant incorporation in the glyceride-glycerol fraction. As opposed to acetate, glucose and pyruvate were mainly converted into glyceride-glycerol fractions, with lesser incorporation into CO_2 and fatty acid fractions. A similar pattern of utilization of these molecules by adipocytes or adipose fragments was noted previously in many studies (16,17,22,23).

The average cell population from older rats was two times larger than that derived from young rats (A vs B preparations from Table 1). As noted earlier by Francendese and DiGirolamo (16) and May (17), the larger adipocytes from older rats showed diminished rates of fatty acid synthesis from various precursors compared to adipocytes from younger animals. However, this impairment in the synthesis of fatty acid was not limited only to larger adipocytes but was also observed in the smaller adipocytes from old rats (an adipocyte preparation from old rats similar in size present in young rats, A vs C preparation). Therefore, it is likely that the diminished capacity of fatty acid synthesis in older animals noted here and reported previously (16,17) is related to age rather than to cell size. The rates of glyceride-glycerol and CO_2 formation did not change with age as noted in other studies (16,17).

TABLE 1

Effect of Adipocyte Size and Animal Age on Substrate Utilization

Age (wks)	Body wt (g)	Adipose wt (g)	Cell population	Adipocyte size (μm)	Acetate			Glucose			Pyruvate			Palmitate	
					CO_2	GG	GFA	CO_2	GG	GFA	CO_2	GG	GFA	CO_2	Total lipid
6–8 (n=16)	199±32	0.92±0.28	A	46.5±3.41	9.8±3.5	—	10.07±1.9	24±6.9	179±33	12.4±3.1	96±7.34	165±45	68.9±19.9	23.15±11.65	158±25
56–60 (n=4)	531±39[a]	5.59±1.68[a]	B	83±11.0	6.73±1.18	—	0.90±0.44	21.5±6.47	86±14	0.67±0.2	105±15	131±15	3.95±1.37	21.42±12	159±24
			C	50.5±7.93	9.5±1.7		0.84±0.31	18.17±8.2	93±20	0.57±0.2	88±24	134±43	3.19±2.1	22.05±12.75	106±18
P value between	A and B			<.01	ns		<.005	ns	<.05	<.001	ns	ns	<.005	ns	ns
	A and C			ns	ns		<.01	ns	<.05	<.001	ns	ns	<.005	ns	<.05
	B and C			<.01	ns		ns	ns	ns	ns	ns	ns	ns	ns	<.05

Adipocytes were isolated from young and old animals. Each value is the mean ± SD from four different experiments. Rates of utilization of substrates are expressed as nmol/hr/10^6 adipocytes. CO_2, carbon dioxide; GG, glyceride-glycerol; GFA, glyceride fatty acids. A, average cell size population of young rats; B, average cell population of old rats. C, population of adipocytes obtained from old rats which is of similar size in young rats. The smaller adipocytes obtained from young rats were similar in size as in A, therefore, data are not reported. ns = Not significant.

[a]Body weights and adipose tissue weights of older rats are significantly different than young rats: $P < .001$.

TABLE 2

Effect of Adipocyte Size and Age on Glycerolipid-Synthesizing Enzymes

Age (wk)	Body wt (g)	Adipose wt (g)	Adipocyte preparations[b]	Adipocyte size (μm)	Enzyme activities (nmol/min/mg protein)[c] GPAT	PPH	DGAT	MGAT	Protein (mg/10^6 adipocytes)
6-9	210±40[a]	1.91±0.23[a]	A	52.8 ±1.5	22.27±2.9	61.9 ±3.31	15.15±6.20	2.3 ±0.16	0.34±0.11
			B	42.5 ±3.69	8.67±2.9	30.75±4.88	6.20±1.5	1.80±0.2	0.39±0.1
56-60	456±42	4.01±1.14	C	79.3 ±4	6.85±0.97	26.55±6.65	2.44±0.65	1.93±0.82	0.43±0.15
			D	46.75±2.5	2.53±2.0	6.54±3.03	2.54±1.2	0.90±0.65	0.21±0.02

Adipocyte homogenates from young and old rats were used to measure various enzyme activities. Each value is the mean ± SD from four different experiments using 25 young and 8 old male Sprague-Dawley rats.

[a]Body and adipose tissue weights from young rats were significantly different from old rats: $P < 0.005$. The level of significance for other values is given in the text.

[b]A, average adipocyte preparation from young rats; C, average adipocyte preparation isolated from old rats; B and D represent the population of adipocytes of similar sizes obtained from young and old rats, respectively.

[c]GPAT, glycerophosphate acyltransferase; PPH, Mg^{2+}-dependent phosphatidate phosphohydrolase; DGAT, diacylglycerol acyltransferase; MGAT, monoacylglycerol acyltransferase.

Palmitate incorporation into lipid was similar in the adipocytes derived from young and old rats, although adipocytes from older rats were two times larger than those from young rats (A and B preparations, Table 1). When similar cell size preparations from young and old rats were used (A and C preparations), the rates of lipid synthesis from palmitate were significantly lower in the adipocytes from older rats. It is apparent, therefore, that palmitate incorporation into lipid is influenced by both adipocyte size and age of the animal, as noted previously in other studies (10,12,32). A major portion (90% or more) of [^{14}C]palmitate was incorporated into triacylglycerol, with 5-8% in diacylglycerol and 1-2% in phospholipid fractions. This proportion of palmitate incorporation into different lipid fractions did not change significantly as a function of age (data not shown).

Palmitate incorporation into lipid could be influenced by a variety of factors, including substrate concentration, number of fatty acid transporters on the surface of adipocytes (33), labile cell associated fatty acid pool and activity and amount of the various esterifying enzymes. The present studies demonstrate that alteration in the rates of palmitate esterification which occurs as a result of changes in cell size and age may partly be mediated through changes in the activities of various esterifying enzymes (noted below).

Influence of age and cell size on triacylglycerol formation. Table 2 shows the effects of cell size and animal age on triacylglycerol formation from both *sn*-glycerol-3-phosphate and monoacylglycerol. As noted in the previous studies (11), the activities of *sn*-glycerol-3-phosphate acyltransferase, Mg^{2+}-dependent phosphatidate phosphohydrolase and diacylglycerol acyltransferase increased with increase in the cell size in young rats (A and B preparations, $P < 0.01$, Table 2). This effect of the cell size on enzyme activities was also apparent in the older animals, except for diacylglycerol acyltransferase, the activity of which did not increase with increase in cell size as observed in young animals (C and D preparations, Table 2, P for GPAT and PPH activities <0.01). When comparisons were made between young and old animals, the activities of various enzymes from *sn*-glycerol-3-phosphate pathway (GPAT, PPH and DGAT) were significantly reduced in the adipocytes from older rats, irrespective of their sizes (A and B vs C and D preparations, $P < .005$). The monoacylglycerol transferase did not change with either cell size or animal age. These measurements of the individual enzyme activities provide evidence that the entire pathway of esterification via *sn*-glycerol-3-phosphate is diminished in the adipocytes from older rats, without any effect on triacylglycerol formation from monoacylglycerol. This may mean greater contribution of monoacylglycerol pathway for the synthesis of triacylglycerol in older adipocytes.

Adipocyte homogenates incubated in the presence of [^{14}C]glycerol-3-phosphate, palmitate, ATP, CoA and Mg^{2+} formed phosphatidate, diacylglycerol and triacylglycerol as the major reaction products (Table 3). The proportion of the phosphatidate and triacylglycerol formed changed significantly with changes in the adipocyte size and animal age. The neutral lipid to phospholipid (NL/PL) ratio, an indicator of phosphatidate phosphohydrolase activity, decreased with both decreased cell size and with animal age. These changes in NL/PL ratio were consistent with changes in the Mg^{2+}-dependent phosphatidate phosphohydrolase measured directly (Table 2).

Although present studies clearly show that changes in triacylglycerol-forming enzymes from *sn*-glycerol-3-phosphate do occur in response to cell size and age, the underlying mechanism which brings about these changes remains unclear. Mixing experiments did not indicate the presence of an inhibitor of glycerolipid-forming enzymes in old animals. Therefore, alteration of the triacylglycerol-forming enzymes under these conditions may be related either to their concentrations or activity through changes in the concentration of various substrates and activators of triacylglycerol formation.

It is surprising to note that, in spite of this low potential for fatty acid and triacylglycerol synthesis from *sn*-glycerol-3-phosphate, the adipocytes from older rats continue to grow in size and accumulate lipid (34). Since lipoprotein lipase activity increases as the adipocyte

TABLE 3

Incorporation of [^{14}C]Glycerol-3-phosphate into Lipids as a Function of Adipocyte Size and Age

Age (wk)	Adipocyte sample	Adipocyte size (μm)	Distribution of αGP into			
			PL	DG	TG	NL/PL
6-9	A	52.8 ±1.5	56±5	8±2	36±5	0.78±0.14
	B	42.5 ±3.69	63±4	10±2	27±3	0.54±0.09
56-60	C	79.3 ±4	71±11	9±4	21±12	0.42±0.2
	D	46.75±2.5	87±7	5±3	7±5	0.14±0.08
P value between	A and B	<.05	<.05	ns	<.05	<.05
	A and C	<.005	<.05	ns	<.05	<.05
	A and D	<.005	<.001	ns	<.001	<.001
	B and C	<.001	ns	ns	ns	ns
	B and D	ns	<.001	ns	<.001	<.001
	C and D	<.005	<.05	ns	ns	<.05

sn-Glycerol-3-phosphate acyltransferase was assayed in the presence of [^{14}C]glycerol-3-phosphate, palmitate, ATP, CoA and Mg^{2+} by using adipocyte homogenates as an enzyme source. Rates of glycerolipid formation and other details of the experiment are given in Table 2. The relative distributions of [^{14}C]glycerol-3-phosphate into phospholipids (mainly phosphatidate, indicated as PL), diacylglycerol (DG) and triacylglycerol (TG) are given as percentages. Each value is the mean ± SD from four different experiments with assays carried out in duplicate. NL, sum of DG and TG. ns, Not significant.

enlarges with age (35), it is likely that the continued deposition of triacylglycerol occurs in these cells through the uptake of exogenous fatty acids formed by the action of lipoprotein lipase and their subsequent esterification with monoacylglycerol (formed during lipolysis and by lipoprotein lipase action) or with *sn*-glycerol-3-phosphate formed from different sources, such as glucose, pyruvate, lactate and glycerol (present studies,16,17,36). Thus, if the ample supply of substrates (fatty acids, *sn*-glycerol-3-phosphate and monoacylglycerol) is available, the existing potential for triacylglycerol formation, although low, is sufficient to maintain the continued deposition of triacylglycerol in the adipocytes from older animals. In conclusion, the present studies clearly demonstrate that the decreased capacity of fatty acid and triacylglycerol synthesis from *sn*-glycerol-3-phosphate pathway observed in the adipocytes of older rats is related to the age of the animal rather than to the cell size. The increased deposition of triacylglycerol in the large adipocytes is mediated through increasing supply of substrates for triacylglycerol formation and sustained activities of various enzymes involved in triacylglycerol formation from monoacylglycerol and *sn*-glycerol-3-phosphate.

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Distribution of Vitamins A and E in Blood and Liver of Rats Depleted of Vitamin A or Vitamin E

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Young Sprague-Dawley male rats (n = 150) were fed a semipurified diet, either without vitamin A (VA), without vitamin E (VE) or supplemented with both vitamins A and E (control). At the end of weeks 0, 1, 3, 5, 6, 7, 8 and 9, groups of rats were anesthetized with methoxyflurane, and blood was collected by cardiac puncture until the rat was exsanguinated. The liver was excised. Whole blood (WB) from each rat was fractionated into plasma (PLA), leukocytes (LEU), platelets (PLT) and erythrocytes (RBC). Each blood component was extracted with heptane and livers were extracted with $CHCl_3/CH_3OH$ (2:1, v/v). The extracts were analyzed for VA and VE by high performance liquid chromatography. The relationship among blood components in the loss of VA was PLT = LEU > WB > PLA. The relationship among blood components in the loss of VE was PLA > RBC > WB > LEU = PLT. VA and VE levels in other blood components decreased precipitously between weeks 0 and 4 in the animals placed on deficient diets. These results and correlation analyses between vitamin contents of blood components and of livers indicate inadequacies for the use of certain blood components as monitors of lipid-soluble vitamin status in the rat.

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Plasma levels, urine levels or hair assessed by usual static biochemical procedures are uncertain indicators of fat-soluble vitamin status or other nutrient deficiencies in individuals. During the past decade there has been an increasing awareness of the limitations of concentrations in serum to assess vitamin and micronutrient nutritional status (1–3). Reports continue to be published in which the only determinations made are of plasma, urine or perhaps hair. In pure diet-induced deficiency, for example, plasma value for zinc is an excellent indicator of the onset of negative zinc balance but will return to normal with only partial repletion of body zinc (2). However, in the context of disease or even altered normal physiology such as in pregnancy, plasma zinc does not appear to be an indicator of zinc status (2). Currently a low plasma zinc, as well as other nutrients (4), must be carefully considered in relation to the history, diet and physical findings in each patient. Measurement of such levels limits our interpretation of nutrient status because such levels are affected by a multitude of biological factors. Factors include the homeostatic regulation of circulating vitamins when reserves of a vitamin may be depleted before measureable change in circulating levels takes place.

In spite of the problems associated with the use of plasma levels of nutrients for assessment, peripheral blood is still the best material available from patients to study. This has caused investigators (4,5) to focus on cellular analyses to provide potentially a better understanding about the total body status of concentration. From blood, peripheral white blood cells may well be an alternative accessible body tissue that turns over rapidly and which can be isolated and/or cultured and subsequently tested for response to specific nutritional changes (5).

In an attempt to define the usefulness of isolated leukocytes or other blood cell components as suitable indices of vitamin A (VA) and/or vitamin E (VE) status we studied the temporal relationships of retinol, retinyl palmitate and α-tocopherol in rats fed VA- or VE-deficient diets. Correlations between various blood components and alterations in hepatic vitamin concentrations losses were determined.

MATERIALS AND METHODS

Animals and diets. One hundred-fifty young male Sprague-Dawley rats (Bantin-Kingman, Fremont, California), aged 30–35 days and weighing 90–125 g, were housed individually in stainless steel wire-bottom cages. The cages were suspended from racks that were equipped with an automatic watering system, and the animal room was maintained at 25 C, 65% relative humidity, with a 12-hr light-dark cycle (light from 7 a.m. to 7 p.m.). The animals were fed a standard stock diet (Rodent Chow, Ralston Purina Co., St. Louis, Missouri) for three days after arriving from the supplier. After the adjustment period, they were divided randomly into three groups and fed a semipurified AIN-76 diet (Dyets Inc., Bethlehem, Pennsylvania) with or without α-tocopherol or retinol, or other forms of VE (6) as shown in Table 1. Tap water was allowed ad libitum throughout the experiment. Body weights were measured two times each week throughout

TABLE 1

Composition of Semipurified Diet[a]

Ingredients	Percentage
Casein	20.0
DL-methionine	0.3
Cornstarch	15.0
Sucrose	50.0
Fiber (cellulose)	5.0
Corn oil	5.0
AIN mineral[b]	3.5
AIN vitamin[b]	1.0
Choline bitartrate	0.2

[a]Dietary constituents were obtained from Dyets Inc., Bethlehem, Pennsylvania.

[b]As specified by American Institute of Nutrition. Control diet contained (per kg) 8 mg of retinyl palmitate and 200 mg of dl-α-tocopheryl acetate.

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the study. Total food intake and food spillage were measured over a 24-hr period several times each week throughout the study.

Blood and liver preparation. Four rats were randomly selected from each dietary group at week 0, 1, 3, 5, 6, 7, 8 and 9 after starting the dietary regimen; the rats were weighed and anesthetized with methoxyflurane (Metofane, Pitman-Moore Inc., Washington Crossing, New Jersey). The thorax was opened, and blood was collected into heparinized tubes by cardiac puncture until the rat was exsanguinated. The liver was excised, cleaned, resected of extraneous material and weighed.

An aliquot of blood from each rat was used to determine hematocrit values. The remaining blood was used to isolate individual components. One ml of whole blood (WB) was set aside for total hematology evaluation.

Leukocyte separation. Leukocytes were isolated as previously described (7). Four ml of whole blood were carefully layered on 4 ml of a leukocyte separation mixture (2% methyl cellulose in 0.9% saline:33.9% sodium metrizoate, 16:10 parts) in a 15-ml graduated conical centrifuge tube. Separation was complete within 30 min, whereupon the upper layer, which contains leukocytes (LEU) and platelets (PLT), was removed and centrifuged in another conical centrifuge tube for 15 min at 1400 × g at 4 C. The LEU pellet was resuspended and homogenized in a known volume and extracted for vitamin analysis.

Plasma, platelet and erythrocyte isolation. Ten ml of whole blood were centrifuged in 16-ml plastic centrifuge tubes for 15 min at 150 × g at 4 C. The platelet-rich plasma (PRP) was transferred to another 16-ml plastic centrifuge tube and the buffy coat/erythrocyte (RBC) layer recentrifuged at 1400 × g for 15 min at 4 C. The plasma (PLA) was saved frozen for vitamin analysis and the lower layer used to isolate RBC. The PRP was recentrifuged at 150 × g for 15 min at 4 C, the pellet was discarded and the supernatant was centrifuged at 800 × g for 15 min at 4 C (8). The subsequent pellet of PLT was washed in phosphate buffer and resuspended for analysis.

For RBC, the buffy coat was removed and the remaining cells were washed three times with phosphate-buffered saline (PBS 150 mM NaCl in 5 mM phosphate, pH 8.0), resuspended and prepared for vitamin analysis. RBC, PLT and LEU counts were done by Coulter Counter, and protein analyses of various blood components were done by the procedure of Miller (9). Data for VE and VA concentrations were normalized per mg of protein for LEU and PLT and per ml for whole blood, RBC and PLA. Recovery and cross-contamination values were similar to those reported by others (8).

Liver extraction. Liver samples weighing between 0.5–1.0 g were excised from the right lobe of each liver to minimize interlobular variability in vitamin A distribution (10). Samples were then cooled on dry ice and minced with a razor blade on a glass plate over crushed ice. Minced liver was transferred to a glass culture tube, 18 × 150 mm, containing 10 ml of absolute ethanol, 0.1% BHT, and was homogenized with a Polytron (Brinkman Instruments Inc., Westbury, New York) using a PT-10 probe (speed no. 6) for 15 sec. Homogenate was poured into a 16 × 125 mm tube with Teflon-lined screw cap, the probe was rinsed twice for 5 sec with 5 ml ethanol/BHT, and the rinses were added to the homogenate.

Homogenate was centrifuged for 15 min at 4 C, 1300 × g, decanted into a 40-ml glass round-bottom centrifuge tube and evaporated in a vacuum centrifuge (Savant Instruments Inc., Hickville, New York). The pellet was extracted with 10 ml $CHCl_3/CH_3OH$ (2:1, v/v), 0.2% BHT by vortexing for 1 min and was centrifuged as above. The supernatant was combined with the ethanol layer for evaporation and the extraction was repeated once more. The combined residue was redissolved in 2 ml CH_3OH, filtered, and 25 μl was injected for analysis by high performance liquid chromatography (HPLC).

Vitamin A and vitamin E analyses. Analysis was by reversed-phase HPLC as described previously (6) with the following modifications: The system used a Microsorb™ (Rainin Instrument Co. Inc., Woburn, Massachusetts) C-18 15-cm column equipped with a cartridge-type guard column (Brownlee Labs, Santa Clara, California). PLA, WB and cellular fraction analyses were carried out isocratically with mobile phase of methanol/water (93:7, v/v), 1.0 ml/min ultraviolet detection at 292 nm. Liver extracts were analyzed on the same column with programmed mobile phase and flow rate changes as follows: methanol/water (90:10, v/v), 1.0 ml/min, changed to methanol/tetrahydrofuran/water (65:30:5, v/v/v), 1.5 ml/min at 11 min, controlled by a programmable pump (Model 590, Waters Associates, Milford, Massachusetts). Retinol, α-tocopherol and retinyl palmitate were eluted at ca. 9.10, 21.2 and 28.5 min, respectively. Quantitation was by peak area, and recovery was greater than 96% in all cases, as determined by vitamin addition.

Statistical analysis. Data were analyzed by analysis of variance procedures and regression evaluation. Each time point represents 3–4 animals. Logarithmic transformations were made to provide a better evaluation of data (11).

RESULTS

Figure 1 illustrates weekly body weight changes and food intake values. Body weights and food intakes were similar at the start of the experiment. Final body weights were significantly less ($P < 0.05$) between rats fed VA-deficient diets and those fed VE-deficient or control diets. Food intake was not significantly influenced by various diets devoid of VA or VE. There was no significant difference for hematocrit values between the three treatment groups. The largest decrease in VE among the three blood components was found in PLA. WB and RBC "seem" to maintain their VE. VA levels of plasma parallel the WB. There was no detectable VA in erythrocytes.

VA depletion had little influence on LEU VE levels. After five wk of VA depletion, LEU VA levels fell to nondetectable concentrations. VE depletion did have an influence on LEU VA levels. LEU VA levels fell as low as 28% (range 28–66%) of control after seven wk of VE depletion. PLT levels of the vitamins were highly variable in our hands. Only PLT VE seemed to be related to VE depletion.

The relationship among blood components in the loss of VA was PLT = LEU > WB > PLA. The relationship among blood components in the loss of VE was PLA > RBC > WB > LEU = PLT.

Liver levels of free VA and VE decreased in rats fed VA- or VE-deficient diets, respectively. Retinyl palmitate levels also decreased in rats fed VA-deficient diets.

The linear response of various blood component VE

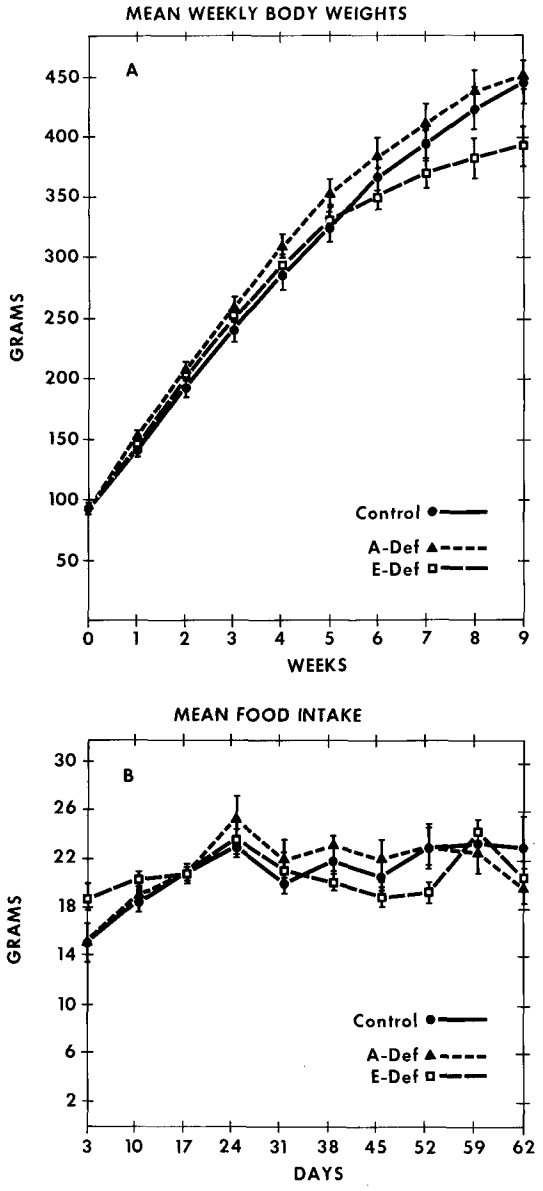

FIG. 1. Mean weekly body weights (A) and food intake (B) of rats fed diets deficient in vitamin E or vitamin A. Mean ± S.E.M., N = 4.

TABLE 2

Correlations of Vitamin E Levels in Blood Components with Vitamin E Levels in Livers of Rats

Blood component[a]	r	m[b]	y[c]	p
Whole blood	0.6044	0.17135	1.9655	<0.001
Plasma	0.5113	0.13727	3.1564	<0.001
Red blood cells	0.5125	0.05823	1.0537	<0.001
Leukocytes	0.6678	0.14388	0.1186	<0.001
Platelets	0.3702	−0.00021	0.3292	<0.001

[a]VE units for whole blood, μg/ml; PLA, μg/ml; RBC, μg/ml; LEU, μg/mg protein; PLT, μg/mg protein.
[b]Slope.
[c]Intercept.

content and liver VE is shown in Table 2. Calculations were based on direct measurements of blood values compared to liver levels from the same period. The response for WB, PLA, RBC and LEU VE was strongly linear with change of liver VE. PLT VE values did not correlate with liver VE. In Table 3 correlations were determined using logarithmic transformed data. Transformation of data resulted in better correlations between blood component VE and liver VE content.

The relationship between VA content in various blood components with liver VA is shown in Table 4. WB or PLA VA values demonstrated good correlations with liver but LEU and PLT produced poor linear relationships. When blood component and liver VA values were transformed to logarithms no correlations could be found (Table 5).

DISCUSSION

Fat-soluble vitamin status has often been estimated by measuring the level of that vitamin in plasma. Caution has been expressed by several investigators (3,13-15) regarding the validity of such estimates and the meaning of such results. In a clinical situation, low levels of plasma vitamins A or E more often are due to redistribution than depletion, and may reflect stress rather than true deficiency. With VE, early work has shown that PLA VE was correlated with plasma lipids (12) and in the rat

TABLE 3

Correlations of Vitamin E Levels in Blood Components with Vitamin E Levels in Livers of Rats: Logarithmic Transformed Data

Blood component[a]	r	m[b]	y[c]	p
Whole blood	0.6195	0.46318	0.2087	<0.001
Plasma	0.6281	0.71387	−0.1839	<0.001
Red blood cells	0.3165	0.39501	−0.1306	<0.001
Leukocytes	0.7068	0.61932	−0.3010	<0.001
Platelets	0.7097	0.19625	−0.8703	<0.001

[a]VE units for whole blood, μg/ml; PLA, μg/ml; RBC, μgl/ml; LEU, mg protein; PLT, μg/mg protein.
[b]Slope.
[c]Intercept.

TABLE 4

Correlations of Vitamin A Levels in Blood Components with Vitamin A Levels in Livers of Rats

Blood component[a]	r	m[b]	y[c]	p
Whole blood	0.5505	0.05968	0.0629	<0.001
Plasma	0.4544	0.10897	0.1521	<0.001
Leukocytes	0.0401	0.00851	0.0271	NS
Platelets	0.1405	0.01754	−0.0097	<0.05

VA units for whole blood, μg/ml; PLA, μg/ml; LEU, μg/mg protein; PLT, μg/mg protein.
[b]Slope.
[c]Intercept.

TABLE 5

Correlations of Vitamin A Levels in Blood Components with Vitamin A Levels in Livers of Rats: Logarithmic Transformed Data

Blood component[a]	r	m[b]	y[c]	p
Whole blood	0.0599	0.13560	0.7302	NS
Plasma	0.0516	0.09945	−0.4126	NS
Leukocytes	0.0916	−0.49927	−1.1575	NS
Platelets	0.1018	0.67913	−1.7441	NS

[a]VA units for whole blood, μg/ml; PLA, μg/ml; LEU; μg/mg protein; PLT, μg/mg protein.

[b]Slope.

[c]Intercept.

it has been evaluated that the best guide to VE status is analysis of the actual tissue content of the vitamin.

Direct analysis of tissue content of the vitamin is the best guide to VE status. However, access to tissues, with the exception of biopsy, is not possible in human nutritional studies. A component of blood remains the best alternative. PLA or serum VE may indicate the onset of negative balance for VE, particularly for uncomplicated dietary deficiency, but it does not correlate with total body VE and better measures are needed. Currently, a low plasma VE and perhaps VA must be carefully considered in relation to the history, diet and physical findings in each patient or subject.

These findings support a previous report (15) that illustrated PLT VE can be correlated well with tissue and dietary intake of VE. It is also apparent from the present results that blood LEU VE reflects liver vitamin E closely. Close correlations were found when VE values were transformed to the corresponding logarithmic values. From the present study, we conclude that LEU VE is a likely blood component to monitor rat VE status. Adequate cell separation without some PLT and LEU cross-contamination with limited amount of blood is difficult. Some investigators have attempted to correct for PLT contamination by a variety of mathematical considerations; however, these correction factors have been inconsistent. Our cell separation method was similar to others (8) which have indicated a yield of 68–74% for each fraction with less than 2% PLT contamination. VE increases the efficiency of antibody production in animals whose immune response functions normally (16,17). Such response may be related directly to lymphocytes and/or chemotactic activities of neutrophils. VE-deficient animals have reduced neutrophil chemotaxis (18).

When liver stores of VA are reduced, PLA VA concentrations tend to fall (19). This explains the strong correlation that was found between PLA VA and liver VA. WB VA correlated well with liver VA. This is a reasonable finding since erythrocytes had no detectable VA content; therefore, WB VA values are essentially PLA measurements. LEU and PLA VA did not correlate well with liver VA, although direct correlation between PLT VA and liver VA was at borderline significance ($P < 0.05$, $r^2 = 0.1405$). Like VE, PLA VA values can be influenced by other factors in addition to VA status itself, e.g., protein-calorie malnutrition, the ingestion of toxic substances and infestation with parasites (20). Also, since liver contains 90% or more of the total stores of VA and PLA, VA values may be limited for their use in nutritional assessment.

There is more than a casual interrelationship between VA and VE. Retinol and its esters are extremely susceptible to oxidative damage (18). Total VA in liver and plasma are decreased during VE deficiency. Also, liver stores of VA are increased during vitamin supplementation (21–23). Olsen (20) suggested that this was not the result of the antioxidant properties of VE because the synthetic substitute N,N′-diphenyl-p-phenylenediamine cannot replace VE in these effects. We found that LEU VA levels were lower in VE-deficient rats. This may explain why LEU and perhaps PLT VA do not reflect liver levels of VA because of VA loss through oxidation. Similarly to recent findings, we failed to find an effect of VE depletion on PLA retinol (24) and liver retinol for the limited time of depletion that we investigated.

To conclude, logarithmic transformed data of LEU and/or PLT VE correlate well with liver VE changes during depletion. This was not true for direct or logarithmic transformed data of LEU and/or PLT VA values. Plasma VA values best reflect changes of liver VA levels.

Our findings support the findings reported by Bieri et al. (25): we detected no VA in isolated rat RBC.

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COMMUNICATIONS

Identification of 5,9-Hexadecadienoic Acid in the Marine Sponge *Chondrilla nucula*

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The fatty acid 5,9-hexadecadienoic was identified in the sponge *Chondrilla nucula*. This is the shortest fatty acid with the Δ5,9 unsaturation yet isolated from a marine sponge.
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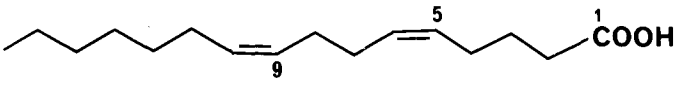
Since the pioneering work of Bergmann (1), phospholipid fatty acids from marine sponges have received considerable attention. Interesting has been the finding of marine phospholipid fatty acids (C26-C30) with an unusual Δ5,9 unsaturation (2). This unusual unsaturation in the fatty acids of marine sponges has always been attributed to the very long chain phospholipid fatty acids, but no report to date mentions such unsaturation for other fatty acids. In this work we present the isolation and characterization of a medium chain fatty acid possessing the unusual Δ5,9 unsaturation from the marine sponge *Chondrilla nucula.* The latter sponge is an interesting ubiquitous sponge from the Caribbean; unlike other common sponges it is very dense, and in shape, size and color is reminiscent of a chicken liver.

EXPERIMENTAL PROCEDURES

Chondrilla nucula was collected in La Parguera, Puerto Rico, during November 1985. The sponge was washed in seawater, carefully cleaned of all nonsponge debris and cut into small pieces. Immediate extraction with chloroform/methanol (1:1, v/v) yielded the total lipids. The neutral lipids, glycolipids and phospholipids were separated by column chromatography on ammonium hydroxide-treated silicic acid (100-200 mesh) using the procedure of Privett et al. (3). The fatty acyl components of the phospholipids were obtained as their methyl esters by reaction of the phospholipids with methanolic hydrogen chloride (4) followed by purification on column chromatography eluting with hexane/ether (9:1, v/v). The resulting methyl esters were analyzed by gas chromatograph-mass spectrometry (GC-MS) using a Hewlett Packard 5995A gas chromatograph-mass spectrometer equipped with a 30 m × 0.32 mm fused silica column coated with SE-54. For the location of double bonds, N-acyl pyrrolidide derivatives were prepared by direct treatment of the methyl esters with pyrrolidine/acetic acid (10:1, v/v) in a capped vial (1 hr at 100 C) followed by ethereal extraction from the acidified solution and purification by preparative layer chromatography (PLC). Hydrogenations were carried out in 10 ml of absolute methanol and catalytic amounts of platinum oxide (PtO_2). Mass spectrometry results for the 5,9-hexadecadienoic acid (Scheme 1) follow.

5,9-Hexadecadienoic acid methyl ester. MS m/e (rel int) 266 (M^+,7), 150 (14), 149 (10), 141 (24), 140 (10), 136 (14), 135 (13), 124 (16), 121 (12), 110 (26), 109 (63), 99 (18), 96 (22), 95 (26), 94 (15), 83 (25), 82 (34), 81 (100), 80 (23), 79 (25), 74 (22), 69 (44), 68 (25), 67 (69), 59 (17), 55 (70), 54 (43), 53 (16).

5,9-Hexadecadienoic acid pyrrolidide. MS m/e (rel int) 305 (M^+,7), 181 (3), 180 (17), 141 (2), 140 (2), 139 (2), 127 (6), 126 (17), 114 (9), 113 (100), 110 (3), 98 (19), 85 (10), 79 (4), 72 (12), 71 (2), 70 (10), 60 (3), 56 (7), 55 (19).



SCHEME 1

TABLE 1

Identified Phospholipid Fatty Acids from *Chondrilla nucula*

Fatty acids	Abundance (%)
Tetradecanoic (14:0)	2.0
13-Methyltetradecanoic (15:0)	7.0
Pentadecanoic (15:0)	3.0
5,9-Hexadecadienoic (16:2)	12.0
9-Hexadecenoic (16:1)	20.0
Palmitic (16:0)	26.0
15-Methylhexadecanoic (17:0)	2.0
Heptadecanoic (17:0)	3.0
11-Octadecenoic (18:1)	7.0
Octadecanoic (18:0)	8.0
Behenic (22:0)	2.0
Tricosanoic (23:0)	0.8
Lignoceric (24:0)	4.0
Pentacosanoic (25:0)	1.0
Hexacosanoic (26:0)	0.8

RESULTS

Our results are presented in Table 1. As can be seen, the major acids in our *C. nucula* specimen were palmitic (16:0), 9-hexadecenoic (16:1) and 5,9-hexadecadienoic (16:2). The latter acids accounted for more than 50% of the total mixture. Only saturated very long chain fatty acids were encountered in the *C. nucula* that we collected. The longest acids were pentacosanoic (25:0) and hexacosanoic (26:0). The phospholipid mixture was shown by thin layer chromatography (TLC) to be phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE).

For the characterization of the 5,9-hexadecadienoic acid, capillary GC combined with MS was used. The fatty acid methyl ester (FAME) of the 5,9-hexadecadienoic acid had an equivalent chain length (ECL) value of 15.55 in capillary GC. ECL clearly expresses where a FAME

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elutes with respect to the series of straight chain saturated methyl esters in a temperature-programmed run. Furthermore, upon catalytic hydrogenation (PtO_2), the hexadecadienoic acid methyl ester was converted to palmitic acid methyl ester, thus excluding the possibility of any branching. The mass spectrum of the hexadecadienoic acid methyl ester presented an M^+ peak at m/e 266 revealing the presence of two double bonds. To exactly locate the double bonds, a pyrrolidide derivative of the fatty acid methyl ester was then prepared. Typical fragmentations in the mass spectra of fatty acid pyrrolidides are to be found at m/e 113 (base peak) and m/e 126. These fragmentations arise from cleavage at the carbon-carbon bonds α and β to the carbonyl (McLafferty rearrangement). While the latter peaks for the 5,9-hexadecadienoic acid pyrrolidide were observed, a peak at m/e 180 (not present in other fatty acid pyrrolidides in the mixture) was observed. The latter peak is typical when two double bonds are located at the 5 and 9 positions resulting from allylic cleavage. The m/e 180 peak is not present in the mass spectrum of other hexadecadienoic acids, i.e., acids with double bonds at positions 2,4; 6,9; 9,12; 5,10; etc. (5). Therefore, there is no doubt that the fatty acid isolated was the 5,9-hexadecadienoic (Scheme 1).

DISCUSSION

Litchfield et al. (6) surveyed fatty acid chain lengths in several sponges and found out that a specimen of *C. nucula* collected by V. Vicente in the Bahia de Jobos near Guayama, Puerto Rico, in December 1974 contained 38% C30 chains in its total fatty acids. Later, Litchfield et al. (7) characterized the C30 acid as 5,9,23-triacontatrienoic (30:3 Δ5,9,23). He also found the common palmitic-C16 (25%), stearic-C18 (10%) and eicosanoic-C20 (11%) acids. To our surprise, no triacontatrienoic acid was detected in our *C. nucula* despite the fact that the analysis was done three times with a SE-54 column heating at 290 C for 1 hr after elution of the final acid reported in this work. We attributed this finding to seasonal variations known to change fatty acid distribution in marine sponges.

While the 5,9-hexadecadienoic acid (Scheme 1) is unprecedented in marine sponges, it is known to occur in nature. However, its occurrence is quite rare and, to the best of our knowledge, it has been isolated only from the cellular slime mold *Dictyostelium discoideum* (8). The latter mold was shown to biosynthesize the 5,9-hexadecadienoic acid from the direct and sequential desaturation of palmitic acid at the 9-position first and subsequently at the 5-position.

Our finding of the presence of this 16:2 acid in *C. nucula* clearly opens a new field for biosynthetic speculations. Is the mode of biosynthesis of the hexadecadienoic acid the same in the sponge as in the mold? Is this acid a true sponge metabolite or is it coming from bacterial symbionts? Work is in progress to elucidate these questions. Whatever the answers, the report of this acid opens a new dimension to the postulation that only very long chain fatty acids in marine sponges possess the Δ5,9 unsaturation.

ACKNOWLEDGMENTS

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A High Cholesterol/Cholate Diet Induced Fatty Liver in Spontaneously Hypertensive Rats

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A high cholesterol diet was found to induce fatty liver in spontaneously hypertensive rats. Although cholesterol ester and triacylglycerol accumulated in large amounts in liver, the increases of these lipids in plasma were relatively small and no increase in cholesterol and cholesterol ester was observed in aorta. In rats fed normal diet, plasma cholesterol ester mainly consisted of arachidonate species; however, oleate and linoleate esters became the most prominent species in rats fed a high-cholesterol diet. The amounts of oleate and linoleate at the 2-position of phosphatidylcholine in both plasma and liver were increased slightly, but the fatty acids of aorta lipids changed little by feeding a high cholesterol diet. These results indicate that the livers of rats fed the high cholesterol diet do not secrete cholesterol ester and triacylglycerol with altered fatty acids as rapidly as they are synthesized and that the increased levels of cholesterol oleate in liver and plasma are not directly correlated with atherogenic lesions under these conditions.
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Spontaneously hypertensive (SH) rats, a strain originally developed by Okamoto and Aoki (1) have been used widely in hypertension research (2,3). Some abnormalities in lipid metabolism have also been noted in these rats (4-9); for example, when fed atherogenic diets, SH rats develop hypercholesterolemia more easily than conventional strains (4,7). In human atherosclerotic lesions, the major lipid component has been shown to be cholesterol ester (ChoE) (10-17). ChoE present in lesions has a higher oleate and lower linoleate content than ChoE present in uninvolved regions of the artery (10-18). Similar changes in the fatty acid compositions of hepatic lipids have been observed in experimental animals (19-24). It has been proposed that ChoE hydrolase is involved in ChoE accumulation (25-27), although there is some controversy about this hypothesis (28-32). Elevations of acyl-CoA:cholesterol acyltransferase (ACAT) occur in rats and rabbits fed high cholesterol diets (33-42) while, in monkey, increases in endogenous cholesterol but not ACAT activity have been reported (43). Some differences in the levels of other enzymes have also been noted in both SH and normotensive rats: lower hepatic cholesterol synthesis in SH than in Wistar/Kyoto (WKY) rats when both were fed an atherogenic diet (6); higher biliary secretion of cholate in SH than in WKY rats fed either a normal or a high cholesterol diet (7); and higher prostanoid synthesis (44,45) and higher phospholipid turnover in aorta (46,47) of SH rats than that of WKY rats fed a normal diet. However, the causal relationship between atherosclerosis and anomalous lipid metabolism has not been fully elucidated. It is also unclear how the development of hypertension and hypercholesterolemia may be related in SH rats.

In an effort to gain more insight into the mechanism by which increases in ChoE, particularly cholesterol oleate, occur, we have examined the effect of a high cholesterol diet on the fatty acid compositions of both hepatic and plasma lipids of SH rats. Aorta lipids were also analyzed to see the effects of the diet-induced changes in hepatic and plasma lipids on this tissue. We have also analyzed the molecular species of ChoE produced by plasma LCAT to estimate roughly the contribution of LCAT in the synthesis of plasma ChoE.

MATERIALS AND METHODS

SH rats (male, five wk of age, Charles River of Japan, Kanagawa, Japan) were fed either a conventional diet MF (Oriental Yeast Co., Tokyo, Japan) or a high cholesterol diet supplemented with 5% cholesterol and 0.5% cholate for up to 65 days. The diet MF contained by weight 24% protein, 5.1% fat, 6.2% minerals, 3.2% fibers and 54.5% non-nitrogenous compounds, with vitamins D_3 and K_3 supplemented. The major fatty acid constituents were linoleate (50%), oleate (22%), palmitate (16%), linolenate (4%) and stearate (2%). Tail systolic blood pressure was measured by the plethysmographic tail method with an apparatus produced by Natsume Co. (Tokyo, Japan).

After fasting overnight at the indicated days of test diets, three rats for each group were killed by decapitation, and livers, blood samples, abdominal and thoracic aortas were removed. Livers and plasmas from the blood samples were kept frozen at −80 C. Aortas were freed of surrounding fat tissue under the microscope and then kept frozen at −80 C. Lipids were extracted with chloroform/methanol according to the method of Bligh and Dyer (48). Neutral lipids and phospholipids were separated by chromatography on silica gel thin layer plates (Merck 60) which were prewashed with the developing solvents (petroleum ether/diethyl ether/acetic acid [80:30:1, v/v/v] or chloroform/methanol/water [70:30:5, v/v/v], respectively). The fatty acid composition of esterified lipids was determined by gas chromatography (GC) of fatty acid methyl esters using heptadecanoic acid as the internal standard. The amounts of lipids were expressed as mg of heptadecanoic acid per g of wet wt or per dl of plasma. Cholesterol was quantitated by GC of its trimethylsilyl ether using ergosterol as the internal standard. Phosphatidylcholine (PC) was hydrolyzed by phospholipase A_2 (*Crotalus adamanteus* venom) as described elsewhere (49) to identify and quantitate the fatty acids at the 1- and 2-positions separately. The lecithin:cholesterol acyltransferase (LCAT) assay was based essentially on the methods of Stokke and Norum (50) and Albers et al. (51), the details of which are described elsewhere (52). Briefly, plasmas kept frozen at −80 C were thawed and preincubated with a sulfhydryl reagent to inhibit LCAT activity, and then preincubated with [^{3}H]cholesterol (Amersham, Buckinghamshire, England) to equilibrate with endogenous cholesterol. The reaction was initiated by adding excess 2-mercaptoethanol to reactivate the LCAT. Molecular species of ChoE

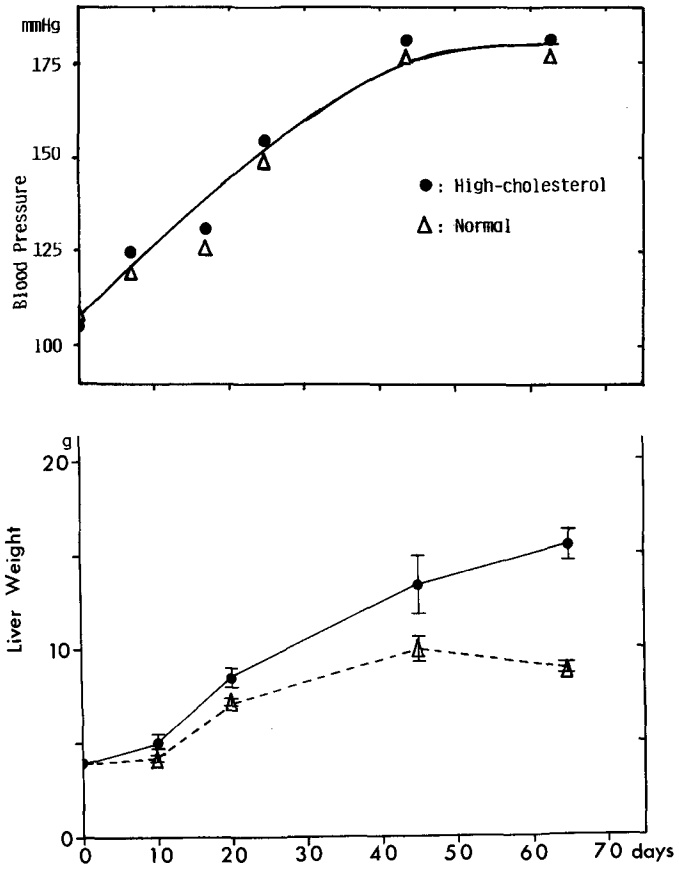

FIG. 1. Effect of a high cholesterol diet on blood pressure and liver weight. Rats (SH, five wk of age, male) were fed either a normal or a high cholesterol diet for the indicated periods. Tail systolic blood pressure was measured by the plethysmographic tail method. Each point represents an average of determinations for three rats (± S.E.).

synthesized by LCAT were determined by $AgNO_3$-silica gel thin layer chromatography.

RESULTS

General observation. Male rats (SH, five wk of age) were fed either the high cholesterol diet or the normal diet for up to 65 days. The differences in the diets did not affect body weight, blood pressure or relative microsomal protein content of liver. Rats in both groups developed a typical hypertension with systolic blood pressures of 150 mm Hg and 185 mm Hg at day 25 and 45, respectively, of the test diets (Fig. 1). On the other hand, significant differences between the liver weights of rats in the two dietary groups were apparent by day 20; liver weights of rats fed the high cholesterol diet were 50% greater by day 65 than those of control rats fed the normal diet. Accompanying the increases in liver weight was a fading of liver color to that typical of fatty liver.

Hepatic and plasma lipid compositions. As shown in Figure 2, the levels of ChoE increased greatly in rats fed the high cholesterol diet. After feeding the high cholesterol diet to rats for 45 days there was a 200-fold increase in the liver ChoE content. Free cholesterol and triacylglycerol increased 1.5- to threefold and fourfold, respectively. The amounts of free fatty acid, phosphatidylcholine and phosphatidylethanolamine were relatively unchanged. We emphasize that liver weight per rat was increased significantly by feeding the high cholesterol diet (Fig. 1) and hence the increases in hepatic lipids per rat were quite pronounced.

The plasma lipid composition was different from that of liver. There is more free cholesterol than cholesterol ester (Fig. 2). The high cholesterol diet elevated plasma free cholesterol 1.8-fold at day 20. The increase in ChoE was slightly less (1.5-fold). Both cholesterol and ChoE in

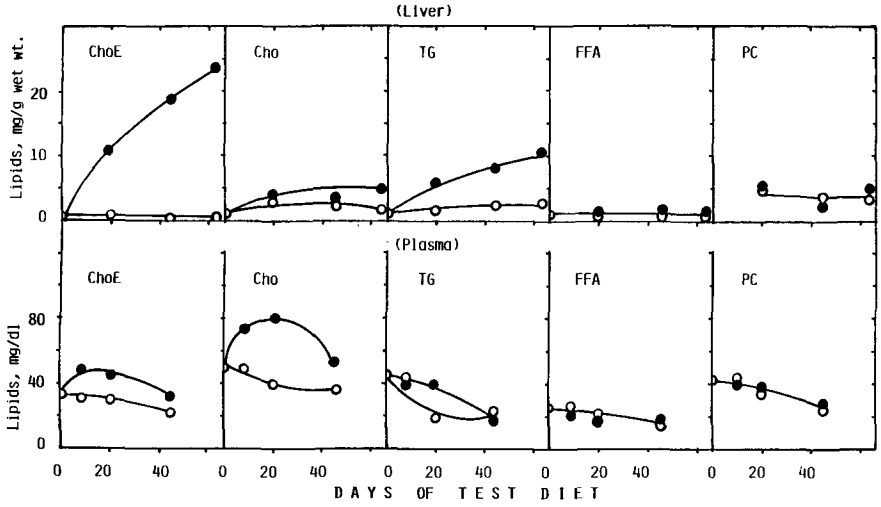

FIG. 2. Effect of a high cholesterol diet on hepatic and plasma lipid contents. After overnight fasting, three rats for each group were killed at the indicated days, samples of livers and plasmas were removed and lipids were extracted with chloroform/methanol. Individual lipid classes were separated by silica gel thin layer chromatography. Lipids containing fatty acids were quantitated by gas chromatography (GC) with heptadecanoic acid as internal standard. Cholesterol was quantitated as its trimethylsilyl ether by GC using ergosterol as internal standard. Each point represents average of determinations for at least two rats. The maximal deviation from mean was 24% of the value given. —•—, High cholesterol diet group; —○—, normal diet group.

plasma tended to decrease by day 45, regardless of the diet. Plasma triacylglycerol levels actually decreased after day 20 on the normal diet and after day 45 on the high cholesterol diet. The high cholesterol diet did not affect the total plasma free fatty acid or PC content, which tended to decrease after day 20. Thus, the 200-fold increase in liver ChoE and fourfold increase in liver triacylglycerol were not directly reflected by corresponding changes in plasma ChoE and triacylglycerol in the high cholesterol diet group, although the sizes of the ChoE pools in plasma and liver are comparable in rats fed the normal diet.

Fatty acid compositions of hepatic and plasma lipids. The fatty acid compositions of ChoE, free fatty acid, triacylglycerol and PC in liver from rats fed the test diets for 20 days are shown in Figure 3. Because the specificity of LCAT is related to the composition of fatty acids at the 2-position of PC (53–58), fatty acids at the 1- and 2-positions of PC were analyzed separately after phospholipase A_2 hydrolysis. As is well-known, each lipid exhibits a characteristic fatty acid pattern. The high cholesterol diet induced a striking change in the fatty acid composition of ChoE: the amounts of saturated and polyene fatty acids decreased while the quantities of oleate and linoleate increased 3.3- and 2.4-fold, respectively. Similar but slightly less pronounced changes were observed in the free fatty acid fraction. Nevertheless, there were only small changes in the fatty acid compositions of triacylglycerol, PC and phosphatidylethanolamine.

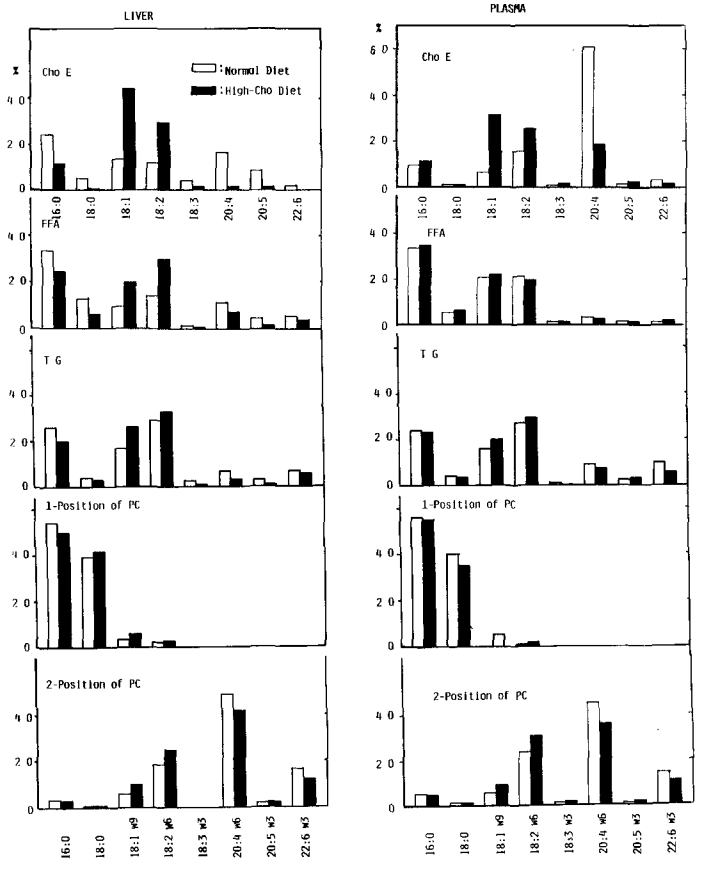

FIG. 3. Effect of a high cholesterol diet on fatty acid pattern of hepatic and plasma lipids. Rats fed test diets for 20 days were used. Fatty acids of hepatic and plasma lipids were analyzed by gas chromatography as methyl esters. Phosphatidylcholine (PC) was hydrolyzed with snake venom phospholipase A_2 to determine the fatty acids at the 1- and 2-positions, separately. Fatty acids were designated by carbon chain length:number of double bonds, and the position of double bond (indicated as ω3 [w3] or ω6 [w6]). Averages of values from two rats are presented. The maximum deviations from the means were 26 and 31% of the values when they were over 10% and 5% of the total, respectively.

The fatty acid compositions of plasma lipids are also shown in Figure 3. In comparing the fatty acid patterns of hepatic and plasma lipids, the following features should be noted: (i) In the normal diet group, plasma ChoE had fatty acid moieties quite different from those of plasma free fatty acid and hepatic ChoE; the plasma ChoE contained relatively more arachidonate and relatively less palmitate and ω3 fatty acids than hepatic ChoE; and the pattern of plasma ChoE fatty acids more closely resembled that at the 2-position of PC. (ii) In the high cholesterol diet group, the fatty acid pattern of plasma ChoE was more similar to that of hepatic ChoE and more unlike that of the 2-position of PC. The fatty acid composition of plasma ChoE differed significantly from that of the normal diet group; oleate and linoleate were increased and arachidonate was decreased by feeding the high cholesterol diet. (iii) The fatty acid compositions of plasma and hepatic PC were quite similar in spite of differences in diets (although the high cholesterol diet did cause a slight increase in oleate and linoleate and a concomitant decrease in arachidonate).

Comparisons of the fatty acids of hepatic and plasma lipids described above were made using livers and plasmas from the rats fed the test diets for 20 days. The time courses for the changes in the fatty acid compositions of hepatic ChoE are shown in Figure 4. The changes in the fatty acid compositions of hepatic ChoE induced by different diets were almost maximal by 20 days of feeding. There were significant decreases in saturated fatty acids and increases in ω9 fatty acids. The positions of double bonds in fatty acids have not been determined, and hence oleate described here represents octadecenoic acids (oleic and *cis*-vaccenic acids). Omega-9, ω6 and ω3 correspond to n-9, n-6 and n-3 series. The proportion of ω6 fatty acids as a whole did not vary significantly, although there were increases in linoleate and corresponding decreases in arachidonate induced by the high cholesterol diet.

The changes in the fatty acids of plasma ChoE were almost maximal after 20 days on the diets (Fig. 4). In fact, the maximum effect was observed even at day 9 of the diet. In contrast to the situation with hepatic ChoE, the proportions of saturated and ω3 fatty acids of plasma ChoE did not decrease but the proportion of ω6 fatty acids decreased.

Since the fatty acid composition of plasma PC was changed by feeding the high cholesterol diet, the molecular species of ChoE produced by plasma LCAT would also be expected to vary. To determine the extent of this variation, the molecular species of ChoE produced by plasma LCAT were analyzed in an in vitro assay system presumed to mimic that occurring in vivo (50–52). As shown in Figure 5, the LCAT showed some preference for arachidonate and less preference for linoleate at the 2-position of PC, but newly formed ChoE roughly reflected the changes in the fatty acid compositions of

PC induced by feeding different diets; the proportions of monoene and diene species increased and that of tetraene decreased by feeding the high cholesterol diet. However, these variations were much smaller than the diet-induced variations in the molecular species of plasma ChoE (see Discussion).

Aorta lipids. Lipids of the abdominal and thoracic aortas at day 20 and day 45 of the test diets were determined. The difference of the diets did not affect the amounts of PC (~1.4 mg/g). The triacylglycerol content was slightly higher in the high cholesterol diet group at day 45 (~2.6 mg/g) than in the control group (~1.5 mg/g). No accumulations of ChoE (~0.05 mg/g) and cholesterol (~1.2 mg/g) were observed in the aorta. The major fatty acids of PC were palmitate (39%), stearate (21%), oleate (11%), linoleate (4%) and arachidonate (18%) while those of triacylglycerol were palmitate (33%), palmitoleate (9%), stearate (5%), oleate (26%) and linoleate (19%). Again, the high cholesterol diet did not induce any significant changes in the fatty acid compositions of these lipids in aortas under the conditions examined.

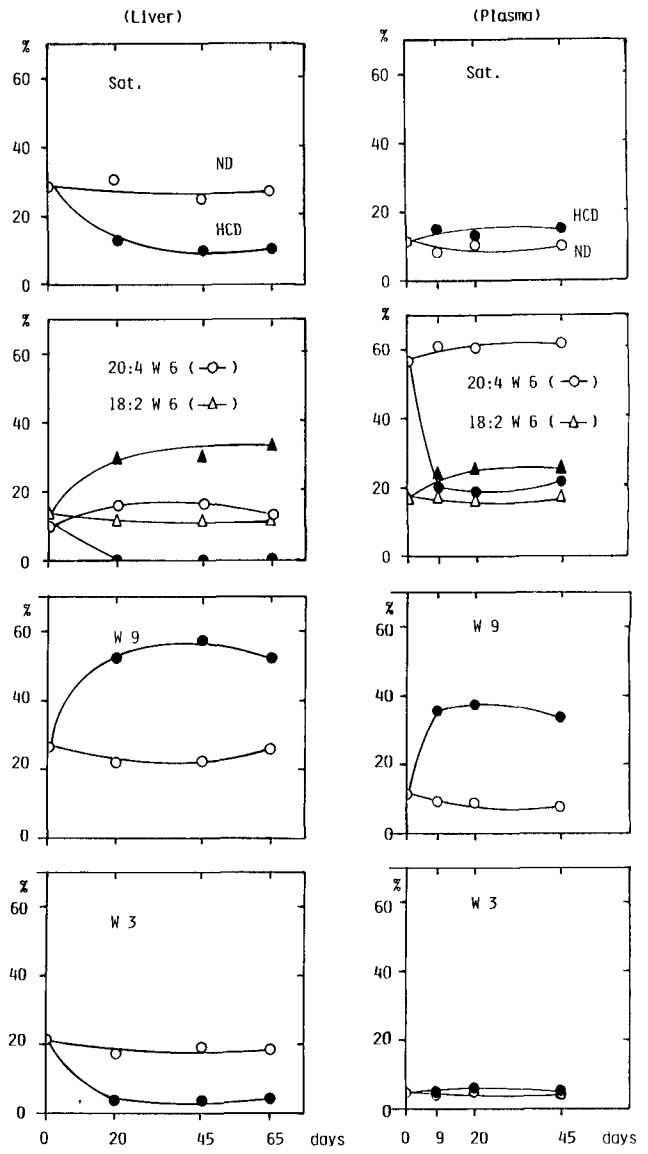

FIG. 4. Time course of the effect of a high cholesterol diet on fatty acid pattern of hepatic and plasma cholesterol ester. Fatty acids of ChoE were grouped into saturated (Sat., mainly 16:0 and 18:0), ω6 (linoleate, 18:2w6, and arachidonate, 20:4w6), ω9 (w9 and w7, mainly 18:1) and ω3 (w3, mainly 18:3w3, 20:5w3 and 22:6w3). ○, △, Normal diet group; •, ▲, high cholesterol diet group.

DISCUSSION

ChoE is thought to be formed mainly through the actions of LCAT in plasma and ACAT in liver and intestine. Since the rats in the present experiment were fasted overnight before plasma and liver samples were removed, the contribution of intestinal ACAT is probably relatively small under these conditions. LCAT utilizes fatty acids from the 2-position of PC, producing only unsaturated ChoE. ACAT is reported to be specific for monoene, saturated and diene species of acyl-CoAs (34,59-62). In the rats fed the normal diet, plasma ChoE consisted mainly of tetraene (arachidonate) species (60% of the total). Monoene (30%) and diene (26%) ChoE were the major species of plasma ChoE in rats fed the high cholesterol diet (Fig. 3). Since plasma LCAT synthesized mainly tetraene species regardless of the diet (Fig. 5), we simply surmised that plasma ChoE was synthesized mainly by LCAT in the normal diet group while the contribution of ACAT increased in the high cholesterol diet group. However, this needs to be examined more carefully.

Plasma free cholesterol, ChoE and triacylglycerol levels were elevated by the high cholesterol diet, but these

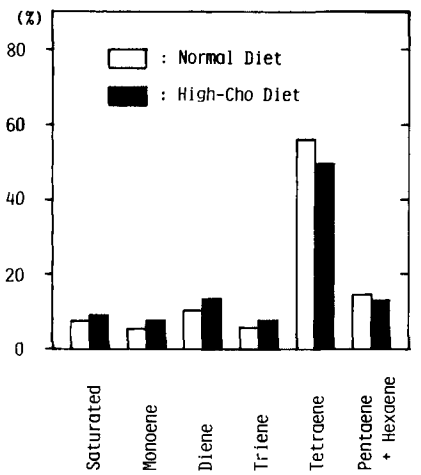

FIG. 5. Selectivity of plasma lecithin:cholesterol acyltransferase. Plasma samples from rats at 20 days of test diet were preincubated with an LCAT inhibitor, 5,5'-dithiobis(2-nitrobenzoic acid), and then with [^{3}H]cholesterol. Incubation was initiated by adding excess 2-mercaptoethanol to reactivate the LCAT. Molecular species of ChoE synthesized from [^{3}H]cholesterol and endogenous acyl donor (PC) were separated by $AgNO_3$-silica gel thin layer chromatography. Radioactivity in each spot was determined by liquid scintillation spectrometry. Although the LCAT inhibitor was not present in blood during the preparation of plasma, the amount of PC used by the LCAT reaction during this period is calculated to be much less than 0.5% of the total. Averages of values for two rats, each assayed in duplicate, are presented. The maximal deviations from means were 6% of the values given for saturated, monoene, diene and tetraene species and 20% for the pentaene plus hexaene species.

changes did not reflect directly the large increases in hepatic lipids. These findings suggest that a step after the synthesis and prior to secretion of ChoE and triacylglycerol is limited in the livers of rats fed the high cholesterol diet. The relative increase in linoleate with a concomitant decrease of arachidonate in ChoE raises the possibility that the elongation and/or desaturation systems became impaired during the development of fatty liver. On the other hand, the ω6 and ω3 fatty acid esters of cholesterol in liver and plasma responded differently to the dietary conditions (Fig. 4). Thus, the increase in ω9 fatty acids and the corresponding decrease in the proportions of polyene fatty acids must not be the only mechanism responsible for the changes in the fatty acid pattern of lipids under these conditions.

Although cholesterol oleate is the major lipid in human atherosclerotic lesions and the levels of hepatic and plasma cholesterol oleate increased significantly, no accumulation of cholesterol oleate was observed in the abdominal and thoracic aortas of SH rats fed a high cholesterol diet (5% cholesterol and 0.5% cholic acid) for up to 45 days. Our preliminary experiments revealed that plasma ChoE was even higher in normotensive WKY rats than in SH rats when the high cholesterol diet was fed for 45 days. On the other hand, in a substrain selected from SH rats, arteriolipidosis-prone (AL) rats, the plasma cholesterol level was reported to reach 500 mg/dl by feeding a high fat/cholesterol diet (20% suet, 5% cholesterol, 2% cholic acid) for one week (63). Fat deposits were noted in arteries but no significant difference was observed in the hepatic cholesterol levels in AL rats and normotensive controls under such conditions (63). Whether the difference in fat deposits in arteries is due to the difference in strains or the difference in diets is to be examined.

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Substrate Specificity of Lysosomal Cholesteryl Ester Hydrolase Isolated from Rat Liver

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The effect of various physicochemical forms of substrate on the activity of acid cholesteryl ester hydrolase isolated from rat liver lysosomes was studied. The amount of sodium taurocholate was varied in the substrate mixture which contained constant amounts of egg phosphatidylcholine (PC) and cholesteryl oleate. The resulting substrate forms produced were PC vesicles, PC vesicles with incorporated sodium taurocholate, mixed micelles, and mixed micelles together with free bile salt micelles. Gradually increasing amounts of sodium taurocholate activated cholesteryl oleate hydrolysis until the molar sodium taurocholate/PC ratio of ca. 0.6; thereafter hydrolytic activity decreased rapidly. The presence of sodium taurocholate micelles clearly inhibits cholesteryl oleate hydrolysis. We therefore propose that the activation observed at low bile salt concentrations depends on bile salt interaction with the substrate vehicle, whereas the inhibition observed at high bile salt concentrations depends on sodium taurocholate interacting with the enzyme. When comparing different phospholipid components in the supersubstrate, the enzyme activity was highest in the presence of dioleyl PC and decreased when present with dipalmitoyl PC and egg PC. Egg lysoPC completely inhibited the enzyme activity. A net negative charge on the surface of the vesicle substrate increased cholesteryl ester hydrolase activity while a net positive charge on the surface inhibited the enzyme activity. Only part of the product inhibition of cholesteryl oleate hydrolase caused by Na-oleate was reversible when tested with bovine serum albumin present in the incubation mixture.
Lipids 21, 481-485 (1986).

Acid cholesteryl ester hydrolase (E.C. 3.1.1.13) has been found in many different tissues, including the liver and arteries (1-8). Studies on the enzyme isolated from liver tissue have shown the presence of cholesteryl ester hydrolase activity in the lysosomal (7,9), microsomal (10) and cytosol fractions (10,11). Sterol ester hydrolase plays an important role in the metabolism of exogenous cholesteryl esters from lipoproteins (12) and in the utilization of endogenously formed sterol esters (12,13). Decreased activity of lysosomal cholesteryl ester hydrolase leads to the accumulation of cholesteryl esters. This results in various disorders of the human body and the accumulation of cholesteryl esters in liver lysosomes in Wolmans disease (14). It may also be an important factor in the accumulation of cholesteryl esters in atherosclerotic arteries (12,15,16). Apart from resulting from genetic disorders, decreased sterol ester hydrolase activity can be caused by alteration in lipid composition and other properties of the substrate vehicle or by the interaction of the lipids with the enzyme (17-19). The importance of investigating the effect of substrate properties on cholesteryl ester hydrolase is well known and has been stressed by several authors (11,20).

By using lipid model systems with defined physicochemical properties as substrate forms, one can investigate the effect of individual lipids on cholesteryl ester hydrolase activity and also elucidate the mechanism by which the enzyme hydrolyzes the ester linkage. One important prerequisite for these studies is the use of a pure and stable enzyme (11,20). Bile salts have been reported to increase cholesteryl ester hydrolase activity in liver (8) and arterial tissues (6), although high concentrations are inhibitory (5,8). The question remains, however, how bile salts affect the enzymatic reaction: by affecting the substrate or the enzyme or both? The activation has mostly been confirmed as depending on the fact that bile salts activate the enzyme by altering the substrate (21). Bile salts can, however, also bind to the enzyme molecule, thereby affecting enzyme activity by allosteric binding (22) and causing aggregation of pancreatic cholesteryl ester hydrolase (23,24).

This report is a follow-up of a previous study (21) on the effect of the properties of the substrate on cholesteryl ester hydrolase activity. It deals mainly with the effect of sodium taurocholate (NaTC) on enzyme activity.

MATERIALS

The following reagents were used: cholesteryl [1-^{14}C]oleate (CO), sp act 34 mCi/mmol (The Radiochemical Centre, Amersham, United Kingdom), cholesteryl oleate (Merck, Darmstadt, Federal Republic of Germany), sodium oleate (Calbiochem, Lucerne, Switzerland), sodium taurocholate, phosphatidic acid (PA), dipalmitoyl phosphatidylcholine (DPPC), dioleoyl phosphatidylcholine (DOPC) (Sigma Chemicals, St. Louis, Missouri) and stearylamine (1-aminooctadecan) (Koch-Light Laboratories, Haverhill, England). Egg phosphatidylcholine (EPC) and egg lysophosphatidylcholine (ELPC) were prepared in our own laboratory (25).

Cholesteryl oleate and the phospholipids were proved to be chromatographically pure by thin layer chromatography on silica gel. Sodium taurocholate was purified by recrystallization (26). Other reagents were of ordinary laboratory grade.

METHODS

Preparation of cholesteryl ester hydrolase. Cholesteryl ester hydrolase was isolated from the livers of adult Sprague-Dawley rats. The livers were homogenized in 0.01 M Tris-HCl buffer (pH 7.5), 0.5 M sucrose, 1 mM EDTA and the lysosomes were fractionated as described earlier (21). Hereafter, the lysosomal pellet has been prepared as described previously (27). The subsequent steps are as follows: The lysosomal pellet was exposed to osmotic shock in 0.01 M Tris-HCl (pH 7.5) and then to alternate freezing and thawing (three times). This solution was used for ammonium sulfate precipitation. The fraction precipitating between 10-70% $(NH_4)_2SO_4$ (w/v)

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was collected, applied to a Sepharose CL-6B column (2.5 × 50 cm) and eluted with 0.05 M Tris-HCl buffer (pH 7.0). These procedures yielded an approximate 40-fold increase in specific activity. After concentration with an Amicon ultrafiltration cell using a PM-30 filter membrane (Amicon Corp., Lexington, MA), the enzyme preparation was stored at −20 C in 0.25 M sucrose, which has been shown to restore the activity effectively (27). This preparation is hereafter referred to as the partially purified cholesteryl ester hydrolase and has been used throughout this study. Protein was determined according to Lowry et al. (28).

Preparation of the substrates. The substrates were prepared in thin-walled glass ampuls. The desired lipid composition of the substrate was obtained by adding known amounts of lipid, phospholipids and cholesteryl ester in benzene and bile salt in methanol from a stock solution to the ampuls. After lyophilization, 1 ml of 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.4), 0.02% NaN_3 was added, and the ampuls were filled with nitrogen, stoppered and shaken by vortexing. Vesicles were prepared by sonication with a bath-type Branson Sonifier for 20 min at 25 C. Substrates forming micellar dispersions were not sonicated, but shaken for 10 min, whereafter the substrate was at ambient temperature for a minimum of 1 hr prior to starting the assay. When studying the effect of different components in the assay mixture on cholesteryl ester hydrolase activity, the basic vesicle substrate contained, per ml, 20 mg EPC and 500 μg cholesteryl [1-^{14}C]oleate. In experiments testing the effect of different substrate compositions on the enzyme activity, the amount of cholesteryl oleate was kept constant but the amount of phospholipid was varied with or without addition of a third component.

Cholesteryl ester hydrolase assay. For each assay, 25–100 μl of the prepared substrate and 150 μl of 0.15 M acetate buffer pH 5.0 was used. The volume was adjusted with distilled water to give a final assay volume of 400 μl after the reaction had been initiated by adding 25–100 μl of enzyme. Incubation was carried out for 15 min at 37 C in a metabolic shaker. Measurement of the released oleic acid was performed as described earlier (21). All measurements were made in duplicate.

RESULTS

Effect of taurocholate on cholesteryl ester hydrolysis. The effect of the anionic detergent sodium taurocholate present in the substrate on the activity of the partially purified cholesteryl ester hydrolase was tested by preparing substrate dispersions with different molar ratios between EPC and NaTC. When increasing amounts of NaTC were added to the basic EPC vesicle substrate the following different physicochemical forms were obtained: EPC vesicles with incorporated NaTC, mixed micelles and mixed micelles together with pure taurocholate micelles (29). The saturation curves obtained with different substrates and the corresponding Lineweaver-Burk plots are shown in Figure 1 (A–F). The results show that cholesteryl ester hydrolysis is clearly affected by decreasing the molar ratio of EPC/NaTC in the substrate. The results also show that the enzyme becomes activated by the addition of a substrate consisting of EPC vesicles and vesicles with incorporated sodium taurocholate (EPC/NaTC = 2:1). With mixed micellar substrates (EPC/NaTC = 1.4:1, 1:1 and 0.7:1), one sees the more typical hyperbolic behavior characteristic of enzyme activity upon substrate addition. Whereas with substrate preparations consisting of a mixture of mixed micelles and bile salt micelles (EPC/NaTC = 0.35:1), one can observe the enzyme inhibition caused by addition of this substrate.

The results of the saturation experiments were plotted as Lineweaver-Burk plots in Figure 1 in order to obtain values for V_{max}. The values obtained for V_{max} (in nmol × mg^{-1} × h^{-1}) were as follows: EPC/NaTC = 1:0 (1798), EPC/NaTC = 2:1 (630), EPC/NaTC = 1.4:1 (1018), EPC/NaTC = 1:1 (514), EPC/NaTC = 0.7:1 (300) and EPC/NaTC = 0.35:1 (222). Increasing the amount of sodium taurocholate in the substrate clearly decreases the maximum velocity of cholesteryl ester hydrolysis. In Figure 2 the results of the saturation experiments are plotted as enzyme activity vs molar ratio EPC:NaTC, with 118.7 μM cholesteryl oleate concentration in the substrate. The observation can be made that the physicochemical form of the substrate clearly affects the activity of cholesteryl ester hydrolysis. The transition between the different substrate forms is pointed out in the figure according to Mazer et al. (29). The results show that an increasing amount of NaTC, especially free NaTC, inhibits cholesteryl ester hydrolase activity. The same conclusion was reached from the result of preincubation of the enzyme with 14 mmol/l of NaTC which gave an 80% inactivation during 10 min. These observations are confirmed in Figure 3, where we have plotted the hydrolytic activity of partially purified cholesteryl ester hydrolase vs protein concentration with substrates having different EPC/NaTC molar ratios. The activity of the enzyme in the presence of the vesicle substrate shows a slightly higher activity than when present with mixed micellar substrates having molar ratios EPC/NaTC = 1.4:1 and 1:1. Substrates with molar ratios EPC/NaTC = 0.7:1 and especially 0.35:1 clearly show a resulting lower enzyme activity.

Effect of phospholipids and surface charge. The effect of EPC, DOPC, DPPC and ELPC in the substrate on cholesteryl ester hydrolysis was tested with substrates containing 20 mg/ml of each phospholipid, respectively. The partially purified enzyme was used and the results are shown in Table 1.

TABLE 1

Different Phospholipids in the Substrate

Phospholipid component	Specific activity (nmol × mg^{-1} × h^{-1})	Percentage change compared to EPC
EPC	901	100
DOPC	1403	156
DPPC	1050	117
ELPC	0	0

EPC, egg phosphatidylcholine; DOPC, dioleoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; ELPC, egg lysophosphatidylcholine. Effect of EPC, DOPC, DPPC and ELPC in the substrate on the activity of partially purified cholesteryl ester hydrolase. Substrate composition per ml: 20 mg phospholipid and 500 μg cholesteryl [1-^{14}C]oleate.

The highest degree of hydrolysis was obtained with DOPC. DPPC gave a slightly higher value than EPC. ELPC, the phospholipid having the most pronounced detergent properties, completely inhibited cholesteryl ester hydrolase activity.

The effect of a positive and negative charge on the supersubstrate surface was tested with stearylamine and phosphatidic acid respectively. The charge on the substrate surface was obtained by incorporating different amounts of these components in the basic vesicle substrate. The enzyme used was partially purified cholesteryl ester hydrolase and the results are shown in Figure 4A.

A moderate negative charge on the substrate surface enhanced the enzyme activity whereas a positive charge had a strong inhibitory effect.

Product inhibition by oleate. Product inhibition of sodium oleate on cholesteryl ester hydrolysis was tested in the presence and absence of bovine serum albumin. The result is shown in Figure 4B.

Sodium oleate inhibits cholesteryl oleate hydrolysis by

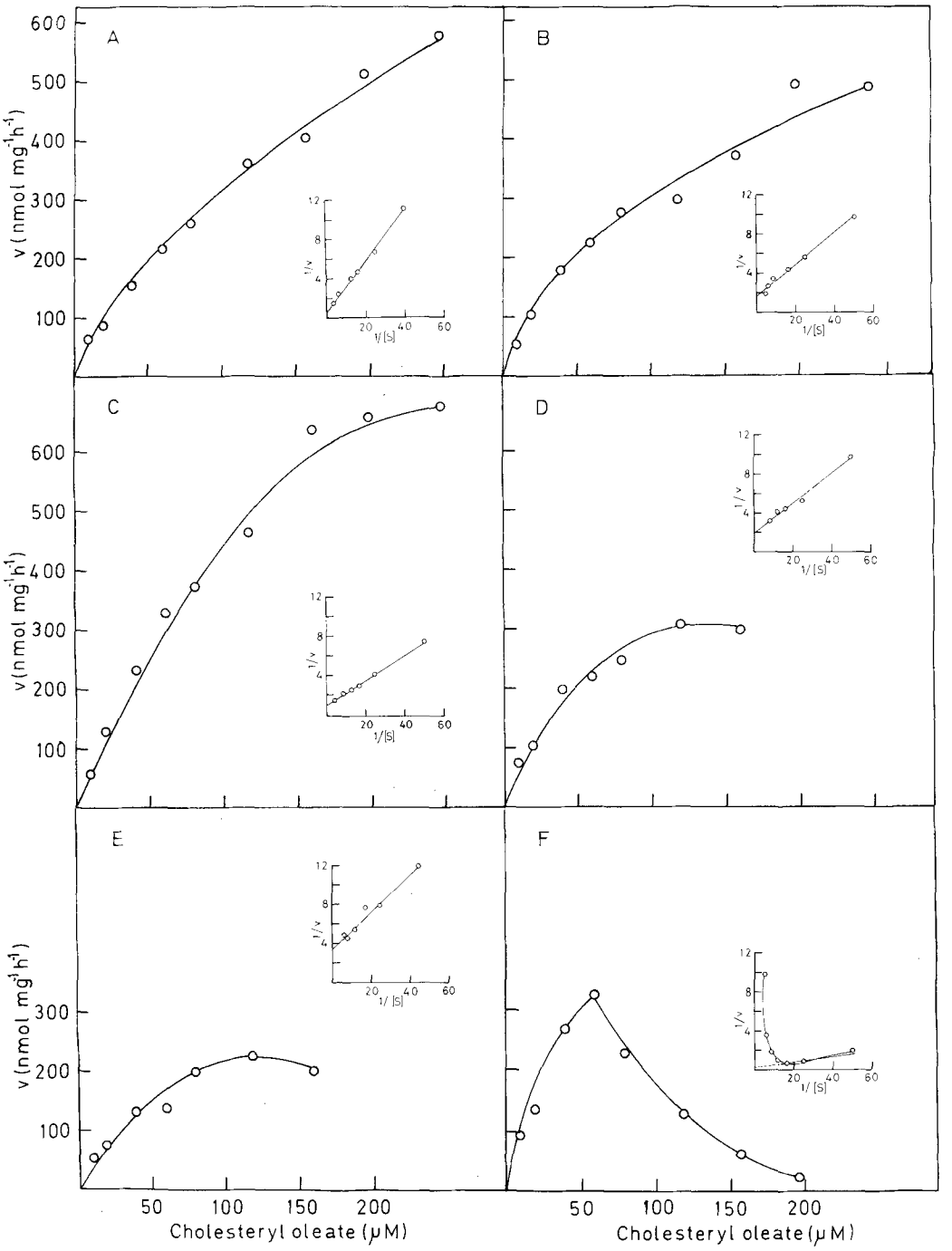

FIG. 1. Saturation curves and corresponding Lineweaver-Burk plots for substrates containing increasing amounts of NaTC. The molar ratios EPC/NaTC in the saturation experiments were as follows: A) 1:0, B) 2:1, C) 1.4:1, D) 1:1, E) 0.7:1 and F) 0.35:1. Each point is the mean of at least two measurements.

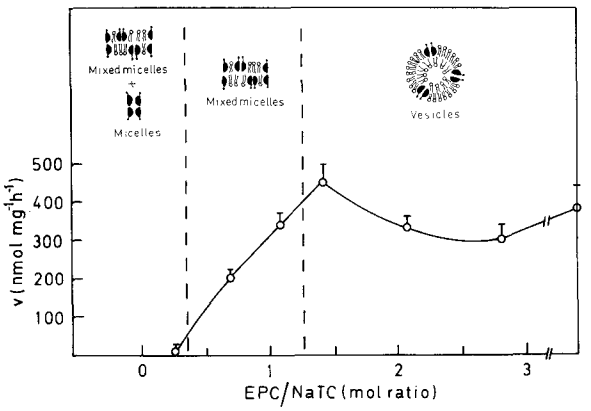

FIG. 2. Effect of physicochemical properties of the supersubstrate on the hydrolysis of cholesteryl oleate. Transition between different supersubstrates are represented by dotted lines. Partially purified enzyme with 118.7 μM cholesteryl oleate was used. Results presented as the mean ± SEM for three experiments.

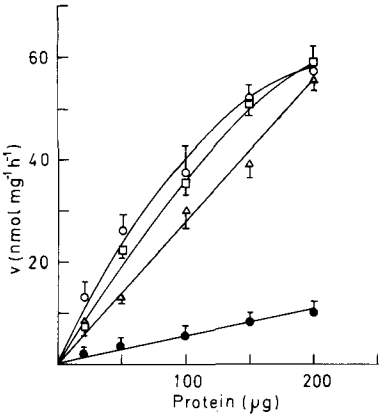

FIG. 3. Effect of increasing protein concentration on cholesteryl oleate hydrolysis with the following EPC/NaTC molar ratios in the supersubstrate: 1.4:1 (□), 1:1 (○), 0.7:1 (△) and 0.35:1 (●). Each point shows the mean ± range of two experiments.

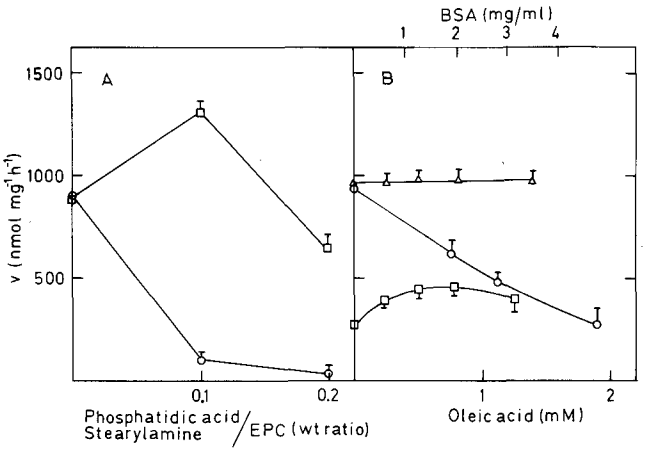

FIG. 4.A. Effect of phosphatidic acid (□) and stearylamine (○) on the hydrolysis of cholesteryl oleate incorporated in the vesicle supersubstrate. B. Effect of sodium oleate on the hydrolysis of cholesteryl oleate in the absence (○) and presence (□) of BSA with 1.9 mM Na-oleate and effect of BSA on the hydrolysis of cholesteryl oleate (△). The routine vesicle substrate was used and the compounds tested were added in aqueous solutions. After two min preincubation the reaction was started by adding the enzyme. Each point shows the mean ± range of at least two measurements.

up to 70% at a final assay concentration of 1.9 mM. Only part of the inactivation can be recovered by BSA addition. Albumin does not appear to inhibit cholesteryl ester hydrolase activity.

DISCUSSION

The primary function of cholesteryl esters is to provide a circulatory transport form and intracellular storage form for cholesterol. The role of cholesteryl ester hydrolase is to mobilize cholesterol for re-esterification, steroidogenesis and other functions. Lysosomes contain the major enzyme activity of acid cholesterol esterase and acid lipase (11,30), and the role of lysosomes in the metabolism of exogenous cholesteryl esters and triglycerides in liver cells (14) and arterial tissue (12) is well documented. The broad substrate specificity of acid lipase indicates that one enzyme is responsible for the hydrolysis of cholesteryl esters, triglycerides, diglycerides and monoglycerides in lysosomes (20,30,31).

The enzymatic hydrolysis of cholesteryl esters is a heterogenous reaction because the enzyme is water-soluble but the substrate is not. The enzyme-substrate interaction takes place at a lipid-water interface in two distinct steps; adsorption of enzyme to the interface followed by substrate hydrolysis within the interfacial plane (32). Adsorption and catalytic activity are independent of each other and localized to different regions. The activating effect caused by the negatively charged phosphatidic acid indicates that charge interactions might play an important role in the formation of a functional enzyme-substrate complex. The interface binding surface of cholesteryl ester hydrolase might include positively charged residues, i.e., lysine and arginine. This suggestion is further supported by the inhibitory effect of the positively charged stearylamine. However, the experiments of binding cholesteryl ester hydrolase to neutral vesicles indicated that the enzyme is not irreversibly bound to the substrate and that the on/off rate for binding is fast. This result is in agreement with a report showing low binding of the enzyme to cholesteryl ester droplets stabilized by phospholipids (33). A relatively weak binding of pancreatic cholesterol esterase to a lecithin monolayer compared to oleic acid monolayers has also been reported (34). A similar high affinity seems to exist between fatty acid and acid cholesteryl ester hydrolase, since only part of the activity inhibited by Na-oleate can be restored by albumin.

The physical form of the substrate will be important for the hydrolytic activity of cholesteryl ester hydrolase (22). In spite of the obvious importance, the physical state of the substrate is rarely determined in most studies of cholesteryl ester hydrolysis. For example, the physical state of cholesteryl esters after being added in acetone to the aqueous system is not known, although this method for substrate presentation has been used frequently. In a previous study, we have shown that PC vesicles, containing 4 mol % cholesteryl oleate, were a suitable substrate form for lysosomal cholesteryl ester hydrolase from rat liver (21). In the present report, the same substrate form was chosen as the basic one, which

has modified mainly by addition of NaTC. It is commonly assumed that the adsorbed enzyme penetrated the surface monolayer to hydrolyze bulk cholesteryl esters (35). However, carbon-13 nuclear magnetic resonance (NMR) results have shown that a cholesteryl ester has a "horseshoe" conformation in PC bilayers, such that the carbonyl group is close to the aqueous interface (36). Such a conformation makes it possible for the enzyme to reach the ester bonding without penetrating the surface monolayer. In fact, the phase specificity of adsorbed enzyme strongly suggests that it functions only at the lipid-water interface (37). The higher activity of cholesteryl ester hydrolase with the unsaturated phosphatidylcholine DOPC recorded in this study, compared with that obtained with DPPC, may in accordance with this concept depend on a higher carbonyl group concentration in contact with the water for the more expanded unsaturated phospholipid.

Bile salts have been widely used for substrate preparations. The effect of bile salt on liver cholesteryl ester hydrolase has been reported to be either activating (11) or inhibitory (5). This study clearly demonstrates that the effect of NaTC on the enzyme activity is dependent on the relative concentrations of phospholipid and bile salt. The incorporation of small amounts of NaTC into the EPC vesicles gave a small decrease in activity. This effect may be explained by the condensing effect of the bile salt on the PC bilayer, which makes the packing density of the EPC bilayer similar to that of DPPC (38). The activation of the enzyme at EPC/NaTC molar ratios around 1.5 may result from the negative charge caused by the bile salt, in parallel with the effect of phosphatidic acid, or by the faster release of reaction products from the substrate (39).

When the proportion of NaTC compared to EPC is increased beyond the border between vesicles and mixed micelles, the activity begins to fall. The structure of the mixed micelles is not uniform throughout the whole region, but two essentially different mixed micelles exist. At EPC/NaTC ratios higher than ca. 0.5 lamellar particles similar to PC bilayers exist, while at ratios lower than 0.5 particles of globular shape dominate (40). The conclusion seems to be that the lamellar micelle is a suitable substrate form, but the globular one is not. At the same time as the structure of the mixed micelle changes with an increasing amount of NaTC, the concentration of free NaTC also increases. Equilibrium dialysis has shown that only about 20% of the NaTC is bound to the mixed micelles at 38 C (41). Studies of pancreatic lipase have indicated that sodium taurodeoxycholate inhibits hydrolysis primarily by preventing the adsorption of the enzyme at the lipid-water interface (42). Such an effect may also be active in the inactivation of cholesteryl ester hydrolase by NaTC.

This study affirms the importance of physical state and molecular organization of the substrate for the activity of acid cholesteryl ester hydrolase. Bile salts traditionally have been widely used for substrate preparations. The results presented here point out that bile salts must be used with caution because of their dramatic effect on the expressed activity of the enzyme. The most favorable substrate form which can be deduced from this study is mixed vesicles of EPC and NaTC in a 1.5:1 molar ratio. Such vesicles give a high activity at the same time as they are easy to prepare in a reproducible way.

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Lipids Containing Isoricinoleoyl (9-Hydroxy-*cis*-12-octadecenoyl) Moieties in Seeds of *Wrightia* Species

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Composition of lipids in mature seeds of *Wrightia tinctoria* and *Wrightia coccinea*, which contain large proportions of isoricinoleic acid (9-hydroxy-*cis*-12-octadecenoic acid) as constituent fatty acid beside the common long chain fatty acids, is reported. The major classes of acyl lipids in the seeds of the two *Wrightia* species, identified by chromatographic and mass spectrometric analyses, are found to be triisoricinoleoylglycerol and diisoricinoleoylacylglycerols. Phospholipids and glycolipids contain only small proportions of isoricinoleoyl moieties. Among two possible precursors for the biosynthesis of isoricinoleic acid, 9-hydroxystearic acid, but not *cis*-12-octadecenoic acid, is detectable in the seed lipids.
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The presence of small proportions of hydroxy acids as constituent fatty acids of vegetable fats is quite common, but only a few plant species are known in which the hydroxy acids form a major constituent of the storage triacylglycerols (1). The analysis of triacylglycerols containing hydroxy acids by thin layer and gas chromatography is difficult because of the polar nature of the hydroxyl group and the thermal instability of these substances. Moreover, the presence of glycerolipids containing more than three acyl moieties, such as estolides, which are formed by acylation of the hydroxyl groups of the constituent hydroxy fatty acids pose additional analytical problems (2-5).

Only a few studies have been made on the distribution of hydroxyacyl moieties in the molecular species of triacylglycerols. Triacylglycerols of castor oil containing about 90% ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid) consist of 68.2% triricinoleoylglycerol, 28.0% diricinoleoylacylglycerols, 2.9% monoricinoleoyldiacylglycerols and small amounts of triacylglycerols containing common long chain acyl moieties only (6). In both diricinoleoylacylglycerols and monoricinoleoyldiacylglycerols, the ricinoleoyl moieties are located preferentially at the *sn*-2 position (6). Gunstone and Qureshi (7) have examined the triacylglycerols of four *Strophanthus* seed oils containing 6-15% isoricinoleic acid (9-hydroxy-*cis*-12-octadecenoic acid). They found that the triacylglycerols containing hydroxyacyl moieties are only composed of monoisoricinoleoyldiacylglycerols, in which the *sn*-2 position is mainly composed of isoricinoleoyl moieties.

We report here the composition of seed lipids of *Wrightia tinctoria* and *Wrightia coccinea*, both of the Apocynaceae family, which are known to contain 70% and 76% isoricinoleic acid, respectively (8). The structure of minor constituents of these seed lipids, which could be possible precursors in the biosynthesis of isoricinoleic acid, is also determined.

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MATERIALS AND METHODS

Materials. Mature seeds of *W. tinctoria* and *W. coccinea* were supplied by the National Botanical Research Institute (Lucknow, India). All reagents and adsorbents were from E. Merck (Darmstadt, Federal Republic of Germany). Solvents were distilled before use. Column packings for gas chromatography (GC) and lipid standards were purchased from Applied Science Laboratories Inc. (State College, Pennsylvania). Castor oil (DAB grade), used as a reference mixture, was obtained from a local pharmacy.

Lipid extraction and derivatization. The mature seeds were finely ground and heated with isopropanol, and the lipids were extracted with chloroform/methanol (2:1, v/v) as described elsewhere (9).

Total lipids or lipid fractions were converted to methyl esters by transmethylation (10). Methyl esters were purified by preparative thin layer chromatography (TLC) on Silica Gel H using hexane/diethyl ether (90:10, v/v). The zones corresponding to methyl isoricinoleate and methyl esters of common long chain acids were located by spraying the chromatoplates with 0.2% ethanolic 2′, 7′-dichlorofluorescein and viewing under UV light. These zones were then scraped off, combined if required and eluted with water-saturated diethyl ether. After removal of diethyl ether in a stream of nitrogen, the methyl esters were dissolved in hexane and the residual fluorescent dye was removed by washing with 0.1 M Tris/HCl, pH 7.4, followed by water.

Lipid fractions containing hydroxyl groups were converted to acetate (11) or trimethylsilyl ether (12) derivatives, which were subsequently purified by TLC as described above.

Fractionation and analysis of lipids. Total lipids from the seeds of *W. tinctoria* or *W. coccinea* were applied on layers of Silica Gel H and the chromatoplates developed twice with diethyl ether up to a height of 2 cm from the origin. The zone migrating with the solvent front (neutral lipids) and that remaining at the origin (phospholipids plus glycolipids) were removed by scraping. Alternatively, the chromatoplates were developed first up to a height of 2 cm by developing twice with diethyl ether and subsequently up to a height of 19 cm with hexane/diethyl ether/acetic acid (50:50:1, v/v/v). This resulted in the separation of the individual classes of neutral lipids, whereas the phospholipids and glycolipids did not migrate from the origin. The zone at the origin of the chromatogram was scraped off and the scrapings were extracted twice with 3.8 ml Bligh and Dyer solvent (13); 2 ml each of chloroform and water were added and the resulting chloroform extract containing the phospholipids and glycolipids was fractionated by TLC on Silica Gel H using chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, v/v/v/v/v). The lipid fractions were marked under UV light after spraying the chromatoplates with 0.2% ethanolic 2′,7′-dichlorofluorescein and scraped off.

The fractions of neutral lipids were eluted from the

adsorbent with water-saturated diethyl ether. The fractions of phospholipids and glycolipids were extracted from the scrapings with the Bligh and Dyer solvent mixture and the nonlipid contaminants were removed by partitioning (13). Aliquots of the lipid fractions were then derivatized as described before.

Methyl esters and their acetate or trimethylsilyl ether derivatives were analyzed by GC in a Perkin-Elmer F-22 instrument equipped with flame ionization detectors (Perkin-Elmer & Co GmbH, Überlingen, Federal Republic of Germany). Glass columns (1.8 m × 4 mm), packed with 10% (w/w) Silar 5CP/Gas-Chrom Q, 80–100 mesh, and nitrogen (40 ml/min) as carrier gas were used. The column temperature was programmed from 190 C to 230 C (2 C/min), after which the temperature was kept at 230 C for 10 min. Peak areas were measured with an integrator (Autolab System IVb, Spectra Physics GmbH, Darmstadt, Federal Republic of Germany). The figures reported are percentages of uncorrected peak areas. The relative proportions of various lipid classes were determined by GC of their methyl esters using methyl heptadecanoate as an internal standard (14).

Lipid fractions were also analyzed by mass spectrometry (MS) in a CH 7 instrument (Varian-MAT, Bremen, Federal Republic of Germany). Samples were introduced from the direct insertion probe at 70 eV. A gas chromatograph coupled with a mass spectrometer (Hewlett-Packard 5992) was also used. An open tubular capillary column, 50 m, coated with Silar 5CP was used for the combined GC-MS. The relative intensities (RI) of the fragments reported are with respect to that of the base peak as 100.

Positional distribution of acyl moieties in fractions of triacylglycerols was determined by hydrolysis with pancreatic lipase (Sigma Chemie GmbH, Munich, Federal Republic of Germany) (15). The resulting 2-acylglycerols were isolated by TLC (16) and transmethylated, and the methyl esters were analyzed by GC as described above.

To detect 9-hydroxystearic acid, the methyl esters of hydroxy fatty acids were isolated from the total methyl esters by TLC as described before. The methyl esters of hydroxy fatty acids were then fractionated by argentation TLC on Silica Gel G containing 20% silver nitrate using hexane/diethyl ether (70:30, v/v) as developing solvent. Methyl 9-hydroxystearate obtained by catalytic hydrogenation of methyl isoricinoleate (17) was used as a reference substance. The chromatoplate was sprayed with 0.2% ethanolic 2′7′-dichlorofluorescein and viewed under UV light. The fraction corresponding to methyl 9-hydroxystearate (Rf 0.3) that migrated just ahead of methyl isoricinoleate (Rf 0.2) was scraped off, eluted with water-saturated diethyl ether and analyzed by GC-MS as described above.

For the detection of *cis*-12-octadecenoic acid, the methyl esters of common long chain acids were isolated from the total methyl esters by TLC, as described before, and then fractionated by argentation TLC as outlined above but by developing twice with hexane/diethyl ether (90:10, v/v). The fraction containing *cis*-monounsaturated methyl esters was scraped off and eluted with water-saturated diethyl ether. From this fraction, methyl *cis*-octadecenoates were isolated by preparative GC and analyzed by reductive ozonolysis followed by GC using the techniques commonly employed in this laboratory (18,19). Detection of *cis*-12-octadecenoic acid was on the basis of the C_{12}-aldester and C_6-aldehyde in the products of reductive ozonolysis.

RESULTS AND DISCUSSION

The composition of acyl moieties of the total lipids of *W. tinctoria* and *W. coccinea,* given in Table 1, shows the predominance of isoricinoleoyl moieties (69 and 74%, respectively), as reported earlier (8).

During the GC analysis of the methyl esters of seed lipids from the *Wrightia* species, it was observed that an

TABLE 1

Composition of Acyl Moieties of Total Lipids and Lipid Fractions in Seeds of *W. tinctoria* and *W. coccinea*

Species/lipids	Percentage of total lipids[a]	Composition (%) of acyl moieties[b]							
		16:0	18:0	18:1	18:2	18:3	20:0	18:1-OH[c]	Others[d]
W. tinctoria									
Total lipids		6	3	8	11	1	<1	69	2
Phospho- and glycolipids	2	23	12	22	29	3	2	8	1
Neutral lipids	98	7	4	7	12	2	<1	67	1
Triisoricinoleoylglycerol	30	3	2	2	<1	<1	<1	92	<1
Diisoricinoleoylacylglycerols	42	7	7	12	9	2	1	61	1
Monoisoricinoleoyldiacylglycerols	4	15	10	19	20	2	1	31	2
W. coccinea									
Total lipids		6	3	6	9	<1	<1	74	1
Phospho- and glycolipids	1	31	13	24	23	1	<1	6	2
Neutral lipids	99	5	3	7	10	<1	<1	73	1
Triisoricinoleoylglycerol	38	2	<1	1	2	<1	<1	94	<1
Diisoricinoleoylacylglycerols	42	11	4	10	10	<1	<1	63	<1
Monoisoricinoleoyldiacylglycerols	2	13	6	23	24	2	2	30	<1

[a]Determined by GC of the methyl esters using methyl heptadecanoate as internal standard (14).

[b]Acyl moieties are designated by the number of carbon atoms:number of double bonds.

[c]Isoricinoleoyl moieties.

[d]Including 20:1, 22:0 and 22:1.

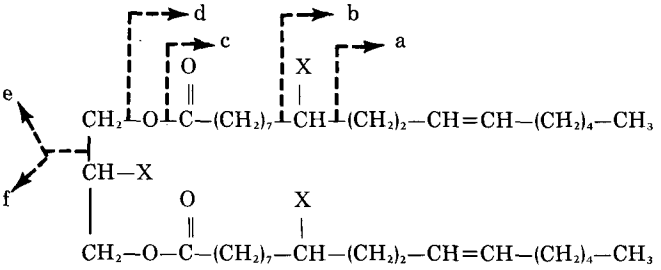
artifact peak (relative retention time 6.1 with respect to methyl palmitate) appears after the emergence of methyl isoricinoleate (relative retention time 5.3 with respect to methyl palmitate). Even after the injection of pure methyl isoricinoleate, the artifact peak is observed which constitutes 5-6% of the peak of methyl isoricinoleate. When methyl isoricinoleate is converted to its trimethylsilyl ether derivative and chromatographed, the artifact peak is no longer observed. It was concluded that the artifact peak was derived from methyl isoricinoleate during GC. Therefore, in all analyses, the area of the artifact peak was added to that of methyl isoricinoleate and the percentage calculated as given in the experimental section.

The level of phospholipids and glycolipids is low in the seed lipids of *W. tinctoria* and *W. coccinea* (Table 1), as found generally in lipids of mature seeds. Analysis of the methyl esters shows that the common long chain fatty acids are the most abundant constituents of phospholipids and glycolipids, whereas isoricinoleic acid is only a minor constituent of these lipids (Table 1). It should be noted in this context that the phospholipids of castor bean do not contain ricinoleic acid (20). The major classes of phospholipids and glycolipids detected in the seeds of both *W. tinctoria* and *W. coccinea* are phosphatidylinositols, phosphatidylcholines, phosphatidylethanolamines, phosphatidylglycerols and monogalactosyldiacylglycerols (data not shown).

The neutral lipids are the predominant constituents of the seed lipids of both *W. tinctoria* and *W. coccinea*, which contain isoricinoleic acid as the major constituent (Table 1). The neutral lipids from both seed species contain a complex mixture of lipid classes, as shown in the thin layer chromatogram (Fig. 1). Such complexities are to be expected due to differences in polarities of the species of triacylglycerols, diacylglycerols or monoacylglycerols containing hydroxyacyl and/or common long chain acyl moieties.

Some of the fractions of neutral lipids were tentatively identified by comparing their chromatographic mobility with the lipid classes of castor oil (Fig. 1). Isoricinoleic acid and its esters were found to migrate on the thin layer chromatogram at a slightly lower rate than ricinoleic acid and its corresponding esters. Fractions 2, 3, 4 and 5 were tentatively identified as triisoricinoleoylglycerol, isoricinoleic acid, diisoricinoleoylacylglycerols and monoisoricinoleoyldiacylglycerols, respectively. Identity of these lipid fractions was established as follows by GC of the methyl esters of their constituent fatty acids and by MS.

A polar fraction (fraction 1) that constitutes 8 and 5%, respectively, of the seed lipids of *W. tinctoria* and *W. coccinea* contains more than 90% isoricinoleoyl moieties (data not shown). This fraction does not appear to be a monoisoricinoleoylglycerol, because it has a higher Rf than monoacylglycerols containing common long chain acyl moieties. The identity of fraction 1 was established as diisoricinoleoylglycerols by MS, as evident from the fragmentation pattern given below.



FRACTION 1

When X=OH: 652 (M^+, RI 0.1), 634 (M-18, RI 0.2), 616 (634-18, RI 1.0), 598 (616-18, RI 0.1), 549 (M-103, RI 0.1), 531 (549-18, RI 0.3), 525 (M-127 [a+2], RI 0.2), 507 (525-18, RI 0.1), 498 (507-18, RI 0.3), 355 (c+74, RI 2), 337 (355-18, RI 6), 298 (d+1, RI 1), 297 (d, RI 3), 281 (c, RI 5), 280 (298-18, RI 7), 262 (280-18, RI 12), 155 (b, RI 18), 55 (base peak, RI 100).

When X=$OCOCH_3$: 778 (M^+, absent), 718 (M-60, RI 0.1), 658 (718-60, RI 0.2), 598 (658-60, RI 0.2), 675 (M-103, RI 0.2), 615 (675-60, RI 0.2), 531 (M-127 + 2 × 60, RI 0.3), 498 (M-b, RI 0.1), 456 (M-322 [c-1], RI 0.7), 439 (M-d, RI 0.4), 438 (456-18 or 498-60, RI 0.3), 425 (f, RI 0.5), 340 (d+1, RI 3), 339 (d, RI 2), 280 (340-60, RI 14), 262 (280-18, RI 7), 43 (base peak, RI 100).

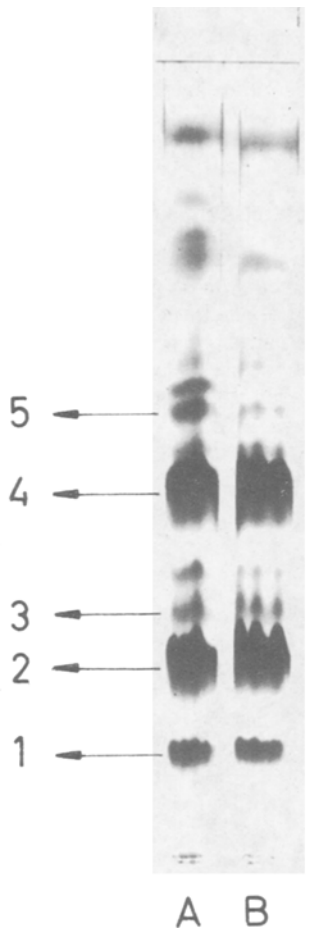
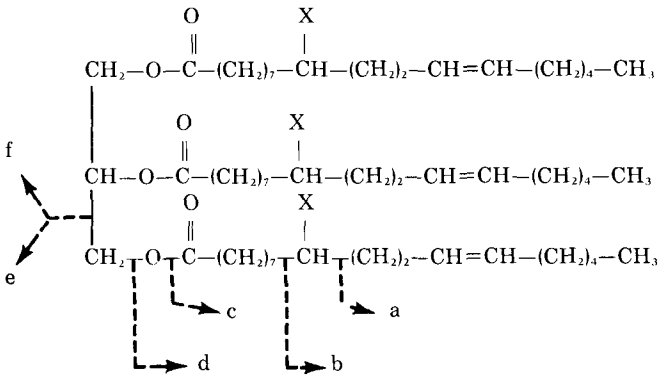


FIG. 1. Fractionation by TLC (Silica Gel H) of total lipids from the seeds of *W. tinctoria* (A) and *W. coccinea* (B). The chromatoplate was developed twice up to 2 cm with diethyl ether, then up to 19 cm with hexane/diethyl ether/acetic acid (50:50:1, v/v/v) and the fractions were visualized by charring. The fractions were identified as 1) diisoricinoleoylglycerol (diacylglycerol); 2) triisoricinoleoylglycerol; 3) isoricinoleic acid; 4) diisoricinoleoylacylglycerols; 5) monoisoricinoleoyldiacylglycerols.

The identity of fraction 1 as a diacylglycerol is clearly apparent from its fragmentation pattern as shown above. The structure of the diacylglycerol as 1,3-diisoricinoleoylglycerol was established from the f and e fragment ions. The absence of fragment ions at m/z 621 and 705, expected from 1,2-diisoricinoleoylglycerol and its acetate derivative, respectively, further confirms the presence of isoricinoleoyl moieties at 1,3-positions.

Fraction 2, a major constituent of the neutral lipids (Table 1), is identified as triisoricinoleoylglycerol by its Rf (Fig. 1), content of isoricinoleoyl moieties (>90%) and the mass spectra of this fraction and its acetate derivative, as shown by the fragmentation pattern given below.



FRACTION 2

When X=OH: 932 (M^+, absent), 896 (M-2×18, RI 0.1), 878 (896-18, RI 0.1), 635 (M-d, RI 0.2), 617 (635-18, RI 0.4), 599 (617-18, RI 0.9), 508 (635-127 [a+2], RI 0.1), 490 (508-18, RI 0.2), 355 (c+74, RI 3), 337 (355-18, RI 10), 298 (d+1, RI 3), 280 (298-18, RI 9), 262 (280-18, RI 12), 155 (b, RI 16), 55 (base peak, RI 100).

When X=$OCOCH_3$: 1058 (M^+, absent), 938 (M-2×60, RI 0.1), 878 (938-60, RI 0.3), 719 (M-d, RI 0.1), 659 (719-60, RI 0.1), 645 (f-60, RI 0.1), 599 (659-60, RI 0.4), 592 (719-127, RI 0.1), 532 (592-60, RI 0.1), 340 (d+1, RI 1), 280 (340-60, RI 25), 262 (280-18, RI 9), 43 (base peak, RI 100).

The mass spectra of fraction 2 and its acetate show no molecular ion peaks at m/z 932 and 1058, respectively, but other highest mass ion peaks due to loss of 2 and 3 molecules of water/acetic acid, respectively, are observed at m/z 896/938 and 878/878. Other characteristic fragments of triisoricinoleoylglycerol and its acetate are shown above.

Fraction 3, which constitutes 2% and 3%, respectively, of the seed lipids of *W. tinctoria* and *W. coccinea*, is identified as unesterified isoricinoleic acid by comparison of its Rf value with that of authentic isoricinoleic acid. Moreover, the methyl esters prepared from this fraction by treatment with diazomethane revealed the presence of more than 90% methyl isoricinoleate when analyzed by GC.

Fraction 4, the most predominant constituent of the neutral lipids of both seed species (Table 1), is identified as diisoricinoleoylacylglycerols by its Rf (Fig. 1) and the content of isoricinoleoyl moieties (about 2/3 of total). The mass spectra of this fraction and its acetate derivative are quite similar to that of fraction 2 (triisoricinoleoylglycerol). The major fragment ions are due to the isoricinoleoyl moieties including m/z 155, which is characteristic for isoricinoleic acid. In addition, very small fragment ions due to common long chain acyl moieties (16:0, 18:0, 18:1, 18:2, 18:3) were observed. The highest mass ion peaks due to loss of two molecules of water or acetic acid are observed at m/z 854 for diisoricinoleoylpalmitoylglycerols, m/z 882 for diisoricinoleoylstearoylglycerols, m/z 880 for diisoricinoleoyloleoylglycerols and m/z 878 for diisoricinoleoyllinoleoylglycerols.

Fraction 5, a minor constituent of the neutral lipids of both seed species (Table 1), is identified as monoisoricinoleoyldiacylglycerols by its Rf (Fig. 1) and the content of isoricinoleoyl moieties (about 1/3 of the total).

In addition to fractions 1 to 5, several fractions of neutral lipids are found in the lipids of both seed species (Fig. 1). These fractions, which comprise 14% and 10%, respectively, of the total neutral lipids of *W. tinctoria* and *W. coccinea* seeds, were not characterized further.

The fractions of diisoricinoleoylacylglycerols (fraction 4) and monoisoricinoleoyldiacylglycerols (fraction 5) were analyzed for the positional distribution of acyl moieties. The data given in Table 2 show that in diisoricinoleoylacylglycerols of both seed species the *sn*-2 position is mainly composed of isoricinoleoyl moieties, while oleoyl and linoleoyl moieties occur preferentially at the *sn*-1,3 positions. In monoisoricinoleoyldiacylglycerols of *W. tinctoria* seeds, the isoricinoleoyl moieties are uniformly distributed between *sn*-2 and *sn*-1,3 positions, whereas in the corresponding lipid class of *W. coccinea* seeds the isoricinoleoyl moieties are somewhat preferentially located at the *sn*-1,3 positions. In this context, it is of interest to note that the hydroxy acids are preferentially esterified at the *sn*-2 position of triacylglycerols of the seeds of castor (6) and *Strophanthus* species (7). It seems that isoricinoleic and ricinoleic acids behave as typical C_{18} unsaturated fatty acids with regard to their positional distribution in triacylglycerols.

The composition of the major molecular species of diisoricinoleoylacylglycerols in the seeds of *W. tinctoria* and *W. coccinea*, respectively, calculated according to Vander Wal (21), are 15.5% and 29.0% for diisoricinoleoylpalmitoylglycerols, 18.3% and 11.4% for diisoricinoleoylstearoylglycerols, 31.8% and 28.0% for diisoricinoleoyloleoylglycerols and 24.4% and 28.4% for diisoricinoleoyllinoleoylglycerols.

To our knowledge, nothing is known so far on the biosynthesis of isoricinoleic acid. Its isomer, ricinoleic acid, seems to be formed by 12-hydroxylation of oleoyl-CoA, possibly via oleoylphosphatidylcholines (22). The following pathways are conceivable in the biosynthesis of isoricinoleic acid (9-OH Δ^{12} 18:1):

(1) 18:0	Δ^9 18:1	$\Delta^{9,12}$ 18:2	9-OH Δ^{12} 18:1
(2) 18:0	Δ^{12} 18:1		9-OH Δ^{12} 18:1
(3) 18:0	9-OH 18:0		9-OH Δ^{12} 18:1

Thus, isoricinoleic acid can be formed by 9-hydroxylation of linoleic acid, a major constituent of the seed lipids of the *Wrightia* species (pathway 1). Alternatively, *cis*-12-octadecenoic acid, derived by Δ^{12} desaturation of stearic acid, can be hydroxylated at C_9 to yield

TABLE 2

Positional Distribution of Acyl Moieties in Diisoricinoleoylacylglycerols and Monoisoricinoleoyldiacylglycerols Isolated from Seeds of *W. tinctoria* and *W. coccinea*

Species/triacylglycerols	Position of acyl moieties	Composition (%) of acyl moieties[a]							
		16:0	18:0	18:1	18:2	18:3	20:0	18:1-OH[a]	Others[a]
W. tinctoria									
Diisoricinoleoylacylglycerols	*sn*-2[b]	5	4	3	3	1	<1	83	<1
	sn-1,3[c]	7	9	17	13	2	1	50	<1
Monoisoricinoleoyldiacylglycerols	*sn*-2[b]	15	13	16	21	3	<1	31	<1
	sn-1,3[c]	15	9	21	19	2	1	32	1
W. coccinea									
Diisoricinoleoylacylglycerols	*sn*-2[b]	11	13	3	2	<1	<1	70	<1
	sn-1,3[c]	10	1	14	14	1	<1	60	<1
Monoisoricinoleoyldiacylglycerols	*sn*-2[b]	37	39	8	3	<1	1	11	<1
	sn-1,3[c]	2	−10	30	34	3	1	39	1

[a]See Table 1.

[b]Determined by hydrolysis with porcine pancreatic lipase and analysis of 2-acylglycerols.

[c]Calculated from the composition of acyl moieties at the *sn*-1,2,3 positions (Table 1) and *sn*-2 position.

isoricinoleic acid (pathway 2). A further possibility of formation of isoricinoleic acid is by Δ^{12} desaturation of 9-hydroxystearic acid which, in turn, can be derived by hydroxylation of stearic acid (pathway 3).

To assess the involvement of the pathways 2 and/or 3 in the biosynthesis of isoricinoleic acid, the seed lipids of *W. tinctoria* and *W. coccinea* were examined for the presence of possible precursors, i.e., *cis*-12-octadecenoic acid and 9-hydroxystearic acid, respectively.

Methyl octadecenoates, isolated from the total methyl esters by argentation TLC and preparative GC, were subjected to reductive ozonolysis and the resulting aldehydes and aldesters were analyzed by GC. Neither C_{12}-aldester nor C_6-aldehyde was detectable in the ozonolysis products, which virtually rules out the presence of *cis*-12-octadecenoic acid in the seed lipids of both *W. tinctoria* and *W. coccinea*.

From the methyl esters of the seed lipids of both *W. tinctoria* and *W. coccinea*, a fraction migrating ahead of methyl isoricinoleate was isolated by argentation TLC and analyzed by GC. This fraction gave a single peak that coincides with the peak of methyl 9-hydroxystearate that had been prepared by catalytic hydrogenation of methyl isoricinoleate. This fraction was also analyzed by GC coupled with MS; its mass spectrum was found to be identical with that of methyl 9-hydroxystearate. The characteristic peaks of methyl 9-hydroxystearate are m/z 187, 158, 155, 127 and 126. Thus, the identity of 9-hydroxystearic acid is unequivocally established in the seed lipids of *W. tinctoria* and *W. coccinea*, and thus the operation of the pathway 3 in the biosynthesis of isoricinoleic acid can be envisaged.

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(24*R*)-14α-Methyl-24-ethyl-5α-cholest-9(11)-en-3β-ol: A New 14α-Methylsterol from *Cucumis sativus*

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The structure of a new 14α-methylsterol isolated from *Cucumis sativus* has been shown to be (24*R*)-14α-methyl-24-ethyl-5α-cholest-9(11)-en-3β-ol. Characterization of the 14α-methyl-Δ$^{9(11)}$ skeletal structure was undertaken based on the comparison with 14α,24-dimethyl-5α-cholest-9(11)-en-3β-ol obtained from 24-methylpollinastanol by acid-catalyzed isomerization. Isolation and identification of an additional 14α-methylsterol, 24-methylenepollinastanol, from the plant also is described.
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Our recent study demonstrated the occurrence of Δ^8-sterols in addition to Δ^7- and Δ^5-sterols in some Cucurbitaceae plants, including cucumber (*Cucumis sativus* L.) (1), representing the first detection of Δ^8-sterols lacking a 4-methyl group in higher plants. This paper describes further study on the sterol constituents of the aerial parts of cucumber, which led to the isolation and characterization of a new 14α-methylsterol, (24*R*)-14α-methyl-24-ethyl-5α-cholest-9(11)-en-3β-ol ([24*R*]-**1a**), together with 14α-methyl-24-methylene-9β,19-cyclo-5α-cholestan-3β-ol (24-methylenepollinastanol, **2b**).

EXPERIMENTAL PROCEDURES

General methods and materials. Recrystallizations were performed in MeOH. Melting points (mp) taken on a heat block were uncorrected. Preparative thin layer chromatography (TLC) on silica gel (0.5 mm thick) (1) and argentation (silica gel/$AgNO_3$; 4:1, w/w) preparative (0.5 mm thick) TLC were developed three times with hexane/EtOAc (6:1, v/v) and four times with CCl_4/CH_2Cl_2 (5:1, v/v), respectively. The bands on silica gel and argentation TLC were observed under UV light (3600 Å) after spraying with a 0.05% solution in EtOH of rhodamine-6G and 2′,7′-dichlorofluorescein, respectively. Preparative high performance liquid chromatography (HPLC) was carried out on a Partisil 5 ODS-2 (25 cm × 10 mm id) (Whatman, Clifton, New Jersey) column with MeOH as a mobile phase (flow rate, 4 ml/min). Gas liquid chromatography (GLC) was performed with a Shimadzu GC-4CM instrument on a SCOT OV-17 glass capillary column (30 m × 0.3 mm id, column temperature 260 C). The Rc-values (relative mobility) in the argentation TLC and the relative retention times (RRT) in the HPLC and GLC were expressed relative to cholesterol acetate (1.00). Mass spectra (EI-MS, 70 eV) were taken on a Hitachi M-80B double-focusing gas chromatograph-mass spectrometer by means of a probe injection. Proton nuclear magnetic resonance (^{1}H NMR) spectra (250 MHz) were recorded on a Hitachi R-250 instrument in a $CDCl_3$ solution, with tetramethylsilane as the internal standard. An authentic specimen of **2b** was isolated from banana peel (2). The origin of authentic (24*R*)- and (24*S*)-24-ethyl-5α-cholest-7-en-3β-ols (**5a**) was described previously (3). (24*R,S*)-24-methylpollinastanol (**2c**) acetate was obtained from **2b**-acetate by hydrogenation in EtOH over PtO_2 at atmospheric pressure and temperature overnight. The isomerization of (24*R,S*)-**2c**-acetate by gaseous HCl in $CHCl_3$ was carried out as described previously (4), yielding a mixture of the acetates of (24*R,S*)-14α,24-dimethyl-5α-cholest-9(11)-en-3β-ol (**1c**), (24*R,S*)-14α,24-dimethyl-5α-cholest-8-en-3β-ol (**3c**) and (24*R,S*)-14α,24-dimethyl-5α-cholest-7-en-3β-ol (**4c**).

Sterol isolation from cucumber. The sterol fraction (3.08 g) obtained from MeOH extract (528 g) of the air-dried aerial parts of cucumber (5.3 kg) was separated by silica gel TLC into two fractions, one containing mainly Δ^5-sterols (fraction A, 74 mg) and another consisting mainly of Δ^7-sterols (fraction B, 2.50 g) (1). Acetylation of fraction A in Ac_2O/pyridine at room temperature overnight gave the acetate (75 mg), which was shown by GLC to contain two unknown sterols with RRT 1.82 (**1a**-acetate) and 1.62 (**2b**-acetate) in addition to several Δ^5-sterols. The acetate fraction A was subjected to argentation TLC to give five fractions (A-1–A-5). Fraction A-3 (10.5 mg) from the third least polar band contained **1a**-acetate, whereas fraction A-5 (8.1 mg) from the most polar band contained **2b**-acetate. Isolation of **1a**- (1.5 mg, RRT = 0.88 in HPLC) and **2b**- (2.1 mg, RRT = 0.98) acetates from each fraction was performed by HPLC. Identification and characterization of other sterols and the composition of sterol fraction of the aerial parts of cucumber were described previously (1).

RESULTS AND DISCUSSION

High resolution MS of **1a**-acetate (mp 97–98 C, Rc = 0.89 in argentation TLC) showed M$^+$ at m/z 470.4109 (rel int 31%, $C_{32}H_{54}O_2$, calcd. 470.4120), with the following prominent fragmentation ions: at m/z 455. 3852 (100%, M$^+$-Me), 410.3876 (3%, M$^+$-HOAc), 395.3852 (24%, M$^+$-Me-HOAc), 329.3852 (3%, M$^+$-side chain [$C_{10}H_{11}$]), 287.2010 (5%, M$^+$-$C_{10}H_{11}$-ring D [C_3H_6]), 273.1835 (6%, m/z 287-CH_2), 269.2242 (7%), 261.1821 (8%), 227.1841 (8%) and 213.1655 (7%), which suggested that the sterol has a saturated C_{10} side chain, and a monounsaturated skeleton with an additional methyl group, probably located at C-14 (5,6). The skeletal ^{1}H NMR signals of **1a**-acetate at δ 0.656 (3H, *s*, 18-H_3), 0.751 (3H, *s*, 32-H_3), 0.978 (3H, *s*, 19-H_3), 2.024 (3H, *s*, 3β-OAc), 4.68 (1H, *m*, 3α-H) and 5.29 (1H, *m*, 11-H) were almost identical with the corresponding signals of authentic (24*R,S*)-**1c**-acetate (Table 1), and hence the 14α-methyl-Δ$^{9(11)}$ skeletal structure was attributed for sterol **1a**. On the other hand, the ^{1}H NMR data suggested the (24*R*)-ethyl substituted structure for the side chain of **1a** by the signals at δ 0.883 (3H, *d*, 21-H_3), 0.838 (3H, *d*, 26-H_3), 0.817 (3H, *d*, 27-H_3) and 0.848 (3H, *t*, 29-H_3), of which the latter three were consistent with those of the (24*R*)-epimer of authentic 24-ethylsterols, **5a**-acetates (1) (Table 1). Thus, the sterol was considered to have the structure

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TABLE 1

^{1}H-NMR Data (250 MHz; $CDCl_3$)[a] of the Acetyl Derivatives of Some 14α-Methylsterols and Two Other Sterols

Acetate		18-H_3[b]	19-H_3[b,c]	32-H_3[b]	21-H_3[d]	26-H_3[d]	27-H_3[d]	28-H_3[d,e]	29-H_3[f]
1a[g]	(24*R*)	0.656	0.978	0.751	0.883 (6.4)	0.838 (6.7)	0.817 (6.7)	—	0.848 (6.0)
2b[g]	—	0.961	0.081 (*d*, 4.3) 0.442 (*d*, 4.0)	0.898	0.896 (6.4)	1.025 (6.7) 1.030 (6.7)		4.663 (*s*) 4.714 (*s*)	—
1c[h]	(24*R*)	0.656	0.978	0.753	0.873 (6.4)	0.853 (7.0)	0.807 (6.7)	0.780 (6.4)	—
	(24*S*)				0.881 (6.7)	0.858 (6.7)	0.785 (6.7)		
2c[h]	(24*R*)	0.958	0.077 (*d*, 3.7) 0.441 (*d*, 4.0)	0.893	0.859 (6.7)	0.856 (6.7)	0.808 (6.7)	0.781 (6.7)	—
	(24*S*)				0.868 (6.4)	0.859 (6.7)	0.785 (7.0)		
3c[h]	(24*R*)	0.705	0.958	0.887	0.890 (6.1)	0.853 (7.0)	0.806 (6.4)	0.780 (6.4)	—
	(24*S*)				0.899 (5.8)	0.856 (7.0)	0.783 (6.7)		
4c[h]	(24*R*)	0.659	0.827	0.985	0.880 (6.7)	0.853 (6.7)	0.807 (6.1)	0.781 (6.7)	—
	(24*S*)				0.889 (6.7)	0.858 (6.7)	0.784 (6.7)		
5a	(24*R*)	0.532	0.809	—	0.925 (6.1)	0.836 (6.7)	0.814 (6.7)	—	0.845 (7.3)
	(24*S*)	0.533	0.809	—	0.930 (6.4)	0.831 (6.7)	0.810 (6.7)	—	0.854 (7.3)

[a]Given as δ values. Figures in parentheses denote *J* values in Hz. Other signals: δ 2.02 (3H, *s*, 3β-OAc) for all sterols; δ 4.80 (1H, *m*, 3α-H) for **2b**- and **2c**-acetates; δ 4.70 (1H, *m*, 3α-H) for the acetates of **1c**, **3c**, **4c** and **5a**; δ 5.29 (1H, *m*, 11-H) for **1a**- and **1c**-acetates; and δ 5.15 (1H, *m*, 7-H) for **4c**- and **5a**-acetates.

[b]Singlet unless otherwise specified.

[c]Cyclo methylene signals as for **2b**- and **2c**-acetates.

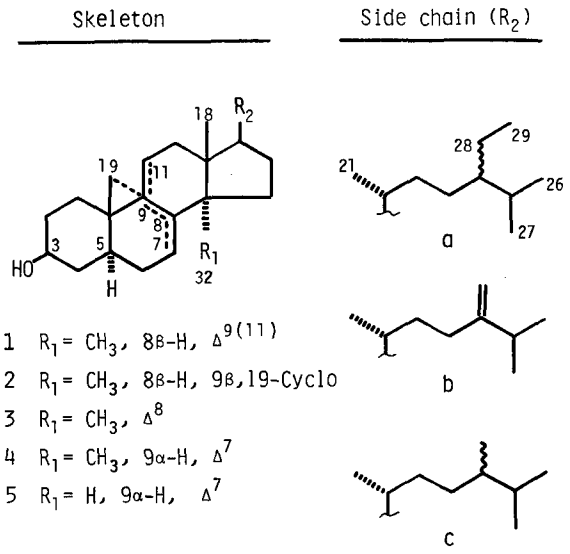
[d]Doublet if not otherwise stated.

[e]Terminal methylene signal as for **2b**-acetate.

[f]Triplet.

[g]Isolated from cucumber in this study.

[h]Mixture of C-24 epimers.



SCHEME 1

(24*R*)-14α-methyl-24-ethyl-5α-cholest-9(11)-en-3β-ol ([24*R*]-**1a**) (Scheme 1). The 20*S*-configuration for the sterol is unlikely since this stereochemistry shifts the 21-H_3 signal to the higher field (7).

MS of **2b**-acetate (mp, not determined; Rc = 0.39 in argentation TLC) showed M^+ at m/z 454 (rel int 12%), with the following prominent ions: at m/z 439 (7%), 411 (4%), 394 (95%), 379 (45%), 351 (6%), 329 (5%), 300 (6%), 273 (3%), 269 (28%) and 55 (100%). The chromatographic data and the MS and ^{1}H NMR spectra (Table 1) were consistent with those of authentic **2b**-acetate (mp 56–57 C) (2), and the sterol was identified as 24-methylenepollinastanol (**2b**). Hydrogenation of **2b**-acetate yielded (24*R,S*)-**2c**-acetate (mp 62–64 C, Rc = 1.20 in argentation TLC, RRT = 1.56 in GLC). High resolution MS of (24*R,S*)-**2c**-acetate showed M^+ at m/z 456.3975 (rel int 7%, $C_{31}H_{52}O_2$, calcd. 456.3965) accompanied with the following prominent ions: at m/z 441 (9%), 396 (80%), 381 (40%), 329 (12%), 302 (7%), 269 (30%), 220 (2%), 213 (7%) and 43 (100%).

9β,19-Cyclosterol is known to yield $\Delta^{9(11)}$-sterol upon acid-catalyzed isomerization (4,8). In order to obtain an authentic 14α-methyl-$\Delta^{9(11)}$-sterol, (24*R,S*)-**2c**-acetate (a

14α-methyl-9β,19-cyclosterol) was subjected to isomerization by gaseous HCl (4,8) which yielded the $\Delta^{9(11)}$-isomer (24*R,S*)-**1c**-acetate (54%, as determined by GLC) in addition to the acetates of (24*R,S*)-**3c** (19%, Δ^{8}-isomer) and (24*R,S*)-**4c** (24%, Δ^{7}-isomer). Argentation TLC of the isomerization product yielded three bands. The least polar band (Rc = 1.18) yielded (24*R,S*)-**3c**-acetate (mp 97–99 C, lit. [9] mp 110–111 C; RRT = 1.32 in GLC). This showed M^+ at m/z 456.3986 (rel int 25%, $C_{31}H_{52}O_2$) in the high resolution MS with the following prominent ions: at m/z 441 (100%), 381 (24%), 329 (1%), 287 (10%), 273 (9%), 269 (4%), 261 (7%), 255 (6%), 227 (15%), 213 (11%) and 201 (16%). The medium polar band (Rc = 1.11) yielded (24*R,S*)-**4c**-acetate (mp 91–93 C, RRT = 1.56 in GLC) which showed M^+ at m/z 456.3995 (rel int 26%) in the high resolution MS with the following prominent ions: at m/z 441 (100%), 381 (48%), 329 (1%), 313 (10%), 287 (11%), 273 (6%), 269 (10%), 261 (9%), 242 (16%), 227 (31%) and 201 (19%). The most polar band (Rc = 0.96) yielded (24*R,S*)-**1c**-acetate (mp 84–86 C, RRT = 1.48 in GLC), which displayed M^+ at m/z 456.3972 (rel int 8%, $C_{31}H_{52}O_2$) in the high resolution MS accompanied with the following prominent ions: at m/z 441 (100%), 381 (28%), 329 (5%), 287 (8%), 273 (6%), 269 (9%), 261 (9%), 255 (6%), 227 (11%), 213 (7%) and 201 (10%). The ^{1}H NMR spectra of (24*R,S*)-**1c**-, **2c**-, **3c**- and **4c**-acetate were listed in Table 1. The skeletal proton signals were assigned by comparison with those of the corresponding double bond isomers of the 4,4-dimethylated derivatives (lanostane-type triterpenes) (4,8), whereas the side chain ^{1}H signals were assigned based on comparison with relevant sterols (1,10–12).

Thus, this study has demonstrated the occurrence of two 14α-methylsterols, **1a** and **2b**, of which the former is considered to be a new sterol, in the aerial parts of cucumber as the minor sterol constituents. Although the occurrence has been reported of 14α-methyl-5α-cholest-9(11)-en-3β-ol (a 14α-methyl-$\Delta^{9(11)}$-sterol) in sea cucumbers (13,14) and of several sterols possessing a 5β,19-cyclo-14α-methyl-$\Delta^{9(11)}$ skeleton in *Nerivilia purpurea* (Orchidaceae) (15), this study seems to be the first detection of a 14α-methyl-$\Delta^{9(11)}$-sterol which lacks a 5β,19-cyclo group in a plant. There are a few other 14α-methylsterols possessing a 9β,19-cyclo (2) (2,5,6,16) and a Δ^{8} (3) (5,9,17), functionalities which have so far been reported to occur in some plants, and sterol **2b** has been detected for the first time in a green alga *Chlorella emersonii* (5) and a higher plant, *Musa sapientum* (6).

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(24S)-14α,24-Dimethyl-9β,19-cyclo-5α-cholest-25-en-3β-ol: A New Sterol and Other Sterols in *Musa sapientum*

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The structure of a new sterol isolated from *Musa sapientum* has been shown by chemical and spectroscopic methods to be (24*S*)-14α,24-dimethyl-9α,19-cyclo-5α-cholest-25-en-3β-ol. In addition, several known (24*S*)-24-methyl-Δ[25]-sterols, their 24-methylene isomers and other sterols (4,4-dimethyl-, 4α-methyl- and 4-demethyl-sterols) together with 3-oxo-4α-methylsteroids were isolated from the plant and identified. The biogenetic implication of these sterols and 3-oxosteroids is discussed.
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Previous studies on the sterol constituents of banana peel have shown the occurrence of two unique sterols, 24ξ-methyl-31-nor-5α-cycloart-25-en-3-one (**1d**) (1), a 24-methyl-Δ[25]-steroid ketone, and 14α-methyl-24-methylene-9β,19-cyclo-5α-cholestan-3β-ol (24-methylenepollinastanol) (**6d**) (2), a 14α-methyl-4-demethylsterol, besides several other usual sterols (4,4-dimethyl-, 4α-methyl- and 4-demethyl-sterols) and 24-methylene-31-nor-5α-cycloartan-3-one (**1d**) (3,4). We have undertaken a detailed reinvestigation of the sterol constituents of banana peel, and this paper describes the isolation and characterization of a new sterol (24*S*)-14α,24-dimethyl-9β,19-cyclo-5α-cholest-25-en-3β-ol ([24*S*]-24-methyl-25-dehydropollinastanol, [24*S*]-**6e**). Isolation and identification of other sterols including (24*S*)-24-methyl-Δ[25]-sterols also are described.

EXPERIMENTAL PROCEDURES

General methods and materials. Recrystallizations were performed in acetone/MeOH. Melting points (mp) taken on a heat block were uncorrected. Preparative (0.5 mm thick) thin layer chromatography (TLC) on silica gel with hexane/EtOAc (6:1, v/v) and argentation TLC (silica gel/$AgNO_3$, 4:1, w/w) with CCl_4/CH_2Cl_2 (5:1, v/v) were developed three and four times, respectively. The bands on silica gel and argentation TLC were observed under UV light (3600 Å) after spraying with a 0.05% solution in EtOH of rhodamine-6G and 2′,7′-dichlorofluorescein, respectively. Preparative high performance liquid chromatography (HPLC) was carried out on a Partisil 5 ODS-2 column (25 cm × 10 mm id) (Whatman, Clifton, New Jersey) with MeOH as a mobile phase (flow rate, 4 ml/min). Gas liquid chromatography (GLC) was performed with a Shimadzu GC-4CM instrument on a SCOT OV-17 glass capillary column (30 m × 0.3 mm id, column temperature 260 C). The Rc-values (relative mobilities) in the argentation TLC and the relative retention times (RRT) in the HPLC and GLC were expressed relative to cholesterol (**7a**) acetate (1.00). Mass spectra (EI-MS, 70 eV) were taken on a Hitachi M-80B double focusing gas chromatograph-mass spectrometer by means of a probe injection. Proton (^1H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a Hitachi R-250 (250 MHz for ^1H) or a JNM FX-100 (Japan Electron Optics Laboratory Co., Tokyo) (25.0 MHz for ^{13}C) instrument in a $CDCl_3$ solution, with tetramethylsilane as internal standard. Hydrogenation was performed in EtOH over PtO_2 at atmospheric pressure and temperature overnight. All other techniques used in this study have already been described (5). Bananas (*Musa sapientum* L.) were purchased at a local market. Two 3-oxo-4α-methylsteroids (**1d** and **1e**; see Table 1 for the systematic and trivial names), three 4,4-dimethylsterols (**2b**, **2d** and **2e**), four 4α-methylsterols (**3d**, **3e**, **4d** and **5d**) and seven 4-demethylsterols (**7a**, **7c**, **7d**, **7e**, **7f**, **7g** and **7h**) used as the reference specimens in this study were described in the previous articles (6,7).

Extraction and fractionation of the sterol mixture. Banana peel (3.7 kg) was air-dried and the tissue (420 g) was extracted with CH_2Cl_2 in a Soxhlet extractor. The extract (25 g) was saponified with 10% KOH in MeOH. The unsaponifiable lipid (13.1 g) obtained was fractionated by TLC, which gave 3-oxo-4α-methylsteroid (Rf = 0.88, 3.09 g, fraction A), 4,4-dimethylsterol (Rf = 0.52, 2.68 g, fraction B), 4α-methylsterol (Rf = 0.42, 1.43 g, fraction C) and 4-demethylsterol (Rf = 0.28, 0.84 g, fraction D). The latter three fractions were acetylated, and fraction A and the acetylated fractions B, C, and D were subjected to argentation TLC for further fractionation. Upon argentation TLC, a portion of fraction A (400 mg) eventually yielded highly pure **1d** (89 mg) and **1e** (24 mg), whereas a portion of the acetylated fraction B (900 mg) yielded **2b**- (120 mg), **2d**- (153 mg) and **2e**-acetates (171 mg). On the other hand, the acetylated fraction C (1.54 g) gave **3e**- (137 mg) and **5d**-acetates (6 mg) and a mixture (376 mg) of **3d**- and **4d**-acetates on argentation TLC. The mixture was then separated into **3d**- (RRT = 0.94 in HPLC, 210 mg) and **4d**-acetates (RRT = 0.76, 2 mg) by HPLC. Finally, argentation TLC of the acetylated fraction D (864 mg) gave **6e**- (5 mg) and **7g**-acetates (156 mg), a mixture (D-1, 243 mg) of **7a**-, **7c**- and **7f**-acetates, a mixture (D-2, 191 mg) of **6d**- and **7h**-acetates and a mixture (D-3, 10.5 mg) of **7d**- and **7e**-acetates. HPLC of the three mixtures enabled separation as follows: D-1: **7a**- (RRT = 1.00 in HPLC, 2 mg), **7c**- (RRT = 1.07, 15 mg) and **7f**-acetates (RRT = 1.18, 101 mg); D-2: **6d**- (RRT = 0.76, 119 mg) and **7h**-acetates (RRT = 1.01, 15 mg); D-3: **7d**- (RRT = 0.85, 2 mg) and **7e**-acetates (RRT = 0.79, 3 mg).

RESULTS AND DISCUSSION

Table 1 showed the mp, chromatographic data and molecular ion (M^+) in MS for the acetyl derivatives (3-ketones for **1d** and **1e**) of sterols identified and characterized in banana peel in this study. Table 1 lists the percentage compositions of sterols in each of the following four fractions: 3-oxo-4α-methylsteroid (A), 4,4-dimethylsterol (B), 4α-methylsterol (C) and 4-demethylsterol (D), which were determined based on the GLC and

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TABLE 1

Compositions of Four Sterol Fractions and Mp, Chromatographic Data and Molecular Ion (M^+) of Sterols from Banana Peel

Compound/Acetate		Mp (C)	RRT[a] (GLC)	Rc[a] (argentation TLC)	M^+ (m/z)	Percentage composition in each fraction
3-Oxo-4α-methylsteroid (fraction A)						
1d	24-Methylene-31-nor-5α-cycloartan-3-one (cycloeucalenone)[b]	85-86	1.51	0.20	424	71.2
(24*S*)-**1e**	(24*S*)-24-Methyl-31-nor-5α-cycloart-25-en-3-one (31-norcyclolaudenone)[b]	133-135	1.47	0.31	424	26.1
	Others, unidentified	—	—	—	—	2.7
4,4-Dimethylsterol (fraction B)						
2b	5α-Cycloart-24-en-3β-ol (cycloartenol)	120-122	1.86	0.80	468	23.1
2d	24-Methylene-5α-cycloartan-3β-ol (24-methylenecycloartanol)	114-116	2.07	0.41	482	39.0
(24*S*)-**2e**	(24*S*)-24-Methyl-5α-cycloart-25-en-3β-ol (cyclolaudenol)	116-118	2.03	0.64	482	37.9
4α-Methylsterol (fraction C)						
3d	Cycloeucalenol	115-116	1.77	0.36	468	70.0
(24*S*)-**3e**	31-Norcyclolaudenol	114-115	1.72	0.56	468	28.6
4d	24-Methylene-31-nor-5α-lanost-8-en-3β-ol (obtusifoliol)	106-109	1.49	0.40	468	1.4
5d	24-Methylene-31-nor-5α-lanost-9(11)-en-3β-ol	—	1.69	0.22	468	Trace
4-Demethylsterol (fraction D						
6d	24-Methylenepollinastanol	56-57	1.61	0.39	454	36.2
(24*S*)-**6e**	(24*S*)-24-Methyl-25-dehydropollinastanol	89-90	1.57	0.53	454	3.8
7a	Cholest-5-en-3β-ol (cholesterol)	—	1.00	1.00	368[c]	0.3
7c	24-Methylcholesterol (24*R* : 24*S* = 60 : 40)	133-135	1.31	1.03	382[c]	7.7
7d	24-Methylenecholesterol	—	1.35	0.18	380[c]	0.5
(24*S*)-**7e**	(24*S*)-24-Methyl-25-dehydrocholesterol	—	1.32	0.20	380[c]	0.3
(24*R*)-**7f**	(24*R*)-24-Ethylcholesterol (sitosterol)	134-136	1.63	1.02	396[c]	26.3
(24*S*)-**7g**	(22*E*,24*S*)-24-Ethyl-22-dehydrocholesterol (stigmasterol)	143-146	1.43	0.93	394[c]	20.7
7h	(24*Z*)-24-Ethylidenecholesterol (isofucosterol)	135-136	1.81	0.50	454	3.1
	Others, unidentified	—	—	—	—	0.8

[a]RRT and Rc were expressed relative to **7a**-acetate.

[b]Physical properties corresponded to the 3-ketone.

[c]M^+-HOAc.

argentation TLC data. Identification (with the exception of the configurational determination at C-24 as for the sterols possessing an asymmetric center at C-24) of the following compounds were carried out based on the comparison of RRT in GLC, Rc in argentation TLC and MS data, and for some compounds, ^{1}H NMR data, RRT in HPLC and mp data with the authentic compounds: **1d** and **1e** from fraction A; **2b**, **2d** and **2e** from fraction B; **3d**, **3e**, **4d** and **5d** from fraction C; and **7a**, **7c**, **7d**, **7e**, **7f**, **7g** and **7h** from fraction D.

High resolution mass spectrometry (MS) of **6d**-acetate showed M^+ at m/z 454.3771 (rel int 12%, $C_{31}H_{50}O_2$, calcd. 454.3807), with the following fragmentation ions: at m/z 439.3619 (7%, M^+-Me), 411.3267 (4%, M^+-C_3H_7), 394.3585 (95%, M^+-HOAc), 379.3332 (45%, M^+-Me − HOAc), 351.3051 (6%, m/z 411-HOAc), 329.2476 (5%, M^+-side chain [C_9H_{17}]), 300.2796 (6%, M^+-ring A [$C_9H_{14}O_2$]), 269.3360 (28%, M^+-C_9H_{17}-HOAc), 267.2111 (14%, m/z 269-2H), 213.1651 (8%, M^+-C_9H_{17}-ring D [C_3H_6]-CH_2-HOAc) and 55.0545 (100%), which suggested that the sterol has a monounsaturated C_9 side chain, and a skeleton with a 9β,19-cyclo group and an additional methyl group, probably located at C-14 (2,8,9). The 9β,19-cyclo structure was supported by the two methine doublets appearing at δ 0.081 and 0.443, which might be due to 19-H_2 group, in the ^{1}H NMR of **6d**-acetate (Table 2). The methyl ^{1}H signals of **6d**-acetate were almost consistent with the corresponding signals of **3d**-acetate, with one exception, that **6d**-acetate had no methyl doublet due to 30-H_3 group, as shown in Table 2. Thus, **6d** was considered to be 14α-methyl-24-methylene-9β,19-cyclo-5α-cholestan-3β-ol (24-methylenepollinastanol) (2,9), the occurrence of which is already known in banana peel (2).

High resolution MS of **6e**-acetate showed M^+ at m/z 454.3816 (rel int 12%, $C_{31}H_{50}O_2$), with the following fragmentation ions: at m/z 439.3626 (5%, M^+-Me), 394.3606 (86%, M^+-HOAc), 379.3348 (44%, M^+-Me-HOAc), 329.2507 (6%, M^+-side chain [C_9H_{17}]), 300.2817 (8%, M^+-ring A [$C_9H_{14}O_2$]), 269.2250 (30%, m/z 329-HOAc), 267.2099 (12%, m/z 329-HOAc-2H), 227.1820 (6%, m/z 269-ring D [C_3H_6]), 219.1231 (6%), 213.1657 (5%, m/z 269-C_3H_6-CH_2) and 69.0715 (100%), which suggested that the sterol has a monounsaturated C_9 side chain, and a skeleton with a 9β,19-cyclo group and an additional methyl group, probably located at C-14 (2,8,9). The ^{1}H NMR of **6e**-acetate showed the skeletal ^{1}H signals (10,11) at δ 0.075 and 0.438 (1H and *d* each, 19-H_2), 0.889 (3H, *s*, 32-H_3), 0.950 (3H, *s*, 18-H_3), 2.023 (3H, *s*, 3β-OAc) and

TABLE 2

^{1}H NMR Data (250 MHz, $CDCl_3$)[a] of the Acetates of (24*S*)-Methyl-Δ^{25}-sterols, Their 25-Dihydro Derivatives and Some 24-Methylene Sterols

Acetate	18-H_3[b]	19-H_2[c,d]	30-H_3[c]	32-H_3[b]	21-H_3[c]	26-H_3[c]	27-H_3[c,e]	28-H_3[c,f]
(24*S*)-**1e**[g,h]	0.997	0.393 (4.3) 0.618 (4.0)	0.989 (6.4)	0.899	0.871 (6.4)	1.639 (*s*)	4.671 (*s*)	1.002 (7.0)
(24*S*)-**2e**[g]	0.953	0.336 (4.3) 0.573 (4.0)	0.844 (*s*)	0.887	0.857 (6.4)	1.640 (*s*)	4.662 (*s*)	0.997 (7.2)
(24*S*)-**3e**[g]	0.959	0.145 (4.0) 0.397 (3.7)	0.843 (6.4)	0.887	0.860 (6.4)	1.640 (*s*)	4.667 (*s*)	0.998 (6.7)
(24*S*)-**6e**[g]	0.950	0.075 (4.0) 0.438 (4.3)	—	0.889	0.859 (6.1)	1.639 (*s*)	4.666 (*s*)	0.997 (6.7)
(24*S*)-**7e**[g]	0.670	1.016 (*s*)	—	—	0.911 (6.4)	1.636 (*s*)	4.661 (*s*)	0.992 (6.7)
(24*R*)-**7e**[i]	0.673	1.007 (*s*)	—	—	0.915 (5.6)	1.650 (*s*)	4.655 (*s*)	0.985 (6.9)
(24*S*)-**7e**[i]	0.671	1.006 (*s*)	—	—	0.910 (6.5)	1.636 (*s*)	4.658 (*s*)	0.992 (6.7)
(24*S*)-**1c**[h,j]	1.003	0.394 (4.0) 0.619 (3.7)	0.990 (6.4)	0.909	0.879 (6.4)	0.862 (7.0)	0.788 (6.7)	0.786 (6.7)
(24*S*)-**2c**[j]	0.958	0.337 (4.6) 0.575 (4.0)	0.845 (*s*)	0.897	0.865 (6.4)	0.859 (6.4)	0.785 (6.7)	0.781 (6.7)
(24*S*)-**3c**[j]	0.964	0.148 (4.3) 0.398 (4.0)	0.843 (6.1)	0.897	0.868 (6.4)	0.859 (6.4)	0.785 (6.7)	0.786 (6.7)
(24*S*)-**6c**[j]	0.955	0.078 (4.3) 0.441 (4.0)	—	0.893	0.868 (6.4)	0.859 (6.7)	0.786 (6.7)	0.781 (6.7)
(24*R*)-**6c**[k]	0.958	0.077 (3.7) 0.441 (4.0)	—	0.893	0.859 (6.7)	0.856 (6.7)	0.808 (6.7)	0.781 (6.7)
(24*S*)-					0.868 (6.4)	0.859 (6.7)	0.785 (7.0)	
3d[g]	0.970	0.150 (4.0) 0.401 (3.4)	0.844 (6.4)	0.902	0.897 (6.4)	1.026 (6.7) 1.031 (6.7)		4.665 (*s*) 4.718 (*s*)
5d[g]	0.657	1.002 (*s*)	0.843 (6.4)	0.784	0.911 (6.4)	1.025 (6.7) 1.031 (6.7)		4.663 (*s*) 4.718 (*s*)
6d[g]	0.963	0.081 (4.3) 0.443 (3.7)	—	0.898	0.898 (6.1)	1.025 (7.0) 1.031 (6.7)		4.664 (*s*) 4.716 (*s*)

[a]Given as δ values. Figures in parentheses denote J values in Hz. Other skeletal methyl signals: (24*S*)-**2e**-acetate, δ 0.887 (3H, *s*, 31-H_3); (24*S*)-**2c**-acetate, δ 0.888 (3H, *s*, 31-H_3).

[b]Singlet.

[c]Doublet if not otherwise specified.

[d]19-H_2 singlet signal as for the acetates of **5d** and **7e**.

[e]27-H_2 singlet signal as for the sterols with side chain **e**.

[f]28-H_2 signal as for the sterols with side chain **d**.

[g]Isolated from banana peel in this study.

[h]Data corresponded to the 3-ketone.

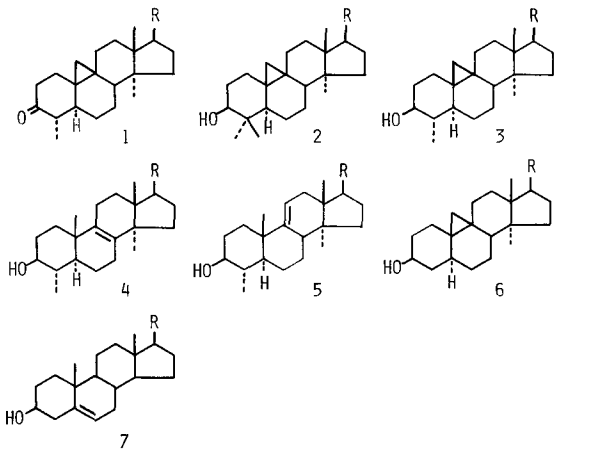
[i]Ref. 10; 360 MHz ^{1}H NMR data as for free sterol.

[j]Hydrogenation product of the Δ^{25}-sterols isolated from banana peel.

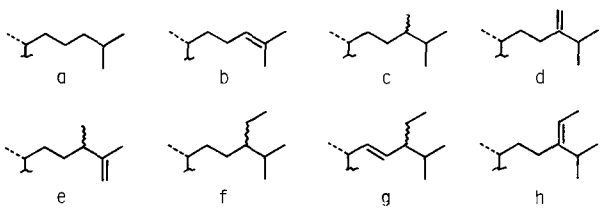
[k]Hydrogenation product of **6d**-acetate. Mixture of C-24 epimers.

4.80 (1H, *m*, 3α-H), which were almost indistinguishable from those of **6d**-acetate (Table 2), indicating the structure of 14α-methyl-9β,19-cyclo-5α-cholestan-3β-ol (**6**) for the skeleton of **6e**. The other ^{1}H signals at δ 0.859 (3H, d, 21-H_3), 0.997 (3H, *d*, 28-H_3), 1.639 (3H, *s*, 26-H_3) and 4.666 (2H, broad *s*, 27-H_2) were consistent with the 24-methyl-Δ^{25} side chain (12–15). The 26-H_3 singlet signal is a diagnostic measure for differentiating the C-24 epimers of 24-methyl-Δ^{25}-sterols (13,15), as has been revealed with the 24*R*- and 24*S*-epimers of 24-methylcholesta-5,25-dien-3β-ol (**7e**) which displayed the 26-H_3 signal at δ 1.650 and 1.636, respectively (13). Taking this into consideration, **6e**-acetate was regarded to have the 24*S*-configuration (26-H_3, δ 1.639). Thus, sterol **6e** was (24*S*)-14α,24-dimethyl-9β,19-cyclo-5α-cholest-25-en-3β-ol (Scheme 1), which is considered to be a new sterol. Hydrogenation of **6e**-acetate afforded **6c**-acetate (mp 61–63 C, M^+ = m/z 456, RRT = 1.56 in GLC). The ^{1}H NMR comparison (16) of the **6c**-acetate with the C-24 epimeric mixture of **6c**-acetate (mp 62–64 C, M^+ = m/z 456), obtained from **6d**-acetate by hydrogenation, supported the 24*S*-stereochemistry (Table 2).

Four other 24-methyl-Δ^{25}-steroids, i.e., **1e**, and the acetates of **2e**, **3e** and **7e**, isolated from banana peel in this



Side chains (R)



SCHEME 1

study, also showed the 26-H_3 signal in the field of δ 1.636–1.640 as shown in Table 2. This was consistent with the 24*S*-stereochemistry, and hence, these 24-methyl-Δ^{25}-steroids (**1e**, **2e**, **3e** and **7e**) were considered to be the 24*S*-epimers. Upon hydrogenation, **1e** and the acetates of **2e** and **3e** yielded **1c** (mp 105–107 C, M^+ = m/z 426, RRT = 1.46 in GLC), **2c**-acetate (mp 134–135 C, M^+ = m/z 484, RRT = 2.01) and **3c**-acetate (mp 119–120 C, M^+ = m/z 470, RRT = 1.71), respectively. The 21-H_3 and 27-H_3 signals in the ^{1}H NMR (Table 2) of the dihydro derivatives (**1c**, **2c** and **3c**) have supported the 24*S*-configuration. On the other hand, the 4-demethyl-Δ^5-sterols, **7f** and **7g**, isolated from banana peel in this study were shown to be the 24*R*(α)- and 24*S*(β)-epimers, respectively, whereas **7c** was shown to be the C-24 epimeric mixture (24*R*:24*S* = 60:40) by ^{13}C NMR spectroscopy (17,18).

This study has thus demonstrated the occurrence of a new (24*S*)-14α,24-dimethyl-Δ^{25}-sterol, (24*S*)-**6e**, accompanied by several 3-oxosteroids and sterols possessing (24*S*)-24-methyl-Δ^{25}-, 24-methylene- and other side chains. 4-Demethylsterols are biosynthesized from 4,4-dimethylsterols (lanostane- and cycloartane-triterpene alcohols) through 3-oxo-4α-methylsteroids and 4α-methylsterols by C-4 and C-14 demethylation (19). It is suggested, moreover, that there are three alternative routes into 24-methyl-Δ^{23}-, (24*S*)-24-methyl-Δ^{25} and 24-methylene-sterols at the first C_1 transfer at C-24 in the biogenesis of the sterol side chain in *Zea mays* (20,21). Taking these into consideration, we have postulated the operation of the following two biosynthetic pathways from **2b** in the tissue of banana peel: the Δ^{25}-route, **2b** → (24*S*)-**2e** → (24*S*)-**1e** → (24*S*)-**3e** → (24*S*)-**6e** →→ (24*S*)-**7e**; and the $\Delta^{24(28)}$-route, **2b** → **2d** → **1d** → **3d** → **6d** →→ **7d**. 4α-Methylsterol **5e** has previously been detected in olive oil from *Olea europaea* L. (5), and this study constituted the second case of its identification (see Table 2 for the ^{1}H NMR data) as the natural product.

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T. Takido permformed the ^{13}C NMR spectra and M. Aimi did the mass spectra.

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Reversibility of Cholinephosphotransferase in Lung Microsomes

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The effect of cytidine 5′-monophosphate (CMP) on the incorporation of cytidine 5′-diphosphate (CDP) [methyl-^{14}C]choline or [1-^{14}C]dipalmitoylglycerol into phosphatidylcholine (PC) catalyzed by rabbit lung microsomal CDPcholine:1,2-diacyl-*sn*-glycerol cholinephosphotransferase (EC 2.7.8.2) was studied. In the presence of 0.85 mM CMP and nonsaturating diacylglycerol concentration, the incorporation of CDP[^{14}C]choline into PC was markedly stimulated, but the incorporation of [^{14}C]dipalmitoylglycerol into PC was inhibited. This was due to the increase of endogenous diacylglycerol generated from microsomal PC by the cholinephosphotransferase reverse reaction. However, the newly synthesized PC was not readily hydrolyzed in the presence of CMP. The results of this study suggest that the endogenous membranous diacylglycerol is utilized more preferentially for PC synthesis than the exogenous diacylglycerol and that the newly synthesized PC could rapidly equilibrate with the endogenous membrane PC pool.
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In mammalian tissues, phosphatidylcholine (PC) is mainly synthesized by the de novo cytidine 5′-diphosphate (CDP) choline pathway in which three steps of enzymatic reactions are involved (1). The third step of the CDPcholine pathway involves a reaction between CDPcholine and 1,2-diacyl-*sn*-glycerol to yield PC and cytidine 5′-monophosphate (CMP) catalyzed by CDPcholine:1,2-diacyl-*sn*-glycerol cholinephosphotransferase (2). Since this reaction is reversible (2–4), it is generally believed that the presence of CMP in the cholinephosphotransferase reaction medium can inhibit PC synthesis when diacylglycerol concentration is at saturation (5). Since endogenous diacylglycerol concentration is far from saturation (6,7), it is not known how CMP could affect PC synthesis under these conditions. Therefore, the purpose of the present study is to investigate the effect of CMP on PC synthesis at nonsaturating diacylglycerol concentrations in the isolated lung microsomes.

MATERIALS AND METHODS

Reagents. CDP[methyl-^{14}C]choline (52.5 mCi/mmol), [methyl-^{14}C]choline (55 mCi/mmol), [9,10-^{3}H]trioleoylglycerol (114 Ci/mmol), [1-^{14}C]dipalmitoyl PC (100 Ci/mol) and [9,10-^{3}H]palmitic acid (20 Ci/mmol) were obtained from New England Nuclear (Boston, Massachusetts). 1,2-Dipalmitoyl-*sn*-glycerol, phosphatidylglycerol (PG) (from egg yolk PC), phosphatidic acid (PA), dithiothreitol (DTT), sodium fluoride, phospholipase D (from peanut) and CMP were products of Sigma Chemical Co. (St. Louis, Missouri). All other chemicals were of reagent grade.

Preparation of lung microsomes. An adult male New Zealand white rabbit was anesthetized with intravenous sodium pentobarbital (20–30 mg/kg body weight) and the thorax was opened. After exsanguination, the lungs were perfused in situ with saline via the pulmonary artery. During this perfusion, the lungs were repeatedly inflated to improve removal of blood. After the lungs were removed from the carcass and dissected free from bronchial tissue, lung tissue was homogenized in three volumes of 0.01 M Tris-HCl/0.33 M sucrose buffer (pH 7.4) in a Potter-Elvehjem homogenizer. Microsomes were obtained from 105,000 × g pellet from postmitochondria and lamellar body fractions. This procedure is detailed elsewhere (8). All steps of subcellular fractionation were carried out at 4 C. Microsomes were stored at −20 C before use.

Cholinephosphotransferase forward reaction with endogenous diacylglycerol. The reaction mixture contained 0.1 mM CDP[Me-^{14}C]choline (1.6×10^4 dpm/nmol), 50 mM Tris-HCl (pH 8.2), 20 mM $MgCl_2$, 1 mM DTT and 50 μg microsomal protein in a final volume of 0.3 ml. The reaction was initiated with the addition of microsomes or CDP[^{14}C]choline and conducted at 37 C in a Dubnoff shaking water bath for 10 min unless otherwise specified. The reaction was stopped by adding 2 ml chloroform/methanol (1:2, v/v) and the lipids were extracted by the method of Bligh and Dyer (9). PC was isolated by thin layer chromatography (TLC) on a precoated Silica Gel 60 plate (E. Merck, Darmstadt, Federal Republic of Germany) with a developing solvent system of chloroform/methanol/water (65:45:5, v/v/v). The plate was exposed in an iodine chamber for a few seconds after development, and the PC spot was scraped into a scintillation vial for radioactivity measurement.

Cholinephosphotransferase forward reaction with exogenous diacylglycerol. This procedure was similar to that described above. A sonicated suspension of 3.3 mM dipalmitoylglycerol, 2.7 mM PG and 0.03% Tween 20 in 0.25 M Tris-HCl buffer (pH 8.2) was prepared as described by Miller and Weinhold (10). The cholinephosphotransferase assay mixture contained 0.5 mM CDP[Me-^{14}C]-choline (1.1×10^3 dpm/nmol), 50 mM Tris-HCl (pH 8.2), 20 mM $MgCl_2$, 1 mM DTT and various amounts of sonicated dipalmitoylglycerol and microsomes (50 μg proteins) in a final volume of 0.5 ml. The reaction was conducted at 37 C for 10 min and stopped with 2 ml chloroform/methanol (1:2, v/v). Lipids were extracted and PC was isolated and counted as described above.

Isolation of [methyl-^{14}C]choline or [9,10-^{3}H]palmitic acid-labeled lung microsomes. A two-month-old New Zealand rabbit was injected with 0.5 mCi [Me-^{14}C]choline or 1 mCi sodium salt of [^{3}H]palmitate in 1 ml saline via the ear vein. After 60 min the animal was killed, the lungs were perfused with saline and removed, and the microsomes were isolated as described above.

Reverse reactions of cholinephosphotransferase and CDPethanolamine:1,2-diacyl-sn-glycerol ethanolaminephosphotransferase. The reverse reaction of cholinephosphotransferase was conducted in a 1.5 ml conical polypropylene centrifuge tube containing 50 mM Tris-HCl (pH 8.2), 20 mM $MgCl_2$, 1 mM DTT, ^{14}C-labeled microsomes with about 50 μg protein (44 nmol PC) and 0.85 mM CMP in a final volume of 0.3 ml (3). The reaction was carried out at 37 C for 10 min and stopped with the addition of 30 μl of 10% trichloroacetic acid (3). Microsomes were precipitated by centrifugation at 10,000 rpm for 5 min with a microfuge. An aliquot of 0.3 ml supernatant was transferred to a scintillation vial and

evaporated to dryness. Its radioactivity was determined. In some studies the radioactivity distribution among CDPcholine, phosphocholine and choline in the reaction mixture was determined as described by Post et al. (6).

The reverse reaction of ethanolaminephosphotransferase was determined by the same method as the cholinephosphotransferase reverse reaction, except that the ^{14}C-labeled microsomes were replaced with ^{3}H-labeled microsomes. At the end of the reaction, lipids were extracted, phosphatidylethanolamine (PE) was isolated by TLC as described above and its radioactivity was determined. The rate of the reverse reaction was measured from the decrease of ^{3}H in PE as compared with the radioactivity in the control which contained no CMP.

Phosphatidic acid phosphatase. [1-^{14}C]Dipalmitoyl PA was prepared from [1-^{14}C]dipalmitoyl PC hydrolyzed by phospholipase D (11). The free acid form of PA was extracted and isolated by silicic acid column chromatography. The substrate [^{14}C]PA was prepared by sonicating 5 mM PA (9336 dpm/nmol) in 0.1 M Tris-HCl (pH 7.4) at 40 W for 3 min in ice water under N_2. The assay of PA phosphatase was conducted in a mixture containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1 mM [^{14}C]PA and about 50 μg microsomal proteins in a final volume of 0.5 ml at 37 C for 10 min (12). Lipids were extracted by the method described above; diacylglycerol and PA were separated by silica gel TLC with a developing solvent of chloroform/methanol/ammonium hydroxide (65:35:5, v/v/v) and their radioactivities determined.

Other microsomal enzyme assays. Acyl-CoA:lysoPC acyltransferase (8), alkaline phosphatase (13) and NADPH cytochrome C reductase (14) were assayed by the established methods. Triacylglycerol lipase activity was determined by measuring the liberated free fatty acids from [^{3}H]trioleoylglycerol substrate under the cholinephosphotransferase assay conditions described above. The substrate of [^{3}H]trioleoylglycerol (3 mM, 1 μCi) was prepared by sonication of this lipid in a 1-ml mixture containing 2.7 mM PG, 0.03% Tween 20 and 0.25 M Tris-HCl (pH 8.2) as similarly described for dipalmitoylglycerol preparation (10). After the lipase assay, lipids were extracted, the free fatty acids were isolated by silica gel TLC with a developing solvent of petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v) and their radioactivities were counted.

Radioactivity determination and other analyses. Radioactivity of the sample was measured in 10 ml of scintillation fluid (3.3 g of 2,5-diphenyloxazole in 400 ml toluene and 200 ml ethylene glycol monoethyl ether) with a BetaTrac 6895 liquid scintillation counter (Tracor Analytic, Elk Grove Village, Illinois), and was expressed as dpm derived via the external standard ratio method with automated quench correction. Protein content of microsomes was measured by the method of Lowry et al. (15) with bovine serum albumin as a standard.

RESULTS

The effect of CMP on the incorporation of CDP[^{14}C]choline into PC. Under optimal assay conditions, cholinephosphotransferase activity with either endogenous or exogenous diacylglycerols was linear for at least 10 min incubation and 60 μg microsomal proteins. With CDP[^{14}C]choline at saturation (0.1 mM) and endogenous diacylglycerol as the cosubstrate, CMP markedly stimulated the incorporation of CDP[^{14}C]choline into PC (Fig. 1). A maximum of 9-fold stimulation at ca. 0.85 mM CMP was observed; the intensity of stimulation started to decline at higher CMP concentrations. At lower CDPcholine concentrations (10 μM), the maximum stimulation decreased to sixfold, at which the optimal CMP level was 0.5 mM, and the maximum stimulation decreased further to 3.7-fold at 2.7 μM CDPcholine, at which optimal CMP concentration was 0.15 mM.

With the presence of exogenous dipalmitoylglycerol, CMP at 0.85 mM had no significant effect on the Vmax (14.28 nmol/min per mg protein without CMP vs 12.50 nmol/min per mg protein with CMP), but it decreased the apparent Km of the enzyme for dipalmitoylglycerol from 0.25 mM to 0.03 mM in the presence of NaF (Fig. 2). CMP, however, decreased the enzyme activity by 30% at 2 mM and 40% at 5 mM at a saturating concentration of dipalmitoylglycerol (data not shown).

The effect of CMP on PC synthesis from CDPcholine and [^{14}C]dipalmitoylglycerol. As described above, CMP at 0.85 mM stimulated the incorporation of CDP[^{14}C]-choline into PC when dipalmitoylglycerol was at concentrations below saturating (Fig. 2). In contrast, CMP at 0.85 mM inhibited the incorporation of [1-^{14}C]dipalmitoylglycerol into PC under the same conditions (Fig. 3). The inhibition was significant at lower dipalmitoylglycerol concentrations (p values at 0.1, 0.2 and 0.5 mM dipalmitoylglycerol are <0.002, <0.002 and <0.10, respectively, as determined by the unpaired Student's t-test).

Reverse reaction of cholinephosphotransferase. Under the cholinephosphotransferase assay conditions, 0.85 mM CMP in the reaction mixture induced the breakdown of microsomal [^{14}C]PC (Table 1), similar to previous observations (3,4,16,17). The amount of ^{14}C radioactivity decreased in PC was approximately equal to that found in the aqueous fraction after the microsomes were precipitated. Over 95% of the radioactivity in the aqueous phase was associated with CDPcholine even after 30 min

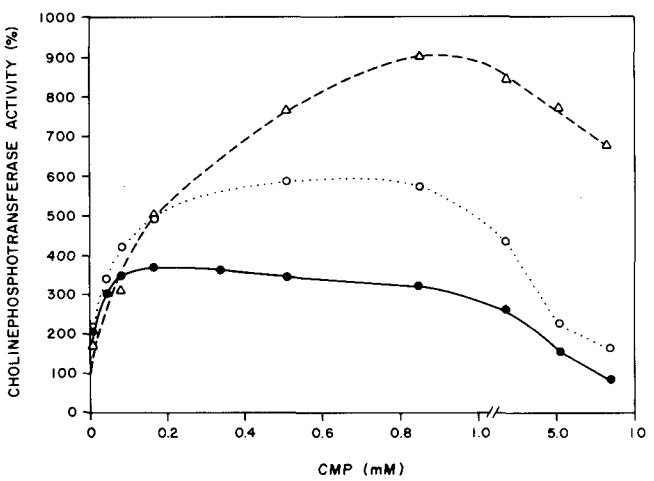

FIG. 1. Effect of CMP on the synthesis of PC from CDP[^{14}C]choline and microsomal endogenous diacylglycerol. Reaction conditions are described in Materials and Methods. The enzyme activities of the controls (at 0 mM CMP) were 63.00 ± 5.53, 87.26 ± 2.07 and 138.98 ± 7.87 pmol PC/min per mg protein (mean ± SD, n = 3) at 2.7 μM (●), 10 μM (○) and 0.1 mM (△) CDP[Me-^{14}C]choline, respectively.

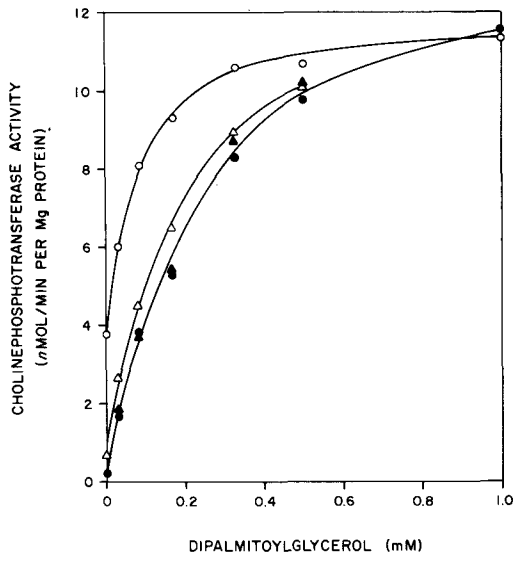

FIG. 2. Effect of CMP on the synthesis of PC from CDP[^{14}C]choline and exogenous dipalmitoylglycerol. Before assay, microsomes were preincubated in the reaction mixture (Tris, Mg^{2+}, DTT) (●) and the reaction mixture containing 0.85 mM CMP (△), 20 mM NaF (▲) or 0.85 mM CMP and 20 mM NaF (○) at 37 C for 15 min. Each point is the average of duplicate determinations.

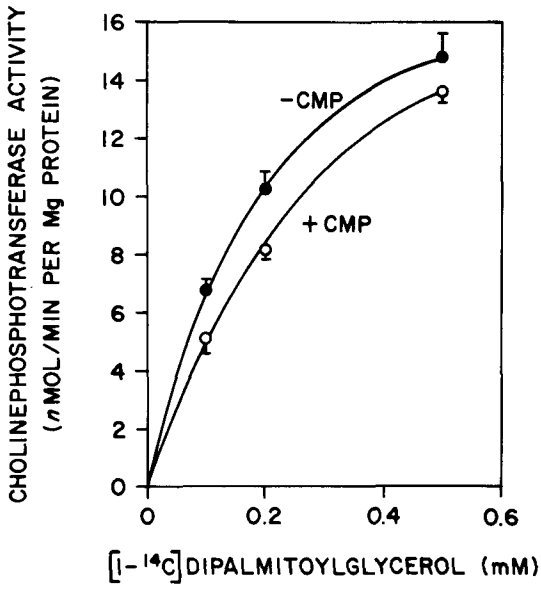

FIG. 3. Effect of CMP on the synthesis of PC from [1-^{14}C]dipalmitoylglycerol. Microsomes were preincubated with 0.85 mM CMP and 10 mM NaF for 15 min at 37 C before adding [^{14}C]dipalmitoylglycerol and 0.5 mM CDPcholine (○). The control contained no CMP or NaF (●). Bars represent mean ± SD of four experiments.

TABLE 1

Effects of CMP on the Activities of Microsomal Enzymes

Enzymes	Enzyme activity[a] −CMP	+CMP (0.85 mM)
Cholinephosphotransferase[b] reverse reaction	n.d.	4.56 ± 0.87
Ethanolaminephosphotransferase[b] reverse reaction	n.d.	0.48 ± 0.16
PA phosphatase[b]	11.20 ± 2.88	11.21 ± 3.12
Triacylglycerol lipase[b]	0.32 ± 0.05	0.214 ± 0.12
Acyl-CoA:lysoPC acyltransferase[b]	3.93 ± 0.06	3.66 ± 0.35
Alkaline phosphatase[c]	0.20 ± 0.03	0.21 ± 0.02
NADPH cytochrome c reductase[c]	2.40 ± 0.32	1.25 ± 0.31

n.d., Not detectable.

[a]Results are mean ± SD of triplicate determinations.

[b]Enzyme activity expressed as nmol product/min per mg protein.

[c]Enzyme activity expressed as OD/min per mg protein.

incubation. No CDP[^{14}C]choline could be detected in control which contained no CMP. This ruled out the possibility of microsomal phospholipase C reaction activated by CMP. The reverse reaction was inhibited by 60% by 0.5 mM CDPcholine, similar to the inhibition observed in liver microsomes (3), and by 45% by 0.3 mM exogenous diacylglycerol.

The effects of CMP on other microsomal enzymes. The level of microsomal endogenous diacylglycerol could be elevated from the hydrolysis of PA or triacylglycerol during the cholinephosphotransferase reaction. However, the activities of PA phosphatase and triacylglycerol lipase were not increased by 0.85 mM CMP (Table 1). It seems that CMP did not affect other microsomal enzymes, such as acyl-CoA:lysoPC acyltransferase, alkaline phosphatase and NADPH cytochrome C reductase (Table 1).

Although the rate of the breakdown of PE measured in this study might represent a total rate from the reactions of the reverse ethanolaminephosphotransferase and phospholipase A, no significant phospholipase A activity

could be detected as assessed by measuring radiolabeled lysoPC. Thus, the breakdown of PE was mainly catalyzed by reverse ethanolaminephosphotransferase, and this reaction rate was much slower than that of cholinephosphotransferase (Table 1). This was similar to the reaction observed in liver microsomes (3). This is comparable to the reaction rates of the forward reactions of both enzymes. In lung, the forward reaction rate of cholinephosphotransferase was about six times faster than that of ethanolaminephosphotransferase (18). Therefore, we concluded that most endogenous diacylglycerol generated by CMP was derived from PC.

DISCUSSION

Two substrates, CDPcholine and diacylglycerol, are required for PC synthesis catalyzed by the microsomal cholinephosphotransferase. The results of this study show that when CDPcholine was radioactively labeled and the cosubstrate diacylglycerol was rate-limiting, the incorporation of CDP[^{14}C]choline into PC was stimulated by CMP at certain concentrations (Figs. 1 and 2). In contrast, when diacylglycerol was radioactively labeled, the incorporation of [^{14}C]dipalmitoylglycerol into PC was inhibited by the same CMP concentration at which the stimulation of the incorporation of CDP[^{14}C]choline was observed (Fig. 3). The stimulation of the incorporation of CDP[^{14}C]-choline into PC affected by CMP could be explained as a result of the increase of endogenous diacylglycerol derived mainly from microsomal membranous PC catalyzed by the cholinephosphotransferase reverse reaction.

Although earlier studies have demonstrated that the CMP-generated diacylglycerol can be reused for PC or PE synthesis, these results were obtained after CMP was removed from the CMP-preincubated microsomes (4,16, 17,19,20). The present findings indicate that the diacylglycerol derived from PC can be immediately reused for PC synthesis even in the presence of CMP, supporting the previous suggestion that cholinephosphotransferase is near equilibrium (21,22).

The inhibition of the incorporation of [^{14}C]dipalmitoylglycerol into PC affected by CMP (Fig. 3) cannot be explained simply as a result of the isotope dilution effect on the specific radioactivity of substrate due to the increase of endogenous diacylglycerol. Under the assay conditions, the amount of endogenous diacylglycerol produced from microsomal PC and 0.85 mM CMP was 2.3 nmol, as estimated from the results in Table 1. This endogenous diacylglycerol was only 2.3% of the amount of total diacylglycerol at 0.2 mM dipalmitoylglycerol but caused a 20% inhibition of the incorporation of [^{14}C]-dipalmitoylglycerol (Fig. 3). On the contrary, the increased 2.3 nmol CDPcholine produced from 0.85 mM CMP did not inhibit the incorporation of CDP[^{14}C]choline (Fig. 1). Therefore, the isotope dilution effect does not seem significant enough to cause an inhibition of the incorporation of the radiolabeled substrate in a homogenous solution. Since endogenous and exogenous diacylglycerols are at a heterogenous physical state, the inhibition of the incorporation of exogenous dipalmitoylglycerol into PC due to the production of a small amount of endogenous diacylglycerol probably occurs because the endogenous diacylglycerol produced from membranous PC is utilized more preferentially than the exogenous diacylglycerol for PC synthesis under the present assay conditions.

The preferential utilization of endogenous diacylglycerol over exogenous diacylglycerol can also be explained from the results of Figure 2. For example, the amount of PC synthesized at less than 0.2 mM dipalmitoylglycerol increased two- to threefold in the presence of 0.85 mM CMP. This is not likely attributable to the slight percentage increase of diacylglycerol pool unless the 2.3 nmol endogenous diacylglycerol produced from membranous PC was preferentially utilized for PC synthesis.

Because of the marked stimulation of the incorporation of CDP[^{14}C]choline into PC in the presence of CMP, one may assume that the newly synthesized PC is not readily hydrolyzed by the reverse reaction while the hydrolysis of endogenous PC continues. This might be explained in that the newly synthesized PC was less than 2.3 nmol in the assayed microsomes which contained about 44 nmol endogenous PC; thus the newly synthesized PC might rapidly equilibrate with the large PC pool in membranes. If this is the case, the hydrolysis of the newly synthesized PC is likely to be negligible.

Ample evidence has suggested that the second step of the CDPcholine pathway catalyzed by CTP:cholinephosphate cytidylyltransferase is the rate-limiting step in PC synthesis (23). However, other factors, such as CMP concentration, might also influence both diacylglycerol and PC concentrations. In this study, we observed that the incorporation of CDPcholine into PC was more effectively stimulated if both NaF and CMP were present in the assay medium (Fig. 2). NaF is assumed to inhibit 5′-nucleotidase activity that hydrolyzes CMP (24). During lung development, the pattern of 5′-nucleotidase activity is similar to the developing profile of the rate of PC synthesis (24). All these seem to suggest that the regulation of CMP level might have influence on the PC content.

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Evidence that Cholesteryl Ester Hydrolase and Triglyceride Lipase Are Different Enzymes in Rat Liver

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Studies on intracellular cholesteryl ester hydrolase (CEH) and triglyceride lipase (TGL) from rat adipose tissue and adrenal cortex have suggested that a single protein is responsible for both activities. To determine whether one hepatic protein catalyzes both reactions, we studied several properties of CEH and TGL in rat liver. During liver perfusion with heparin, perfusate peaks of TGL and CEH did not consistently coincide, and TGL activity was considerably higher and less heat-stable than that of CEH. Significant TGL, but not CEH, activity was released during incubation of isolated hepatocytes. Although microsomes isolated from hepatocytes contained both activities, the specific activities of CEH and TGL in cytosol from hepatocytes were 95% and 3%, respectively, of those found in cytosol from whole liver. Preincubation of liver cytosol with 5 mM Mg^{2+} decreased CEH, but not TGL, activity. Intracellular CEH and TGL activities were completely separated by prep-disc gel electrophoresis. Finally, both cytosolic and microsomal TGL, but not CEH, activities were inhibited by antiserum against rat hepatic TGL. We conclude that extracellular TGL does not have CEH activity and intracellular CEH differs from TGL.
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Hormone-sensitive triglyceride lipase (TGL), purified from rat adipose tissue, also hydrolyzes cholesteryl esters and is activated by phosphorylation by a cyclic-AMP-dependent protein kinase (1). Cholesteryl ester hydrolase (CEH) and TGL activities from adipose tissue have similar patterns of subcellular distribution and copurify extensively (2).

The adrenal cortex contains both TGL and CEH, which provides free cholesterol for steroidogenesis. Both activities from rat adrenal are stimulated by ACTH (3,4), copurify through gel filtration and are activated by phosphorylation (5). CEH from bovine adrenal cortex is activated by phosphorylation (6) and appears to be the same enzyme as hormone-sensitive TGL from rat adipose tissue (7).

Lipoprotein lipase, an extracellular enzyme, is distinct from intracellular TGL/CEH in adipose tissue (8). The adrenals also contain an extracellular lipase which differs from intracellular TGL/CEH (9).

Heparin injections release lipoprotein lipase and hepatic TGL in humans, rats and rabbits (10–12). In contrast with lipoprotein lipase, the metabolic role of hepatic TGL is not well established. The specific activity of TGL in isolated plasma membranes is 10 times that found in liver homogenates; addition of heparin releases TGL from the membranes (13). These observations favor the location of hepatic TGL on the external surface of the cell membrane, where the enzyme may play an important role in the catabolism of lipoproteins before they are taken up by hepatocytes or other cells.

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Little is known about the function of hepatic neutral CEH. CEH activity has been found in human liver (14) and in both the cytosolic (15,16) and microsomal fractions (16) from rat liver. Two studies found no enrichment of neutral CEH activity in isolated plasma membranes from rat liver and provided evidence against the action of a cell surface CEH in the breakdown of the cholesteryl esters before or during the penetration of chylomicron remnants into hepatocytes (16,17).

To examine whether extracellular hepatic TGL has CEH activity and whether intracellular CEH has TGL activity, we compared several properties of the two enzymes.

MATERIALS

Potassium phosphate (monobasic and dibasic), glutathione, cholesteryl oleate, triolein, Triton X-100 and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, Missouri). Heptane, NaOH and EDTA were from Mallinckrodt Inc. (Paris, Kentucky). Tris was obtained from Calbiochem-Behring Corp. (La Jolla, California). Bovine serum albumin was purchased from Accurate Chemical Scientific Corp. (Westbury, New York). Ethanol was from Warner-Graham (Cockeysville, Maryland). Ammonium sulfate was purchased from Bio-Rad Lab. (Richmond, California). Sodium pentobarbital was from Abbott Lab (North Chicago, Illinois). Heparin sodium (beef lung) was obtained from Upjohn (Kalamazoo, Michigan). Collagenase was from Worthington Biochemical Co. (Freehold, New Jersey). [*Carboxyl*-^{14}C]triolein (109 mCi/mmol), cholesteryl [1-^{14}C]oleate (56.6 mCi/mmol) in benzene and Aquasol-2 were purchased from New England Nuclear (Boston, Massachusetts). Sucrose and other solvents and chemicals were obtained from J.T. Baker Chemical Co. (Phillipsburg, New Jersey). Goat antiserum against rat heparin-releasable, hepatic TGL was obtained through Andre Bensadoun, Cornell University (Ithaca, New York).

METHODS

Male Sprague-Dawley albino rats weighing about 225 g were purchased from Charles River Breeding Laboratories (Wilmington, Massachusetts). They were maintained on a reversed 12 hr light-12 hr dark cycle and fed water and Purina rat chow ad libitum.

Preparation of liver homogenates and subcellular fractions. After rats were killed by cervical dislocation, the liver was homogenized in a buffer containing 5 mM glutathione, 25 mM Tris and 225 mM sucrose, pH 7.8. The homogenate was centrifuged at 4 C at 10,000 × g for 20 min in an IEC B-20 centrifuge. The supernatant was then spun at 100,000 × g for 75 min in a Beckman L2-50 ultracentrifuge to isolate microsomes and 100,000 × g supernatant (S_{100}).

Isolation and incubation of hepatocytes. Hepatocytes were isolated by the method of Feldhoff et al. (18) with minor modifications previously described (19).

Approximately four million hepatocytes, with viability greater than 90%, were incubated at 37 C in a total volume of 3 ml in a water shaker bath at 100 cycles/min. After 1 hr cells were separated from the medium by centrifugation in plastic tubes at 4 C at 1000 rpm for 10 min in a model CL International clinical centrifuge. The cell pellet was washed gently twice and resuspended in homogenization buffer. Cells were sonicated with a Biosonik II cell disrupter at 4 C for 30 sec. Hepatocyte microsomes and S_{100} were prepared from the sonicate as described above. The microsomal pellet was washed twice with microsomal suspension buffer containing 100 mM sucrose, 50 mM KF, 40 mM potassium phosphate, pH 7.2, 30 mM EDTA and 20 mM dithiothreitol.

TGL assay. TGL was assayed by the method of Krauss et al. (11) with minor modifications. Labeled triolein (20 μCi) was dried and 1.5 ml cold triolein solution (194.1 mg/ml) was added to make a working stock. Substrate for 30 assays was prepared by drying 220 μl working stock and 10 μl Triton X-100, adding 1 ml 10% bovine serum albumin, 1 ml 0.15 M NaCl and 1 ml 2.0 M Tris HCl, pH 8.2. The solution then was sonicated on ice, 30 sec on and 30 sec off, for 4.5 min with a Model W-225R sonicator from Heat Systems-Ultrasonics Inc. (Plainview, New York). Water (2 ml) was added to make a final substrate volume of 5 ml. Each assay was carried out with 150 μl substrate and 50 to 100 μl enzyme preparation at 30 C in a water bath shaker at 100 cycles/min. After 30 min, the reaction was stopped with 3.25 ml of methanol/chloroform/heptane (3.85:3.42:2.73, v/v/v) and 1.05 ml 0.05 M sodium carbonate, pH 10.5. Each tube was vortexed immediately and the phases were separated by centrifugation for 25 min at 2000 rpm. Supernatant (1 ml) was removed into 6.5-ml scintillation vials. Aquasol-2 (5 ml) was added and samples were counted in a Beckman LS 3150P scintillation counter with an efficiency of 90% for ^{14}C.

CEH assay. CEH activity was measured by a modification of the method used by Khoo et al. (8). Substrate for 30 assays was prepared by drying 21 μl of labeled cholesteryl oleate (2.1 μCi) and adding 1.23 mg cold cholesteryl oleate in 150 μl ethanol. Then 10.5 cc of 56 mM sodium phosphate, pH 7.0, containing 294 mg bovine serum albumin was added. Each assay was carried out with 350 μl substrate and 50–100 μl enzyme preparation at 30 C for 30 min. The reaction was terminated by adding 1.5 ml of the solvent mixture described above and 50 μl 1 N NaOH, pH 11. Each tube was vortexed immediately and the phases were separated by centrifugation for 10 min at 2000 rpm. Supernatant (0.5 ml) was removed into 12-ml scintillation vials and counted after adding Aquasol-2 (10 ml).

Separation of CEH and TGL from the S_{100} fraction of whole liver homogenate. To examine whether intracellular TGL and CEH activity can be separated, S_{100} fraction from whole liver homogenate was applied to the prep-disc gel electrophoresis (purchased from Canalco's Applications Laboratory) and eluted with buffer containing 50 mM triethanolamine, 1 mM EDTA and 5 mM glutathione, pH 7.4. The eluted fractions were assayed for both enzyme activities. A visible protein band retained on the gel was cut out of the gel. This gel slice was homogenized and protein eluted with homogenization buffer was assayed for both activities.

Effect of goat antiserum against rat heparin-releasable, hepatic TGL on TGL and CEH activities. Two hundred μl rat liver cytosol or microsomes were preincubated in the presence or absence of 10 μl of antiserum raised in goats against rat heparin-releasable, hepatic TGL for 60 min at 4 C prior to assays of TGL and CEH activities. One ml of goat antiserum inhibits the activity of hepatic TGL equivalent to 5300 μmol fatty acid released per hour.

Other methods. Liver perfusion is described in the legend for Figure 1. Protein concentrations were determined by the method of Lowry et al. (21).

RESULTS

Time course of heparin release of TGL and CEH during liver perfusion. As shown in Figure 1, no TGL or CEH activity was detected in the perfusate prior to the addition of heparin. A single peak of TGL was released 1.5–2 min after heparin injection in each of the three studies. In contrast, the time course for the release of CEH activity was variable, and several peaks of CEH activity were detected after heparin injection in each study. The highest activities for both enzymes coincided at 2 min of heparin perfusion in one experiment (Fig. 1A). In two other studies (Figs. 1B and 1C) the highest CEH activities (at 0.5 min) were observed earlier than the peak for TGL. The mean peak activity of TGL in the perfusate was 4830 times that of CEH (Table 2).

Incubation of postheparin perfusates at 37 C for 1 hr decreased TGL activity by 80% ($p < 0.01$) but did not significantly alter CEH activity.

Effects of Mg^{2+} and Mn^{2+} on the activities of TGL and CEH in the S_{100} fraction from whole liver homogenates. The TGL and CEH activities of S_{100} from whole liver homogenates were 2690 ± 243 and 24.4 ± 4.7 pmol fatty acid released/mg protein/min incubation, respectively. As shown in Table 1, preincubation of S_{100} at 30 C for 15 min with 5 mM $MgCl_2$ did not change TGL activity but decreased CEH activity by 33% ($p < 0.01$). In similar preincubations with 5 mM $MnCl_2$, TGL activity fell by 17% ($p < 0.01$) while CEH activity increased by 30% ($p < 0.01$).

TABLE 1

Effect of Mg^{2+} and Mn^{2+} on the Activities of Triglyceride Lipase (TGL) and Cholesteryl Ester Hydrolase (CEH) in the S_{100} Fraction from Whole Liver Homogenates

Additions during preincubation	Enzyme activities (% of control)[a]	
	TGL	CEH
None	100	100
Mg^{2+} (5 mM)	99 ± 4	67 ± 14[b]
Mn^{2+} (5 mM)	83 ± 1[b]	130 ± 10[b]

S_{100} fraction from whole liver homogenates (200 μl) was incubated at 30 C for 15 min in the presence and absence of 5 mM $MgCl_2$ or $MnCl_2$ prior to assaying enzyme activities. The enzymatic activities following preincubation in the absence of either metal (control) are presented as 100%. Activities in the controls were 15.4 ± 2.8 pmol/mg/min for CEH and 2787 ± 433 pmol/mg/min for TGL.

[a]The results are given as means ± SD for five experiments.

[b]$p < 0.01$.

TGL and CEH activities in incubation medium and subcellular fractions of isolated hepatocytes. TGL, but not CEH, activity was detected in the medium after incubation of hepatocytes for 1 hr (Table 2). The specific activities of TGL (81 ± 63 pmol/mg/min) and CEH (23.2 ± 4.2 pmol/mg/min) in the S_{100} fraction of hepatocytes were 3% and 95%, respectively, of those found in the S_{100} from whole liver homogenate. Both TGL and CEH activities, 1210 ± 173 and 69 ± 16 pmol/mg/min, respectively, were detected in microsomes from hepatocytes.

Separation of CEH and TGL from S_{100} of whole liver homogenates. CEH activity (79 pmol/mg/min), but no

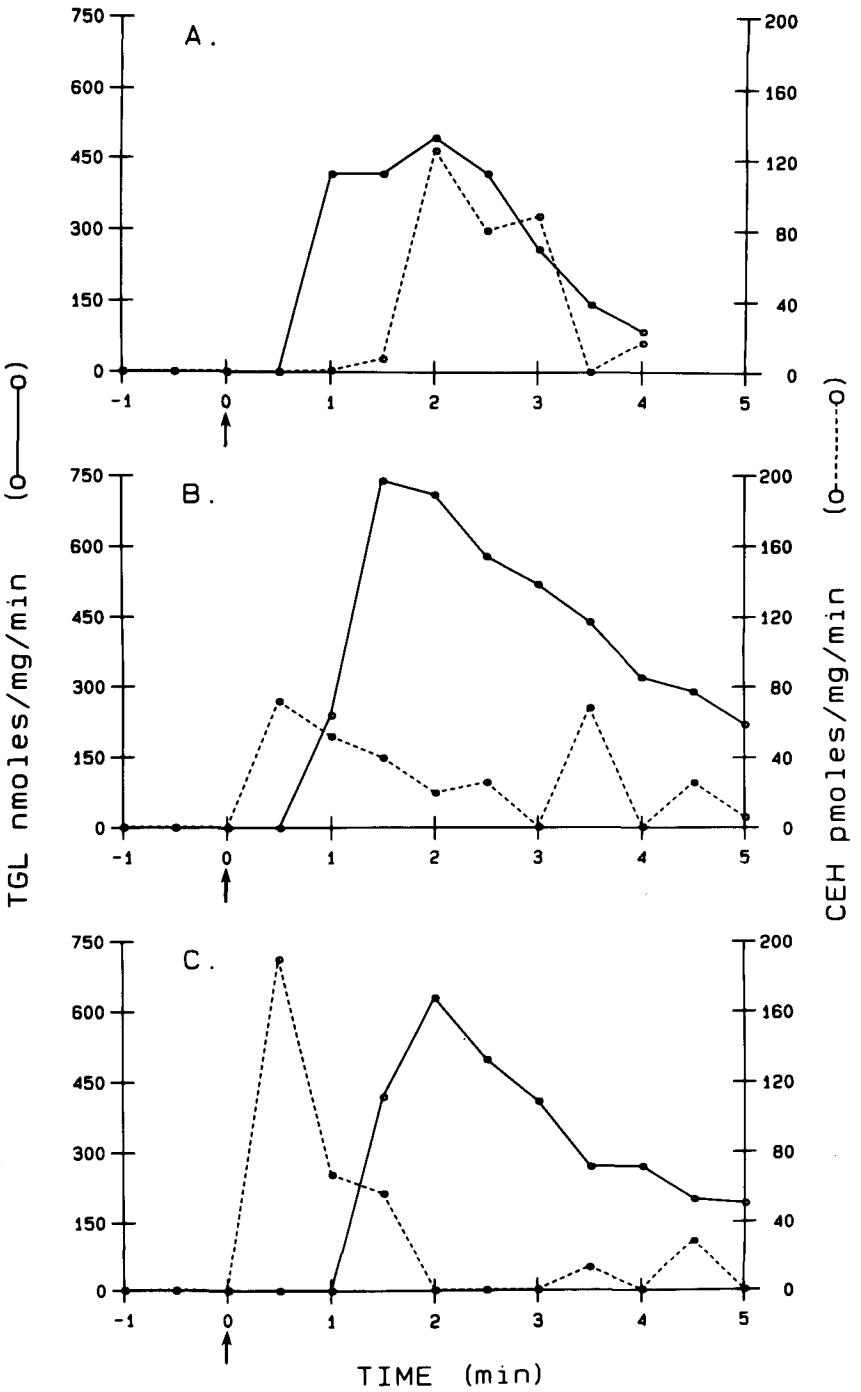

FIG. 1. CEH and TGL activities before and after heparin injection in liver perfusate of (A) first, (B) second and (C) third rat. Heparin sodium (22 units/ml perfusate) was injected at the time indicated by the arrows into the portal vein of three perfused livers prepared by the techniques described by Exton (20). Aliquots, collected every 30 sec for 1 min before and 4–5 min after heparin injection, were assayed for activities of CEH (● – – – ●) and TGL (● —— ●).

TGL activity, was eluted at an elution volume of 660 ml from prep-disc column. The residual protein band retained on the gel contained TGL activity (1610 pmol/mg/min) and no CEH activity.

Effect of goat antiserum against rat heparin-releasable, hepatic triglyceride lipase on TGL and CEH activities. Preincubation of rat liver cytosol and microsomes with antiserum raised in goats against rat heparin-releasable, hepatic TGL reduced cytosolic and microsomal TGL activities by 91% and 78%, respectively, but did not change cytosolic or microsomal CEH activities.

DISCUSSION

Our data provides several lines of evidence that extracellular hepatic TGL does not have CEH activity and that intracellular TGL and CEH are separate proteins. These include different time course, heat stability and relative activity of the two enzymes in postheparin perfusates; different effects of metals (Mg^{2+} and Mn^{2+}) on the enzyme activities; different intracellular/extracellular and parenchymal/nonparenchymal locations; separation of intracellular CEH from TGL during gel electrophoresis; and different effects of antiserum against rat hepatic TGL on the enzyme activities.

Following heparin administration, a single peak of TGL activity was released into the liver perfusate after 1.5–2 min, whereas multiple peaks of CEH activity were released at different times (Fig. 1). The occurrence of multiple peaks of CEH activity in each perfusion study, in contrast to the single peak of TGL activity, makes it difficult to attribute the relatively low CEH activity in the postheparin perfusate to a specific release of CEH by heparin. It is possible that heparin damage of cell membranes causes the release of small amounts of intracellular CEH. Although both enzyme activities peaked at 2 min after heparin injection in one perfusion, TGL, but not CEH, activity declined rapidly during incubation at 37 C for 1 hr.

The observations that the two enzyme activities were separated by prep-disc electrophoresis and that an antibody which inhibited hepatic TGL activity did not reduce CEH activity provided strong evidence indicating they are different proteins.

The ratio of TGL/CEH activities in the postheparin perfusate (Table 2) was much higher than the ratios of TGL/CEH from any other preparation. Although both activities were present in the microsomal fraction of hepatocytes, significant TGL, but not CEH, activity was released into the medium from hepatocytes. Antibody against heparin-releasable, hepatic TGL also inhibited microsomal TGL activity. These observations support the hypothesis that both enzymes are synthesized in hepatocytes, but TGL is secreted from the cell and binds to endothelial cells where it plays a role in the catabolism of lipoproteins (22–26), while CEH acts within the hepatocyte to cleave cholesteryl esters. Little or no CEH activity can be detected in rat liver plasma membrane (16,17), whereas heparin releases TGL from isolated plasma membranes in which TGL activity is 10 times that of liver homogenates (13). TGL is also secreted into the medium from hepatocytes (19,27,28). Jansen et al. reported that the secretion of TGL and its activity within hepatocytes were markedly reduced by cycloheximide, an inhibitor of protein synthesis, and suggested that TGL secretion was dependent on protein synthesis (27).

We found little or no TGL activity in cytosol obtained from hepatocytes. (No activity at all was detected in two experiments.) The average specific activity of TGL in cytosol from hepatocytes was only 3% of that in cytosol from whole liver homogenates; CEH activity in cytosol from hepatocytes was 95% of that in cytosol from liver homogenates (Table 2). It was thus estimated that while the total activities of TGL in hepatocytes only accounted for 2–5% of total TGL activities in whole liver, 51–99% of total CEH activities in liver derived from hepatocytes. This observation provides indirect evidence that most of the TGL activity detected in the cytosol from whole liver homogenates is present in nonparenchymal cells which may play an important role in the catabolism of lipoprotein lipids. Kuusi et al., using immunofluorescence and immunoelectron microscopy to localize heparin-releasable TGL, found TGL exclusively located on the surface of liver endothelial cells and on the surface of developing endocytotic vesicles and intracellular vesicles of endothelial cells. They detected no labeling of hepatocytes or Kupffer cells (29). Nonparenchymal rat liver cells, after intravenous injection of iodinated human HDL and LDL,

TABLE 2

TGL and CEH Activities of the Postheparin Liver Perfusate, Incubation Medium, Microsomes and Cytosol of Isolated Hepatocytes, and Cytosol from Whole Liver Homogenates

	TGL[a] (pmol/mg/min)	CEH[a] (pmol/mg/min)	Ratio of TGL/CEH
Peak activity of postheparin liver perfusate	623,000 ± 98,000	129 ± 48	4830
Medium following incubation of hepatocytes	580 ± 35	0	—
Hepatocyte microsomes	1,210 ± 173	69.0 ± 16.0	18
Hepatocyte cytosol	81 ± 63	23.2 ± 4.2	3
Cytosol from whole liver homogenate	2,690 ± 253	24.4 ± 4.7	110
Ratio of cytosol from hepatocytes/cytosol from liver homogenate	0.03	0.95	—

Liver perfusion described in Fig. 1. Preparation of subcellular fractions and enzyme assays are described in Methods.
[a]The results represent means ± S.D.

contained 4.2 and 6.3 times the amount of radioactivity, respectively, compared to parenchymal cells (30). In vitro nonparenchymal liver cells bound 100 times more hepatic TGL, purified from postheparin rat serum, than did parenchymal cells, and heparin-releasable hepatic TGL is mainly located (85–92%) on the surface of nonparechymal cells (31). In humans, familial deficiency of hepatic TGL is associated with a higher proportion of triglycerides and phospholipids in high density (HDL) and low density lipoprotein (LDL) as well as an increase in very low density lipoprotein (VLDL) with β-mobility (32). Men exhibit a highly significant negative correlation between the cholesterol and phospholipid content of HDL_2 and postheparin hepatic TGL activity (26). Treatment of rats with a specific antibody against hepatic TGL elevates the content of phospholipids in HDL, LDL and intermediate density lipoprotein, the cholesterol content of HDL, and the triglycerides in LDL and VLDL (22–25).

In summary, we conclude that extracellular hepatic TGL does not have CEH activity and intracellular CEH differs from TGL. Our results and previous reports also support the hypothesis that both TGL and CEH are synthesized in hepatocytes. TGL is then secreted and binds to the surface of endothelial cells where, like the model described for lipoprotein lipase in adipose tissue (33,34), it may play an important role in the catabolism of lipoproteins before they are taken up by hepatocytes. CEH remains within hepatocytes where it may regulate the intracellular metabolism of cholesteryl esters.

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The Effect of Feeding Fish Oils, Vegetable Oils and Clofibrate on the Ketogenesis from Long Chain Fatty Acids in Hepatocytes

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Groups of rats were fed diets containing 25% fish oil (FO), 25% soybean oil, 25% partially hydrogenated fish oil (PHFO), 25% partially hydrogenated soybean oil (PHSO), 25% partially hydrogenated coconut oil or 0.3% clofibrate for 3 wk. After the animals were fasted for 24 hr, hepatocytes were isolated and ketogenesis from added palmitate, linoleate *cis* and *trans*, arachidonate and docosahexaenoate was measured. Ketogenesis after oil feeding was significantly stimulated (two- to threefold) only in cells from the PHFO- and PHSO-fed rats. The stimulation was most apparent with the long chain unsaturated fatty acids as substrates. These fatty acids were relatively poor ketone body precursors in control hepatocytes. Essential fatty acid deficiency did not seem to be the reason for this stimulation. Clofibrate also stimulated ketogenesis significantly (1.5- to 3-fold). The degree of stimulation increased with chain length and degree of unsaturation of the substrate. The activity of the enzyme 2,4-dienoyl-CoA reductase was also studied in the same groups. Its activity was stimulated about fourfold in the clofibrate-treated rats and to a lesser extent by the PHFO, PHSO and FO diets. The activity showed no correlation with the content of unsaturated fatty acids in the diet or their oxidation in isolated hepatocytes. The 2,4-dienoyl-CoA reductase, therefore, does not seem to be a regulatory enzyme in the metabolism of dietary polyunsaturated fatty acids. It is concluded that an induction of the peroxisomal β-oxidation system most likely is involved in the reported increases in ketogenesis from very long chain polyunsaturated fatty acids.
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Since Lazarow and de Duve's discovery of a clofibrate-inducible peroxisomal β-oxidation system (1), this inducibility has been extended to drugs structurally unrelated to clofibrate (2) as well as to high fat diets. Particularly, fats containing very long chain fatty acids (C_{22}), such as rapeseed oil and hydrogenated fish oil, have a pronounced effect (3). One striking observation was that clofibrate and partially hydrogenated fish oil (PHFO) specifically increased the capacity of the hepatocyte to oxidize 22:1 fatty acids, most likely because these diets induce an increased chain-shortening capacity by the peroxisomal β-oxidation system (4,5).

Another interesting observation was that both a high fat diet (6) and clofibrate (7) lead to an increased activity of the enzyme 2,4-dienoyl-CoA reductase (E.C. 1.3.1), which is an auxiliary enzyme in the metabolism of polyunsaturated fatty acids (8–11). Liver mitochondria from rats fed these diets show an increased capacity to oxidize polyunsaturated fatty acids, and this increased capacity is at least partially explained by the increased activity of the 2,4-dienoyl-CoA reductase (6,7,9,11).

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In the present study, we have fed rats different fat diets and clofibrate and studied the capacity of isolated hepatocytes from these animals to oxidize fatty acids of different chain lengths and unsaturation.

Fish oil (FO), PHFO, soybean oil (SO), partially hydrogenated soybean oil (PHSO) and partially hydrogenated coconut oil (PHCO) were chosen as fat diets. These diets have been shown to induce widely varying activities of the peroxisomal chain-shortening β-oxidation system (12) and of the 2,4-dienoyl-CoA reductase (6,7).

Palmitate, oleate, linoleate (*cis* and *trans*), arachidonate and docosahexaenoate were chosen as substrates. These fatty acids depend to a varying degree on the peroxisomes for chain shortening and on the 2,4-dienoyl-CoA reductase for their complete β-oxidation.

MATERIALS AND METHODS

Materials. [U-^{14}C]Palmitate and [U-^{14}C]arachidonate were purchased from the Radiochemical Centre (Amersham, England). Unlabeled fatty acids were purchased from Fluka AG, NuChek Prep Inc. (Elysian, Minnesota), and Sigma Chemical Co. (St. Louis, Missouri). β-Hydroxybutyrate dehydrogenase, sorbic acid and coenzyme A were from Sigma. Clofibrate [ethyl 2-(4-chlorophenoxy)2-methyl propionate] was from Fluka AG. All other reagents and chemicals were of analytical grade or the highest purity available. Fatty acids were prepared in a 6% BSA, Krebs/Henseleit buffer (pH 7.4) in a concentration of 4 mM.

Animals and diets. All animals were male rats of the Wistar strain purchased from Møllegaards Breeding Laboratory (Ejby, Denmark). For experiments with normal or clofibrate-treated rats, they were bought as 160–180-g animals and fed a standard pellet diet or a pellet diet containing 0.3% (w/w) clofibrate for 3–4 wk. Rat pellets with 0.3% clofibrate were prepared as described (12).

For the fat-feeding experiments, we purchased weanling rats of ca. 60-g body weight. The animals were kept in grid-bottomed cages with a 12 hr light-12 hr dark cycle; they were fed the standard pellet diet for 5 days after arrival and then the indicated fat diet for 3–4 wk. The following diets (percentage by weight) were used: 25% FO, 25% PHFO, 25% SO, 25% PHSO and 25% PHCO. Five percent SO and 5% SO + 20% PHFO or 5% SO + 20% PHSO were used as control diets in different experiments. The dietary oils, including their analytical data, were obtained from De No Fa and Lilleborg Fabriker A/S (Fredrikstad, Norway). The fatty acid composition of the oil diets is given in Table 1, and the preparation of the semisynthetic diets has been described (13). All diets contained 20% protein, fat as stated in the tables (pellets, 4%) and 50% carbohydrate (low fat, 69%). The 5% SO diet was chosen as control as it had no significant inducing effect on the peroxisomal β-oxidation enzyme system compared to a standard pellet diet (12).

The animals had free access to the diets in the feeding

TABLE 1

Fatty Acid Composition of Dietary Oils[a]

Fatty acid	Fish oil (FO)	Partially hydrogenated fish oil (PHFO)	Soybean oil (SO)	Partially hydrogenated soybean oil (PHSO)	Partially hydrogenated coconut oil (PHCO)
8:0					8.2
10:0					6.4
12:0					45.9
14:0	6.7	6.8			16.8
16:0	14.7	12.8	10.9	10.6	8.7
16:1	9.5	9.9			
18:0	1.0	3.0	3.8	14.0	11.3
18:1	16.1	15.1	24.3	73.8	1.3
18:2	1.6	2.5	52.5		
18:3			7.5		
20:0		2.4			
20:1	12.8	16.4			
20:2	5.4	4.3			
20:5	9.0				
22:0		3.0			
22:1	13.5	14.5			
22:2		1.9			
22:6	6.2				
Trans double bonds (%)		55		55	55

[a]Only major fatty acids (1% or more) are included in the table. Fish oils were obtained from capelin *(Mallotus villosus).*

period (3–4 wk) and were fasted for 24 hr before they were killed. Previous fasting is known to increase the capacity for fatty acid oxidation in isolated hepatocytes (14), and it also reduces the level of inhibiting malonyl-CoA to a minimum (15). All experiments were started between 9 and 10 a.m.

Preparation of liver cells. The rats were anesthetized with ether, and hepatocytes were prepared and purified according to Seglen (16) with minor modifications. Krebs/Henseleit bicarbonate buffer (pH 7.4) containing 0.5 mM $CaCl_2$ and fatty acid-free bovine serum albumin (BSA) (1% and 3.5% in preincubation and incubation with substrates, respectively) was used as cell suspension medium.

Incubation conditions. Incubations were performed in 25-ml Erlenmeyer flasks under 95% O_2/5% CO_2 at 37 C. The cells were preincubated for 20 min with 1 mM (−)carnitine (17) to ensure that low carnitine levels did not limit fatty acid oxidation rates. Incubations were started by mixing 1 ml preincubated hepatocytes with 1 ml of the desired fatty acid at the desired concentration and running for 1 hr unless otherwise stated. The final incubation mixture contained fatty acid at the desired concentration, 0.5 mM (−)carnitine, 3.5% BSA and hepatocytes corresponding to the stated cell protein concentration. The incubation flasks were flushed with 95% O_2/5% CO_2 twice during the incubation and closed with a rubber stopper. Incubations were stopped by pouring the cells into 0.5 ml ice-cold 2 M $HClO_4$ and centrifuging. The supernatant was decanted and neutralized with KOH to pH 7. The samples were stored at −20 C until measured.

Assay procedures. Cell protein was determined by the method of Lowry et al. (18) after sedimentation, resuspension in water and solubilization in 0.1 M KOH. BSA was used as standard. Ketone bodies were measured by the method of Williamson and Mellanby (19). 2,4-Dienoyl-CoA reductase activity was assayed at 30 C in a 0.2% deoxycholate extract of washed cells (8). Protein in the extract was determined according to Whitaker and Granum (20). Sorboyl-CoA was synthesized as described (21), and the concentration was determined by use of a molar extinction coefficient of $23 \cdot 10^3$ cm^{-1} at 300 nm (22). The Wilcoxon two-sample rank test was used for statistical treatment of the results.

RESULTS

Food consumption and weight gain. Food consumption and weight gain of the animals in one experiment were followed. There were no statistically significant differences between the groups, although Figure 1 shows that the 25% PHFO- and 25% PHSO-fed animals possibly gained weight more slowly than the others. The fastest weight gain was obtained with hydrogenated fat supplied with 5% SO. The food consumption for the groups was between 21.4 (25% PHFO) and 22.3 (20% PHFO + 5% SO) g/animal/day.

Ketogenesis as a measure of fatty acid oxidation. Since we wanted to measure the oxidation of fatty acids not available with ^{14}C label, we checked whether ketone body formation could be used as a measure of oxidation rate. Figure 2 shows that the relation between the enzymatically measured ketone bodies and the acid-soluble radioactivity from [U-^{14}C]palmitate and [U-^{14}C]arachidonate calculated as ketone bodies differs depending on the fatty acid concentration in the medium. At the lower concentrations, the acid-soluble radioactivity accounted for only part of the ketone bodies formed, presumably because of a significant dilution by endogenous substrates. However,

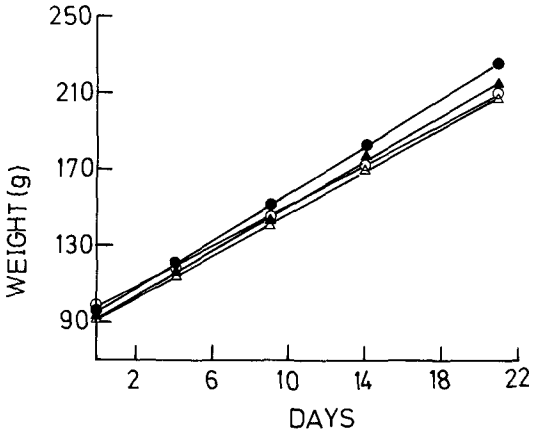

FIG. 1. Weight gain in differently fed animals. Diets: △, 25% partially hydrogenated soybean oil; ▲, 20% partially hydrogenated soybean oil + 5% soybean oil; ○, 25% partially hydrogenated fish oil; ●, 20% partially hydrogenated fish oil + 5% soybean oil.

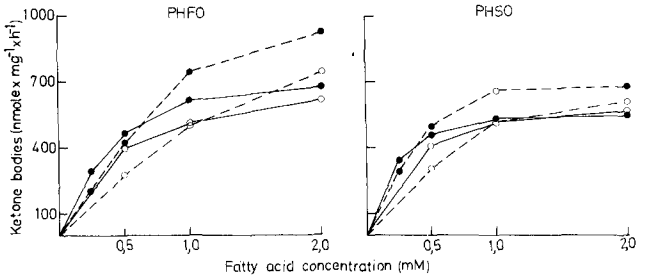

FIG. 2. Ketone body formation at different fatty acid concentrations. Ketone bodies are measured both enzymatically (——) and as acid-soluble radioactivity (— — —). ○, [U-^{14}C]palmitate as substrate; ●, [U-^{14}C]arachidonate as substrate; PHFO, partially hydrogenated fish oil; PHSO, partially hydrogenated soybean oil.

with 2 mM fatty acid in the medium, the formation of ketone bodies calculated from the acid-soluble radioactivity was 15–25% higher than what could be measured enzymatically. By anion exchange chromatography of the acid-soluble palmitate metabolites according to La Noue et al. (23), we found that part of this difference can be explained by about 5% labeled metabolites other than ketone bodies. Some acetyl carnitine formation is also possible. There is also a moderate enzymatic underestimation of ketone bodies because of ≈95% recovery in the enzymatic analysis. The results are presented without correction for these systematic errors. The formation of CO_2 was disregarded since with 1 mM palmitate in the medium only about 9% of the uniformly labeled palmitate oxidized in hepatocytes from fed rats is converted to CO_2, and in fasted rats the contribution from labeled CO_2 is more than halved (24). From these results (Fig. 2) and previous studies (25), we conclude that, with 2 mM fatty acid in the medium, the oxidation of endogenous fatty acids is suppressed and ketogenesis is a reasonably good measure for the capacity of the cells to β-oxidize the fatty acids added. We cannot exclude the possibility that ketogenesis itself may become rate-limiting for the oxidation of palmitate and oleate, which showed the highest oxidation rates. However, a limiting effect of ketogenesis will tend to diminish differences between different fatty acids as ketone body precursors, and therefore cannot explain the dietary effects observed.

Effects of oil diets. Table 2 shows that both 25% PHFO and 25% PHSO diets stimulate the ketogenesis from the incubated fatty acids significantly over the 5% SO control. This stimulation is also significantly higher than the oxidation in cells from 25% FO- and 25% SO-fed rats ($P = 1–5\%$ and $P < 1\%$, respectively). This indicates that hydrogenation of oils containing long chain unsaturated fatty acids gives a product with an effect similar to clofibrate in feeding experiments (Table 4). In support of this, feeding 25% PHCO, which contains short chain saturated fatty acids, gave no stimulation of the β-oxidation (Table 2).

The partially hydrogenated oils contain almost no essential fatty acids (Table 1). To exclude essential fatty acid deficiency as a cause of the observed effects, we also fed rats 20% PHFO + 5% SO and 20% PHSO + 5% SO in a separate experiment. Feeding the hydrogenated oil together with SO ensures an adequate supply of essential fatty acids. In this experiment (Table 3), we obtained a decreased oxidation that is statistically significant for the PHFO/PHFO + SO combination. For PHSO/PHSO+SO, the effect of adding 5% SO was statistically insignificant. Also, Table 2 shows that the diet with 25% PHCO, with no essential fatty acids (Table 1), had only a weak and mostly insignificant effect on ketogenesis capacity.

Effects of clofibrate. Table 4 shows that ketogenesis from palmitate, oleate and linoleate *cis* and *trans* probably is close to maximum rates at 2.0 mM fatty acid in the incubation both for normal and clofibrate cells. For arachidonate and docosahexaenoate, the maximum rate is already reached at 0.5 mM for the normal cells and at 1.0 mM for the clofibrate cells. This means that the capacity for β-oxidation of long chain polyunsaturated fatty acids is increased in the clofibrate cells.

Table 4 also shows that clofibrate stimulates ketogenesis significantly at all the fatty acid concentrations used. For the polyunsaturated fatty acids, the stimulation is most pronounced at the highest fatty acid concentrations, while it is nearly equal at all concentrations of palmitate and oleate. At 2.0 mM, the stimulation of palmitate ketogenesis was about 50%, while ketogenesis from docosahexaenoate increased 200%.

Effects on the 2,4-dienoyl CoA reductase. Table 5 shows that the reductase in clofibrate cells is stimulated about fourfold compared to cells from 5% SO-fed animals. It is striking that 25% PHSO with a low level of unsaturated fatty acids stimulated the enzyme activity, while 25% SO with a high content of unsaturated fatty acids did not. Both FO diets stimulated the enzyme activity significantly.

DISCUSSION

The experiments reported here show that 25% PHFO and 25% PHSO diets increased ketogenesis, especially from the polyunsaturated fatty acids. The effects of the hydrogenated oils are significantly different from those of the corresponding unhydrogenated oils. Experiments with labeled palmitate and arachidonate show that ketone bodies account for most of the fatty acids oxidized.

TABLE 2

Effect of Oil Feeding on the β-Oxidation of Different Fatty Acids

Fatty acid (2 mM)	nmol ketone bodies·mg^{-1}·h^{-1} ± S.E.M.					
	5% SO (4)	25% SO (6)	25% PHSO (4)	25% FO (5)	25% PHFO (4)	25% PHCO (4)
Palmitate (16:0)	253.9 ± 10.8 (1.0)	342.1 ± 31.4 (1.3)	575.9 ± 12.5[a] (2.3)	339.5 ± 34.2 (1.3)	448.1 ± 77.6 (1.8)	301.4 ± 34.7 (1.2)
Linoleate (18:2)	131.6 ± 6.6 (1.0)	265.4 ± 26.6[b] (2.0)	508.5 ± 48.2[a] (3.9)	271.8 ± 39.5[a] (2.1)	495.0 ± 41.5[a] (3.8)	259.4 ± 39.7[a] (2.0)
Linoleate (18:2)tt[c]	186.0 ± 14.4 (1.0)	264.1 ± 24.8 (1.5)	456.3 ± 47.9[a] (2.5)	244.9 ± 30.3 (1.4)	426.2 ± 32.1[a] (2.4)	252.3 ± 29.3 (1.4)
Arachidonate (20:4)	162.5 ± 16.8 (1.0)	213.7 ± 27.3 (1.3)	464.6 ± 29.0[a] (2.9)	240.2 ± 29.4 (1.5)	432.7 ± 30.3[a] (2.7)	204.3 ± 24.8 (1.3)
Docosahexaenoate (22:6)	165.0 ± 15.6 (1.0)	254.4 ± 28.9[a] (1.5)	503.0 ± 50.8[a] (3.1)	259.6 ± 48.3 (1.6)	469.5 ± 13.9[a] (2.8)	227.6 ± 36.1 (1.4)
Endogenous ketone bodies	59.3 ± 3.1	144.6 ± 16.3[b]	107.9 ± 19.3[b]	113.0 ± 11.1[b]	98.5 ± 7.0[b]	82.7 ± 5.8
Cell protein concentration in incubation (mg/ml)	2.6 ± 0.2	1.4 ± 0.2	2.6 ± 0.3	1.8 ± 0.2	3.7 ± 1.7	2.7 ± 0.2

Endogenous values are subtracted from the results. Numbers in parentheses besides oil abbreviations (see Table 1) denote animals in each group. Numbers in parentheses beneath nmol values are ratios compared to the 5% SO-fed control group.

[a]Significantly different from 5% SO-fed group: P = 1-5%.

[b]Significantly different from 5% SO-fed group: P < 1%.

[c]*trans, trans.*

TABLE 3

Effect of Feeding Hydrogenated Marine and Soybean Oils Supplemented with 5% Soybean Oil on Ketogenesis from Different Fatty Acids

Fatty acid (2 mM)	nmol ketone bodies·mg^{-1}·h^{-1} ± S.E.M.			
	25% PHFO (4)	20% PHFO + 5% SO (4)	25% PHSO (4)	20% PHSO + 5% SO (4)
Palmitate (16:0)	619.7 ± 19.3	487.4 ± 26.1[a]	561.8 ± 24.7	529.6 ± 28.3
Linoleate (18:2)	621.8 ± 19.3	518.5 ± 15.2[a]	554.3 ± 19.5	474.6 ± 21.3
Linoleate (18:2)tt[b]	512.3 ± 34.6	423.6 ± 17.8	411.3 ± 22.8	364.4 ± 9.5
Arachidonate (20:4)	683.9 ± 41.8	456.7 ± 21.2[a]	543.2 ± 28.3	445.9 ± 21.4[a]
Docosahexaenoate	549.7 ± 42.4	276.3 ± 11.8[a]	442.0 ± 17.1	315.1 ± 29.8
Endogenous ketone bodies	98.2 ± 9.6	168.3 ± 23.8[a]	105.8 ± 7.4	101.4 ± 7.9
Cell protein concentration in incubation (mg/ml)	3.4 ± 0.2	2.6 ± 0.2	2.7 ± 0.2	3.4 ± 0.5

Endogenous values are subtracted from the results. Numbers in parentheses denote animals in each group (see Table 1 for abbreviations).

[a]Significantly different from corresponding group without 5% SO with P = 1-5%.

[b]*trans,trans.*

High fat diets have been shown to induce increased levels of the ketogenic enzymes, including HMG-CoA synthetase, which is the rate-limiting enzyme in ketogenesis (26). Such an induction evidently can contribute to the increased ketogenesis seen in our experiments. However, it cannot explain the relatively much stronger effect on the ketogenesis from polyunsaturated fatty acids.

From the above-mentioned points, it is evident that the hydrogenated oils and clofibrate induce increased activity of some step(s) prior to the formation of acetyl-CoA in the mitochondria or peroxisomes. What is the nature of this induction and what is its cause?

The possibility of any malonyl-CoA effect on our results is not likely. The malonyl-CoA level in livers from fasted rats is known to be low (15), presumably because fasting increases the intracellular level of long chain acyl-CoA, which inhibits the acetyl-CoA carboxylase. Incubation of the cells with 2 mM fatty acid will also give a high level of acyl-CoA, which competes with malonyl-CoA for its inhibitory binding sites in the mitochondria.

Steroid-like substances (phytosterols) present in vegetable oils could also be suspected to affect ketogenesis. Based on Ref. 27 it can be stated that SO contains 250 mg phytosterols/100 g, while hydrogenated

TABLE 4

Effect of Clofibrate Feeding on the β-Oxidation of Different Fatty Acids

Fatty acid (mM)		nmol ketone bodies·mg^{-1}·h^{-1} ± S.E.M. Normal (6)	Clofibrate (7)	Clofibrate/Normal
Palmitate (16:0)	0.5	192.4 ± 15.0	277.6 ± 39.2[a]	1.4
	1.0	285.0 ± 18.5	434.9 ± 48.0[b]	1.5
	2.0	350.1 ± 28.5	519.8 ± 33.7[b]	1.5
Oleate (18:1)	0.5	192.7 ± 8.4	315.1 ± 27.4[b]	1.6
	1.0	288.5 ± 15.5	502.7 ± 43.8[b]	1.7
	2.0	324.1 ± 26.3	556.4 ± 35.5[b]	1.7
Linoleate (18:2)	0.5	157.5 ± 8.1	241.2 ± 21.5[a]	1.5
	1.0	206.9 ± 12.0	368.1 ± 37.1[a]	1.8
	2.0	264.1 ± 9.4	530.5 ± 62.0[a]	2.0
Linoleate (18:2)tt[c]	0.5	175.0 ± 5.8	250.2 ± 30.5[a]	1.4
	1.0	228.7 ± 6.5	366.2 ± 42.2[a]	1.6
	2.0	234.4 ± 11.6	486.3 ± 52.6[a]	2.1
Arachidonate (20:4)	0.5	172.8 ± 11.8	313.4 ± 33.7[a]	1.8
	1.0	196.5 ± 11.7	451.8 ± 67.8[a]	2.3
	2.0	196.1 ± 13.8	469.8 ± 35.0[a]	2.4
Docosahexaenoate (22:6)	0.5	164.5 ± 14.6	377.9 ± 39.0[b]	2.3
	1.0	165.4 ± 17.8	454.5 ± 45.3[b]	2.7
	2.0	146.8 ± 15.5	453.6 ± 46.1[b]	3.1
Endogenous ketone bodies		47.3 ± 5.6	79.4 ± 11.2	—
Cell protein concentration in incubation (mg/ml)		4.4 ± 0.3	4.4 ± 0.5	

Endogenous values are subtracted from the results. Numbers in parentheses denote animals in each group.

[a]P = 1-5%.

[b]P < 1.

[c]*trans,trans.*

TABLE 5

2,4-Dienoyl-CoA Reductase Activity in Cells from Differently Fed Animals

Diet	nmol NADPH oxidized·mg^{-1}·min^{-1} ± S.E.M.
5% SO (6)	37.5 ± 5.2
25% SO (6)	31.5 ± 3.4
25% PHSO (5)	80.5 ± 9.9[a]
25% FO (7)	72.5 ± 6.4[b]
25% PHFO (4)	70.6 ± 7.1[b]
25% PHCO (6)	68.9 ± 14.8
0.3% Clofibrate (2)	122.6

Numbers in parentheses denote animals in each group. See Table 1 for diet abbreviations.

[a]Significantly different from 5% SO-fed group: P = 1-5%.

[b]Significantly different from 5% SO-fed group: P = < 1%.

SO contains 132 mg/100 g. Thus, Table 2 shows an inverse relationship of phytosterol content to the effect on ketogenesis by the same oils.

Thomassen et al. (12) have reported significant differences in the peroxisomal oxidation between animals fed 25% PHFO, 25% FO, 25% PHSO and 25% SO. In their study on a subcellular fraction enriched in peroxisomes, they found that hydrogenation of three different oils (FO, SO and rapeseed oil) led in every case to a significant induction of the peroxisomal β-oxidation. While we have observed significant stimulation of ketogenesis, but no significant difference between the effects of 25% PHFO and 25% PHSO, they found the 25% PHFO to be more efficient than 25% PHSO. A limiting effect of the enzymes of ketogenesis itself in these high rate groups may be the reason why we observed no significant difference between these high effect diets. Wong et al. (28) found a significant effect of feeding 15% FO compared to 15% safflower oil on endogenous ketogenesis in perfused rat liver. Our results show no difference in the endogenous ketogenesis in cells prepared from animals fed 25% FO and 25% SO, but they are still significantly higher than the 5% SO control (Table 2).

During the preparation of this manuscript, Hertz et al. (29) reported increased peroxisomal β-oxidation-related enzyme activities in cultured rat hepatocytes grown in the presence of bezafibrate or long chain fatty acids. Their results show a specific increase in the peroxisomal β-oxidation-related enzyme activities compared to the mitochondrial ones.

Taken together, our results and a substantial amount of evidence from other studies indicate that it is the peroxisomal β-oxidation that is preferentially induced by the

high fat diets, and that this induction is the main reason for an increased rate of oxidation and ketogenesis, particularly from very long chain fatty acids.

It is striking that the feeding of both hydrogenated oils and clofibrate stimulates to a similar extent the oxidation of docosahexaenoate and erucic acid (22:1) (25), both of which are relatively poor mitochondrial substrates. These results strengthen our previous suggestion about the peroxisomal β-oxidation being responsible for chain shortening of long chain fatty acids that are poorly oxidized by the mitochondria, thus making them better mitochondrial substrates and thereby increasing the overall β-oxidation capacity (4).

Feeding partially hydrogenated marine oil also has been reported to increase the fatty acid oxidation capacity of isolated mitochondria by 50% (3) and of the carnitine palmitoyltransferase in the mitochondria to a similar extent (30). Thus, increased capacity of the mitochondrial fatty acid oxidation system most likely also contributes to the observed effects, particularly in the oxidation of palmitate and oleate, which do not require chain shortening to become efficient substrates for the mitochondrial oxidation.

Our study has confirmed that the activity of the 2,4-dienoyl-CoA reductase is increased in livers from animals fed high fat diets and clofibrate (6,7,31). However, it is a paradox that hydrogenation of SO, which removes most of the unsaturated fatty acids from the diet, increases the activity of the 2,4-dienoyl-CoA reductase, which is an auxiliary enzyme in the oxidation of unsaturated fatty acids. When the groups fed 25% SO and 25% FO are compared, both show a relatively slow oxidation of docosahexaenoic acid (22:6) (Table 2). However, the activity of the 2,4-dienoyl-CoA reductase was doubled in the 25% FO-fed group (Table 5). This enzyme therefore seems not to be rate-limiting for the oxidation of this polyunsaturated fatty acid. These observations suggest that this enzyme, although induced and necessary for the oxidation of the unsaturated fatty acids, has no particular regulatory function in the metabolism of the polyunsaturated fatty acids. An unspecific coinduction with other fatty acid-oxidizing enzymes is a possible explanation for the observed effect. It should be noted that the enzyme also has been found in the peroxisomes (32). The peroxisomes therefore probably can shorten long chain polyunsaturated fatty acids as well as saturated acids (32,33).

The hydrogenated fats contain no, or negligible amounts of, essential fatty acids. Since PHCO, almost without essential fatty acids, proved to be a poor inductor (Table 3) and inclusion of 5% SO with the 20% PHFO and 20% PHSO diets gave only a partial normalization, it is unlikely that a deficiency in essential fatty acids causes the metabolic changes observed.

Finally, it should be pointed out that induction of peroxisomal β-oxidation activity shows a good correlation with the level of long chain acyl-CoA in the livers from rats fed different fat diets (34–36). This supports the idea that the induction is caused by accumulation of CoA esters in the liver cells. Such an accumulation evidently would be more pronounced for diets containing fatty acids poorly oxidized by the mitochondria. Hydrogenation of oils may increase the content of such fatty acids, e.g., by formation of fatty acids with *trans* double bonds and/or long chain, mono- and diunsaturated fatty acids.

Our conclusion, therefore, is that the induction of the chain-shortening capacity of the peroxisomal β-oxidation system represents an important mechanism by which an increased capacity of the hepatocyte to oxidize long chain fatty acids, including the polyunsaturated ones, is obtained.

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Structures of Three New 24,24-Dimethyl-Δ^7-sterols from *Gynostemma pentaphyllum*

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The structures of three new sterols isolated from *Gynostemma pentaphyllum* (Cucurbitaceae) have been shown by chemical and spectroscopic methods to be 24,24-dimethyl-5α-cholest-7-en-3β-ol, (22*E*)-24,24-dimethyl-5α-cholesta-7,22-dien-3β-ol and 24,24-dimethyl-5α-cholesta-7,25-dien-3β-ol.
***Lipids* 21, 515–517 (1986).**

Gynostemma pentaphyllum Makino (Japanese name, Amachazuru), a Cucurbitaceae plant, is known to contain many kinds of dammarane saponins (1, and references cited therein). We have recently shown that (22*E*,24*R*)-24-ethyl-5α-cholesta-7,22-dien-3β-ol (chondrillasterol, [24*R*]-**1d**), along with its (24*S*)-epimer (spinasterol, [24*S*]-**1d**), consists of the most predominant component of the sterol constituents of this plant (2). This paper describes our further study of the sterol constituents of the aerial parts of *G. pentaphyllum*, which led to the isolation and characterization of three new 24,24-dimethyl-Δ^7-sterols.

EXPERIMENTAL PROCEDURES

General methods and materials. Recrystallizations were performed in acetone/MeOH. Melting points (mp) taken on a heat block were uncorrected. Preparative (0.5 mm thick) thin layer chromatography (TLC) on silica gel with hexane/EtOAc (6:1, v/v) and argentation TLC (silica gel/$AgNO_3$, 4:1, w/w) with CCl_4/CH_2Cl_2 (5:1, v/v) were developed three and four times, respectively. The bands on silica gel and argentation TLC were observed under UV light (3600 Å) after spraying with a 0.05% solution in EtOH of rhodamine-6G and 2′,7′-dichlorofluorescein, respectively. Preparative high performance liquid chromatography (HPLC) was carried out on a Partisil 5 ODS-2 column (25 cm × 10 mm id) (Whatman, Clifton, New Jersey) with MeOH as a mobile phase (flow rate, 4 ml/min). Gas liquid chromatography (GLC) was performed with a Shimadzu GC-4CM instrument on a SCOT OV-17 glass capillary column (30 m × 0.3 mm id, column temperature 260 C). The Rc-values (relative mobilities) in the argentation TLC and the relative retention times (RRT) in the HPLC and GLC were expressed relative to cholesterol acetate (1.00). Mass spectra (EI-MS, 70 eV) were taken on a Hitachi M-80B double focusing gas chromatograph-mass spectrometer by means of a probe injection. Proton (^1H) nuclear magnetic resonance (NMR) spectra were recorded on a Hitachi R-250 (250 MHz) or on a JEOL FX-100 (100 MHz, Japan Electron Optics Lab. Co., Tokyo) spectrometer in a $CDCl_3$ solution, with tetramethylsilane as the internal standard. Hydrogenation was achieved in EtOH over PtO_2 at atmospheric pressure and temperature overnight. An authentic specimen of 24,24-dimethyl-5α-lanosta-9(11),25-dien-3β-ol (**2c**) was supplied by W. H. Hui (University of Hong Kong) and its 25-dihydro derivative, 24,24-dimethyl-5α-lanost-9(11)-en-3β-ol (**2a**), was prepared from **2c** by partial hydrogenation.

Sterol isolation from G. pentaphyllum. The sterol fraction (125 mg) obtained from MeOH extract (80 g) of the air-dried aerial parts (leaves and stems) of *G. pentaphyllum* (520 g) (2) was acetylated in Ac_2O/pyridine at room temperature overnight. The acetate fraction (120 mg) recovered was subjected to argentation TLC, which yielded eight bands. The three fractions recovered from the first (Rc = 0.94), third (Rc = 0.65) and seventh (Rc = 0.27) bands down from the solvent front were shown to contain unknown sterols: **1a**- (RRT = 2.02 in GLC), **1b**- (RRT = 1.66) and **1c**- (RRT = 2.00) acetates, respectively. Reverse-phase HPLC of each of three fractions enabled the isolation of the acetates of **1a** (RRT = 1.22 in HPLC, 2 mg), **1b** (RRT = 0.91, 3 mg) and **1c** (RRT = 0.84, 6 mg). Contents of these sterols in the total sterol fraction were determined as **1a** (8.5%), **1b** (3.0%), and **1c** (5.1%) by GLC as the acetyl derivatives. Identification and characterization of the other sterols in the aerial parts of *G. pentaphyllum* have been described previously (2).

RESULTS AND DISCUSSION

The high resolution mass spectrometry (MS) of **1a**-acetate (mp 180–183 C) showed M^+ at m/z 456.3923 (rel int 100%, $C_{31}H_{52}O_2$, calcd. 456.3964) along with the following prominent fragmentation ions: at m/z 441.3717 (17%, M^+-Me), 413.3387 (3%, M^+-C_3H_7), 396.3716 (58%, M^+-HOAc), 381.3511 (12%, M^+-Me-HOAc), 315.2313 (11%, M^+-side chain [$C_{10}H_{21}$]), 288.2114 (8%), 273.1850 (9%), 255.2102 (67%, M^+-HOAc-$C_{10}H_{21}$), 229.1936 (19%) and 213.1627 (19%), which suggested that it was an acetate of a C_{29}-sterol with a monounsaturated skeleton and a C_{10} saturated side chain (3,4). The ^1H NMR spectrum of **1a**-acetate showed signals arising from the conventional 3β-acetoxy-Δ^7 5α-sterol skeleton (2) (Table 1) and those due to the side chain methyl protons at δ 0.761 (6H, *s*), 0.803 (6H, *d*) and 0.917 (3H, *d*). The signals at δ 0.761, which suggests the presence of a gem-dimethyl group on a quaternary center probably located at C-24 (5,6), and 0.803 were consistent with the 28-H_3/29-H_3 (6H, *s*, δ0.766) and 26-H_3/27-H_3 (6H, *d*, δ 0.806) signals, respectively, of **2a**-acetate; hence **1a** was considered to have the structure 24,24-dimethyl-5α-cholest-7-en-3β-ol (Scheme 1).

The high resolution MS of **1b**-acetate (mp 196–198 C) displayed M^+ at m/z 454.3805 (rel int 8%, $C_{31}H_{50}O_2$, calcd. 454.3807), with the following prominent fragmentation ions: at m/z 411.3243 (100%, M^+-C_3H_7), 351.3066 (27%, m/z 411pHOAc), 313.2132 (59%, M^+-side chain [$C_{10}H_{19}$]-2H), 273.1844 (5%), 255.2107 (33%), 229.1955 (5%) and 213.1654 (7%), which suggested that it was an acetate of C_{29}-sterol with two double bonds, one of which is in the skeleton and the other in the C_{10} side chain (3,4). The prominent ions at m/z 411 and 351 were typical for 24-alkyl-Δ^{22}-sterols (3). The ^1H NMR spectrum of **1b**-

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TABLE 1

^{1}H NMR Data (250 MHz; $CDCl_3$)[a] of the Acetyl Derivatives of Some 24,24-Dimethylsterols

Acetate	18-H_3[b]	19-H_3[c]	21-H_3[c]	26-H_3[c]	27-H_3[c]	28-H_3/29-H_3[b]	22-H[d]	23-H[c]	27-H_2[b]
1a	0.529	0.808	0.917 (5.8)	0.803 (6.7)		0.761	—	—	—
1b	0.544	0.812	1.009 (6.4)	0.805 (6.4)		0.891	5.091 (8.5, 15.1)	5.277 (15.6)	—
1c	0.521	0.806	0.911 (6.1)	1.682 (0.6)	—	1.011	—	—	4.659 4.718
2a	0.642	1.067	0.856	0.806 (5.4)		0.766 (7.0)	—	—	—
2c[e]	0.63	1.06	0.86 (5.1)	1.69 (s)	—	1.01	—	—	4.67 4.72

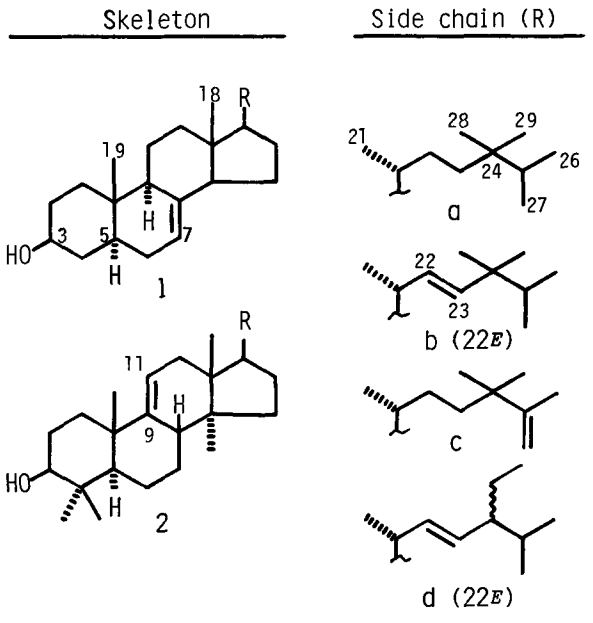
[a]Given as δ values. Figures in parentheses denote J values in Hz. The 3β-acetoxy methyl singlet at δ 2.02, 3α-H multiplet at δ 4.69 and 7-H multiplet at δ 5.14 also were observed for **1a**-, **1b**- and **1c**-acetates.

[b]Singlet.

[c]Doublet if not otherwise specified.

[d]Double doublet.

[e]Determined at 100 MHz.



SCHEME 1

acetate suggested that it possesses the 3β-acetoxy-Δ^7 5α-sterol skeleton (Table 1). On the other hand, the spectrum showed the side chain methyl signals at δ 0.805 (6H, *d*, 26-H_3, 27-H_3), 0.891 (6H, s, 28-H_3, 29-H_3) and 1.009 (3H, *d*, 21-H_3), with two olefinic-proton signals at δ 5.091 (1H, *d*×*d*, J = 8.5 and 15.6 Hz, 22-H) and 5.277 (1H, *d*, J = 15.6 Hz, 23-H) suggesting the 24,24-dimethyl-Δ^{22} side chain structure. The coupling constant (~16 Hz) of the olefinic signals implied that 22-H and 23-H are *trans* oriented (22*E*) (7,8). Thus, **1b** was considered to have the structure (22*E*)-24,24-dimethyl-5α-cholesta-7,22-dien-3β-ol (Scheme 1). Hydrogenation of **1b**-acetate yielded a mixture of the dihydro derivative, **1a**-acetate (30%, as determined by GLC) and unchanged **1b**-acetate (70%), from which was isolated **1a**-acetate (mp 183–185 C) by argentation TLC.

The high resolution MS of **1c**-acetate (mp 193–195 C) showed M$^+$ at m/z 454.3791 (rel int 38%, $C_{31}H_{50}O_2$), with the following prominent fragmentation ions: at m/z 439.3591 (16%, M$^+$-Me), 394.3579 (4%, M$^+$-HOAc), 379.3344 (6%, M$^+$-Me-HOAc), 370.2890 (5%, M$^+$-part of side chain [C_6H_{11}]-1H), 313.2135 (100%, M$^+$-side chain [$C_{10}H_{19}$]-2H), 310.2642 (4%, m/z 370-HOAc), 300.2085 (3%), 288.2083 (12%), 273.1860 (8%), 255.2094 (32%), 253.1952 (7%), 227.1826 (15%) and 213.1651 (25%), which suggested that it was an acetate of a C_{29} sterol with two double bonds, one of which is located in the skeleton and the other in the C_{10} side chain (3,4). The fragmentation ion at m/z 370, due to McLafferty rearrangement involving cleavage of the 23–24 bond with one H transfer from C-22, was typical of Δ^{25}-unsaturated sterols (6). The ^{1}H NMR spectrum of **1c**-acetate suggested that it has the 3β-acetoxy-Δ^7 5α-sterol skeleton (Table 1). The spectrum showed the side chain proton signals at δ 0.911 (3H, *d*, 21-H_3), 1.011 (6H, *s*, 28-H_3, 29-H_3), 1.682 (3H, *s*, 26-H_3), and 4.659 and 4.718 (each 1H and broad *s*, 27-H_2), of which the latter four were consistent with the corresponding signals of **2c**-acetate (Table 1), suggesting the 24,24-dimethyl-Δ^{25} side chain structure for **1c**. Hydrogenation of **1c**-acetate afforded **1a**-acetate (mp 181–183 C), and hence **1c** was considered to be 24,24-dimethyl-5α-cholesta-7,25-dien-3β-ol (Scheme 1).

The three 24,24-dimethylsterols, **1a**, **1b** and **1c**, from *G. pentaphyllum* are considered to be new sterols. The structures of these sterols are unusual in the sense that these contain an acyclic, quaternary carbon in the side chain. Although several sterols from some marine sponges (6,9,10) and triterpenoids including **2c**-methyl ether from *Neolitsea* species (Lauraceae) (5,11) are already known to possess side chains with the quaternary center at C-24, this study seems to be the first demonstration of the

occurrence of 24,24-dimethylated sterols in a higher plant. Sterols with a 24-methyl-$\Delta^{24(25)}$ side chain (12–14) are the possible biosynthetic precursors (6,9,11) to the 24,24-dimethylsterols (**1a, 1b** and **1c**).

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T. Takido performed the ^{1}H NMR (100 MHz) spectra and M. Aimi did the mass spectra.

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METHODS

Mass Spectra of Fatty Acid Derivatives, of Isopropylidenes of Novel Glyceryl Ethers of Cod Muscle and of Phenolic Acetates Obtained with the Finnigan Mat Ion Trap Detector[1]

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The applicability of the Finnigan MAT Ion Trap Detector (ITD) mass spectrometer for structure determination in some selected fatty acids and their derivatives has been investigated. Isopropylidene derivatives of novel glyceryl ethers isolated from cod flesh and of phenolic acetates are included to indicate the potential for diverse structures and to clarify the protonation of ions. The ITD is a simple and unsophisticated gas liquid chromatograph-mass spectrometer, but the spectra obtained are in most respects comparable to those from more conventional electron impact mass spectrometers. However, due to the comparatively high background pressure ($\sim 10^{-3}$ torr) in the ionization chamber of the ITD, there is a tendency for both neutral and ionized molecules to acquire protons from other molecules or fragments through collision. In many cases, the molecular ion was observed as the protonated molecular ion (M+1), as in chemical ionization mass spectrometry. These interactions can be minimized if the sample load is decreased. Phenolic acetates exhibit not only protonation of the molecular ion, but also protonation of stable fragmented neutral molecules or ions.
***Lipids 21,* 518–524 (1986).**

Gas liquid chromatography-mass spectrometry (GC-MS) is an established technique in the analysis of complex mixtures, and is critical to many applications of analytical chemistry because of its combination of sensitivity and a wide range of applicability. Several more sophisticated models of GC-MS instruments have been available to a certain proportion of lipid chemists for approximately two decades, but GC-MS has not been commonly accessible on a direct user basis because of the high cost and complexity of operation. During the last decade the miniaturization of personal computers into data handling microprocessors has proceeded with unexpected rapidity; the union of this development with GC-MS has led to the Finnigan Ion Trap Detector, commonly called an ITD. This is a novel form of mass spectrometer (1,2) available at about one-quarter the price of conventional GC-MS equipment and designed to capitalize on the IBM-PC for control, data storage and data processing. It provides direct comparison with a library of 38,000 spectra on hard disc. This report illustrates some applications appealing to lipid chemists and clarifies the current capability of this relatively inexpensive apparatus.

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EXPERIMENTAL PROCEDURES

Methyl 16-methylheptadecanoate was obtained from Analabs Inc. (North Haven, Connecticut) and pure *cis*-9-octadecenoic acid from Applied Science Lab. Inc. (State College, Pennsylvania). Phenols were purchased from Aldrich Chemical Co. Inc. (Milwaukee, Wisconsin).

Preparation of fatty acid derivatives. The vicinal diol of methyl *cis*-9-octadecenoate was prepared essentially according to the procedure of McCloskey and McClelland (3). The 0-isopropylidene derivative of the diol was obtained by condensing the diol (5 mg) with acetone (1 ml) in the presence of anhydrous copper sulfate (50 mg) upon heating for 2 hr at 50 C. The trimethylsilylether derivative of the diol was prepared by treating the diol (10 mg) with hexamethyldisilazane (0.3 ml) and trimethylchlorosilane (0.1 ml) in the presence of pyridine (1 ml). An N-octadec-9-enoylpyrrolidide of *cis*-9 octadecenoic acid was prepared by heating the methyl ester (0.5 mg) with freshly distilled pyrrolidine (1 ml) and acetic acid (0.1 ml) for 30 min at 100 C (4).

Preparation of phenolic acetates. Phenolic acetates were made by treating the phenols (10 mg) with a mixture of acetic anhydride (1.5 ml), 3 N NaOH (0.5 ml) and crushed ice (5 g) for 5 min (5). The phenolic acetates were extracted with diethyl ether after acidifying with 3 N HCl.

Extraction of cod flesh lipids and preparation of isopropylidene-glyceryl ethers. Lipids from filets (without skin) of cod caught in January 1986 from the Middle Bank off the east coast of Nova Scotia were extracted by the method of Bligh and Dyer (6). The total lipid was separated into polar and nonpolar fractions according to the procedure of Galanos and Kapoulas (7). The nonpolar lipid fraction was subjected to preparative thin layer chromatography (TLC) on "Prekotes" Adsorbosil-5 silica gel TLC plates (20 × 20 cm; Applied Science Laboratories, College Park, Pennsylvania). The development was in hexane/diethyl ether/acetic acid (85:15:1, v/v/v). The bands were visualized by spraying a 0.01% solution of 2′7′-dichlorofluorescein in ethanol and viewing under UV light. The diacylglyceryl ether (DAGE) band was scraped off the TLC plate and extracted from the silica gel using a mixture of $CHCl_3/CH_3OH/H_2O$ (5:5:1, v/v/v), according to the procedure of Fine and Sprecher (8).

The isolated DAGE was saponified with KOH/C_2H_5OH according to the AOCS official method Ca 6b-53. The unsaponifiable matter was extracted with diethyl ether and subjected to preparative TLC as described earlier. The 1-0-alkyl glycerol fraction was isolated and converted to the isopropylidene derivative according to Mueller et al. (9).

GC-MS analyses. GC-MS data was obtained by using a model 700 Finnigan MAT Ion Trap Detector system

controlled by an IBM Personal Computer XT (PC XT). The ITD was connected to a Perkin Elmer 990 gas chromatograph. The wall-coated flexible fused silica GC column was fed through a heated transfer line directly to the ITD gas inlet. Chromatography of the above polar derivatives of octadecenoic acid and of phenolic acetates was executed on a flexible fused silica capillary column (10 m × 0.21 mm) coated with OV-101 methyl silicone. The column temperatures were respectively 130 C and 225 C for the phenolic acetates and the fatty acid derivatives, at a helium carrier gas pressure of 13 kPa. Underivatized fatty acid methyl esters were analyzed on a Supelcowax-10 (bonded Carbowax-20M) fused silica capillary column (30 m × 0.21 mm) operated at 190 C/68 kPa and connected to the ITD in the same fashion. The isopropylidene derivatives of glyceryl ethers were analyzed on a DB-Wax fused silica capillary column (30 m × 0.25 mm; a bonded polyethylene glycol column from J&W Scientific Inc., Rancho Cordova, California) operated at 210 C/68 kPa.

RESULTS AND DISCUSSION

A notable feature of the unit is that the PC XT controls and monitors the vacuum system, system temperatures and scanning of the ITD and also handles all data acquisition and processing.

The principle of the apparatus is described elsewhere (1). The effluent from any gas liquid chromatography (GLC) column, packed or open-tubular, can be accessed via a heated transfer line, but we prefer to pass the exit end of the flexible fused silica column directly into the inlet port of the ITD. The latter operates at high vacuum and the limited capacity of the turbomolecular pump requires that carrier gas flow also be limited. This places minor constraints on GLC operating conditions, but the high thermal stability of bonded liquid phases in flexible fused silica columns (10) permits higher temperatures (up to 280 C) to be used in lieu of high carrier gas flow. In the same way as the phenomenal sensitivity of the Chromarod-Iatroscan TLC-FID system, compared to plate TLC and reagent sprays, requires users to rethink their sample sizes (11), the ITD also functions best with the lowest possible sample entering the system. If the system is overloaded there is a greater tendency for both neutral or ionized molecules to acquire protons from other molecules or fragments, leading to protonation of the parent molecule and other fragments. Figure 1 illustrates this for methyl 16-methylheptadecanoate (iso-18). The comparison shows that in regular electron impact mass

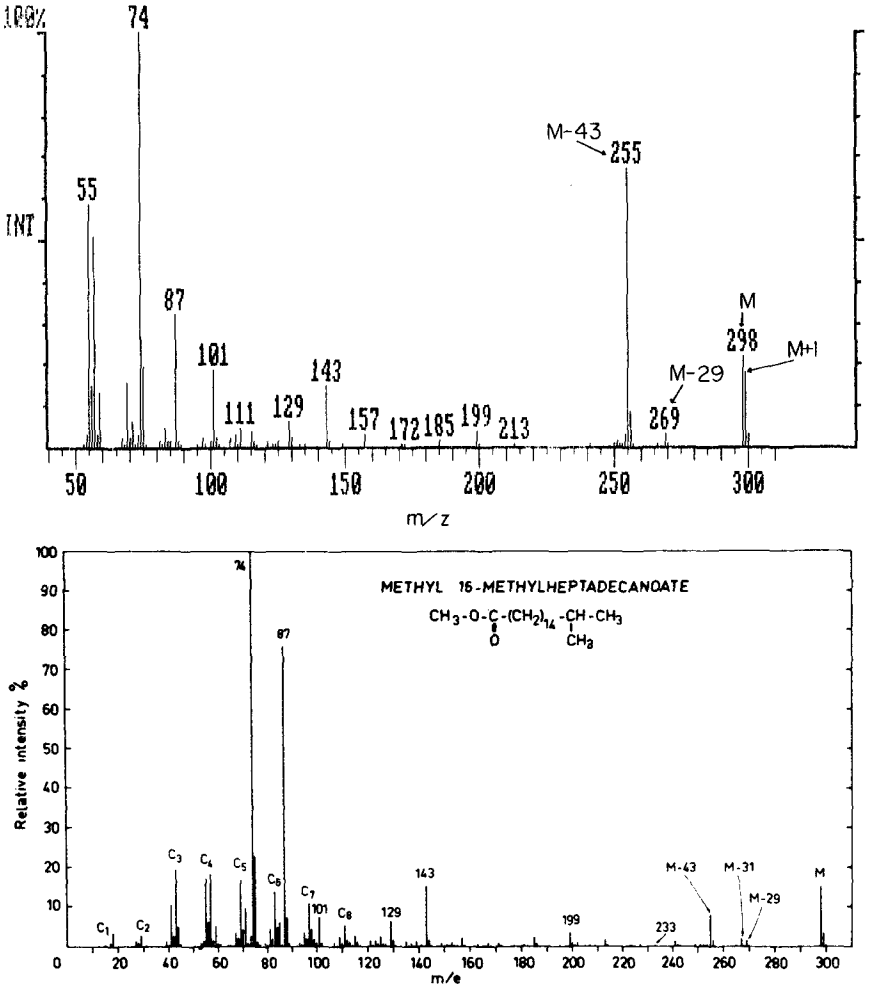

FIG. 1. Mass spectrum of methyl 16-methylheptadecanoate from ITD GC-MS (top) and from a conventional electron impact MS (bottom reproduced from Ref. 8).

spectrometry the ratio of intensities of M^+ to $(M+1)^+$ is 21 (12), which is close to the natural isotope ratio for C_{18}, whereas in the ITD spectrum the molecular ion is accompanied by an almost equal proportion of $(M+1)^+$. However, the ratio of M-43 (m/z 255) to the adjacent M-42 is nearly the same, and most other fragments are similar in proportion in both spectra.

Recently Tulloch and Hogge (13) observed that intensities of $(M+1)^+$ in mass spectra of fatty acid methyl esters exceeded theoretical values on a Finnigan model 4000 GC-MS. Large $(M+1)^+$ ions have been observed commonly with shorter chain methyl esters such as methyl decanoate. They showed with deuterium labeling that a hydrogen radical migrates internally, most favorably from the C-4 position of the fatty acid chain, to the ionized carbonyl oxygen of M^+, and the resultant ion on collision with a neutral molecule transfers the hydrogen as a proton, giving $(M+1)^+$ (Fig. 2). Probably the same mechanism is operating in the ITD to produce the relative abundance of $(M+1)^+$ ions. This proton transfer is more pronounced in the ITD than in the majority of fatty acid spectra obtained by more conventional GC-MS, but is not by any means unique to the ITD. In the ITD the background carrier gas pressure (without the sample) inside the ion chamber is about 10^{-3} torr (14). This is much higher than in the conventional magnetic sector mass spectrometer, which operates around 10^{-6} torr. If the sample pressure inside the ionization chamber exceeds 10^{-2} torr, secondary collisions of the ions or molecules with each other become important (15,16). A high ion residence time (the ions spend time in the ion source and in flight to the detector) could also lead to interaction of ions and neutral molecules. The ion residence time in the ITD is of the order of 10^{-3} seconds (17) compared to 10^{-5}–10^{-6} seconds for many conventional mass spectrometers (15). Thus, any abnormal high intensity or abundance of $(M+1)^+$ in ITD is obviously due to the secondary collisions brought about by the relatively high ion residence time and high sample pressure in the ionization chamber.

The ITD is quite useful in determining the position of double bonds in unsaturated fatty acids. This was demonstrated with the pyrrolidide of *cis*-9-octadecenoic acid and of the isopropylidene and trimethylsilyl ether (TMS) derivatives of the methyl esters of the vicinyl dihydroxy oxidation product. For example, the ITD mass spectrum of methyl erythro-O-isopropylidene-9,10-dihydroxyoctadecanoate (Fig. 3) was almost identical to that published by McCloskey and McClelland (3). The molecular ion and the $(M+1)^+$ ion were absent, but the molecular weight was clearly demonstrated by a loss of a methyl group from the 2,2-dimethyl-1,3-dioxalane ring, giving rise to an intense peak at M-15 (a, m/e 355). Other characteristic fragments appeared at m/z 313 (b), 295 (b′), 281 (c), 263 (d), 256 (x), 246 (e) and 214 (y). The peaks at e and y were one unit higher and the peak at x was one unit lower than those reported in the literature (3). This is probably the result of secondary collisions of fragmented ions. The ions x and y are diagnostic of the original position of the double bond in monoethylenic fatty acids, which are formed by simple cleavage of the C-C bond alpha to the 1,3-dioxalane ring. The ITD-mass spectrum of the TMS derivative of the vicinal diol prepared from methyl *cis*-9-octadecenoate did not exhibit the M^+ or $(M+1)^+$ (Fig. 4), a result similar to that from a conventional mass spectrometer (18). The two ions at m/z 215 and 259, which represent the cleavage between the two carbon atoms which originally constituted the double bond, were

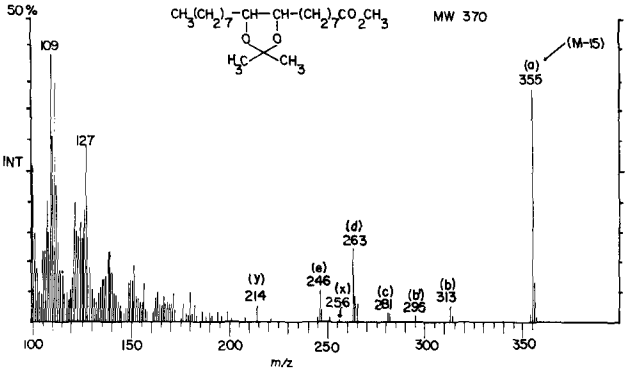

FIG. 3. ITD mass spectrum of methyl erythro-O-isopropylidene-9,10-dihydroxyoctadecanoate.

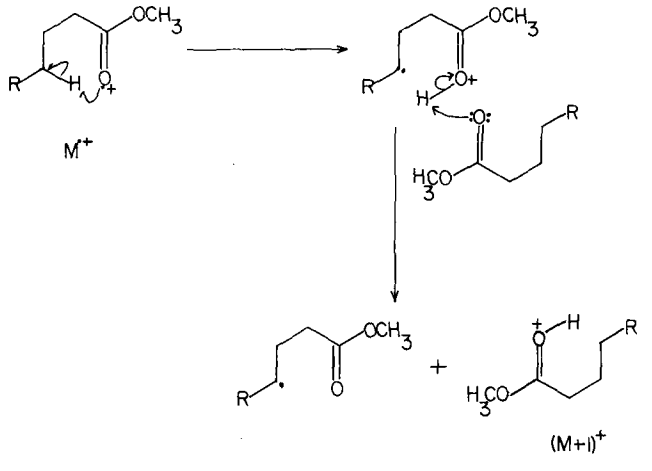

FIG. 2. Postulated mechanism for the formation of $(M+1)^+$ in the ITD mass spectrum of fatty acid methyl esters.

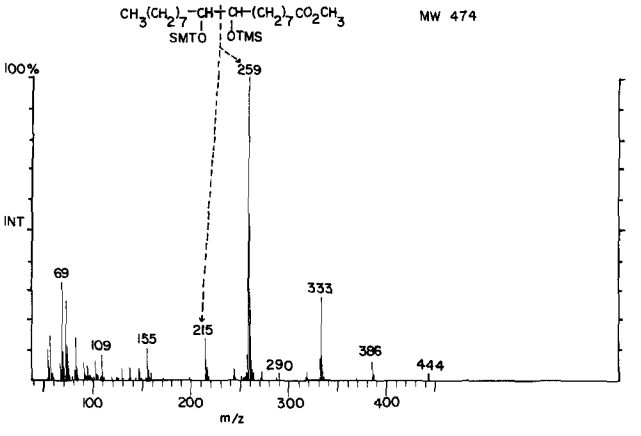

FIG. 4. ITD mass spectrum of the TMS derivative of 9,10-dihydroxyoctadecanoate.

particularly prominent, with m/z 259 being the base peak. Pyrrolidide derivatives of unsaturated fatty acids have distinctive mass spectra with important ions that can be used to locate the position of the double bonds (4,19). Figure 5 (top) illustrates the ITD mass spectrum of the pyrrolidide derivative of *cis*-9-octadecenoic acid. The ITD mass spectrum was almost identical to that obtained on a quadrupole GC-MS (4,19; for an example, see Fig. 5, bottom). The peak at m/z 336 in the ITD spectrum is due to the protonated parent molecule and confirms the molecular weight of 335 for the pyrrolidide derivative. The base peak was the McLafferty rearrangement ion at m/z 113 (not shown in Fig. 5) which is typical of N-acylpyrrolidides (19). Prominent and characteristic fragments appeared at m/z 126, 140, 154, 168, 182, 196, 208, 210, 222 and 236. This series spaced at 14 atomic mass units (amu) is generally observed except in the vicinity of the double bond where the interval is 12 amu, occurring between m/z 196 and 208. According to the rules proposed by Andersson and Holman (4), this confirmed that the double bond resided between carbons 9 and 10 in the N-octadec-9-enoylpyrrolidide.

Protonation is observed not only with the parent molecule, but also with fragmented ions and daughter molecules. During our work with phenols of the suberin of tomato locule protoplasts (5), we observed that this protonation of fragmented ions or molecules is quite pronounced in the ITD spectra of acetylated phenols. The ITD mass spectrum of vanillin acetate is shown in Figure 6. At the time this spectrum was obtained the importance of minimizing sample size was not clearly understood, hence the importance of M+1 (m/z 195). The base peak was at m/z 43, while ions representing acetyl (m/z 151) and ketene (m/z 152) eliminations were about 40 and 50% of the base peak. A similar behavior was also observed with the ITD mass spectrum of p-acetoxybenzaldehyde. A previously published spectrum of vanillin acetate obtained with a conventional mass spectrometer showed that the base peak was at m-43 (m/z 151) due to the elimination of an acetyl radical (20). The peak at m/z 43, due to acetyl ions, was about 85% of the base peak (m/z 151), and the peak at m/z 152, which is due to the elimination of ketene, was around 15% of the base peak (20). The greater intensity of the m/z 43 peak of phenolic acetates in the ITD spectra could be rationalized by considering that the m/z 43 peak is not only due to the acetyl ion but also to a protonated ketene. As depicted in Figure 7, ketene (which is eliminated from the parent molecule) could acquire a proton, through collision, from a fragmented ion such as structure H. The predominance of m/z 43 peak in the mass spectra of phenolic acetates has also been observed by other workers in the GC-MS field (21). The ITD spectrum of vanillin acetate exhibits a prominent peak at m/z 153 (Fig. 6). However, in the reports for magnetic sector MS (20) and in GC-MS (21), the intensity of this ion was much less than that of m/z 151 and m/z 152. The predominance of m/z 153 in the ITD spectrum is obviously due to secondary collisions; it could have formed by the abstraction of a hydrogen radical by the odd-electron ion (B) from another odd-electron ion such as the molecular ion (E). The corresponding ion in the ITD spectrum of p-acetoxybenzaldehyde appeared at m/z 123. It had the same intensity as that of m/z 121.

We have recently examined Atlantic cod flesh lipids by TLC and Iatroscan-TLC/FID (11) and found that cod flesh lipids contain trace levels (<0.05% by wt) of DAGE. There have been no previous reports concerning the existence of DAGE in cod flesh. DAGE are the major lipid components of the livers in some elasmobranch fish such as sharks (22,23). The occurrence of DAGE in marine fish flesh is not commonly mentioned except in some deep-

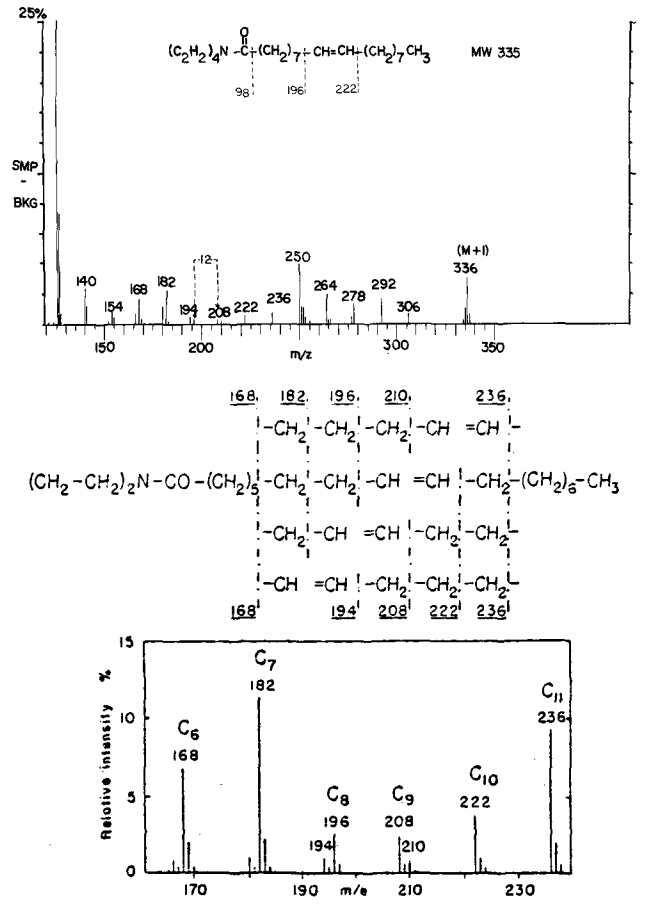

FIG. 5. Mass spectrum of N-octadec-9-enoylpyrrolidide from ITD GC-MS (*top*) and from a conventional GC-MS (*bottom*, reproduced from Ref. 19).

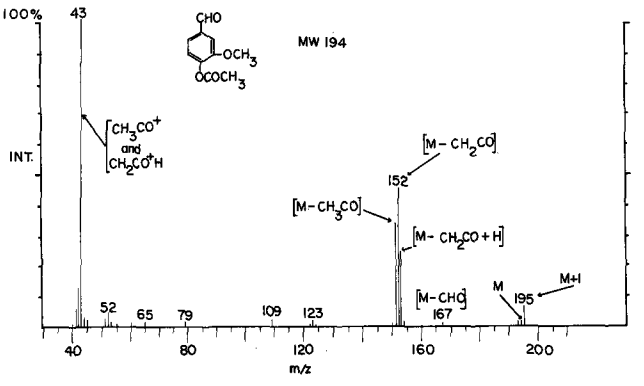

FIG. 6. ITD mass spectrum of vanillin acetate.

sea teleost fish (24,25). The ITD was used to verify, after deacylation, the original existence of DAGE of cod flesh lipids as well as to characterize the individual 1-0-alkyl moieties of DAGE. Figure 8 shows the reconstituted ion chromatogram patterns from the ITD of the isopropylidene derivative of glyceryl ethers, before and after hydrogenation, analyzed on a DB-Wax flexible fused silica capillary column. From the ITD mass spectra, 14:0, 16:0 and 16:1 were identified as the major alkyl chains of the cod flesh glyceryl ethers. ITD mass spectra also showed moderate or minor amounts of 14:1, iso-15:0, anteiso-15:0, iso-15:1, 15:0, 15:1, iso-16:0, iso-16:1, anteiso-17:0, 17:0, 18:0, 18:1 and 18:2 alkyl chains. The mass spectra and the comparison of the peak areas before and after hydrogenation confirmed the identity of all the components. To demonstrate the versatility of the ITD, the mass spectra of a major (1-0-alkyl 16:0, peak no. 10), moderate (1-0-alkyl iso-15:0, peak no. 3) and a very minor (1-0-alkyl 18:2, peak no. 20) component are illustrated in Figure 9. The fragmentation pattern for the isopropylidene glyceryl ethers obtained from the ITD were very characteristic and were not different from those obtained from any conventional GC-MS (9,26). The ITD spectra yielded characteristic ions of M-15 (due to the loss of a CH_3 group from the isopropylidene ring of the parent ion) and m/z 101 (the base peak), both indicative of a glyceryl ether structure (9,26,27). All the major components exhibited the molecular ion and also the protonated molecular ion (e.g., m/z

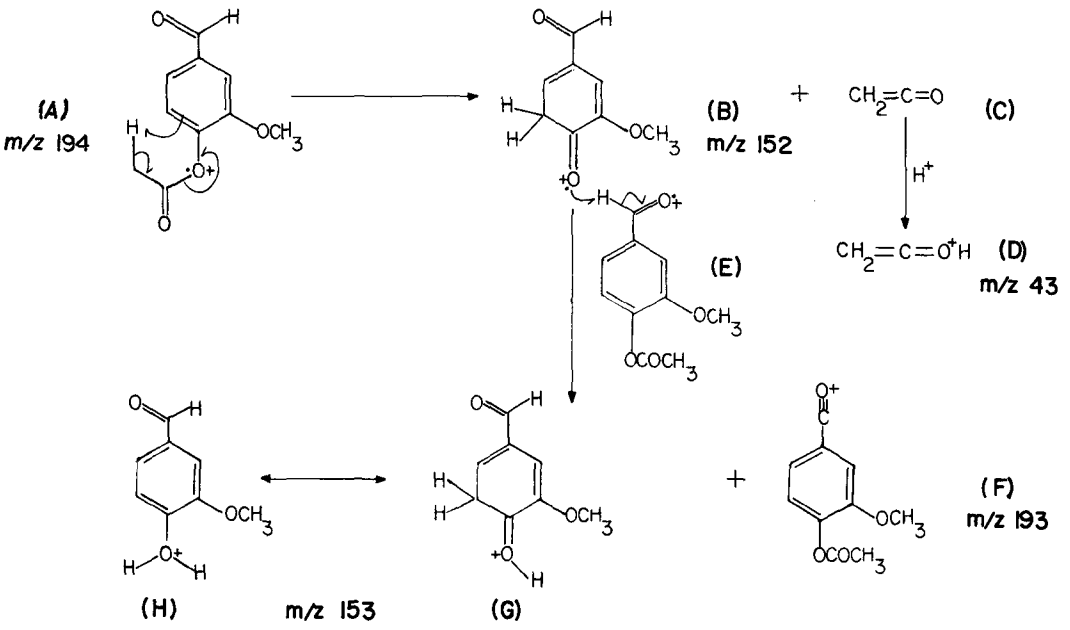

FIG. 7. Proposals for mechanism of formation of m/z 43, 152, 153 and 193 ions in the mass spectrum of vanillin acetate.

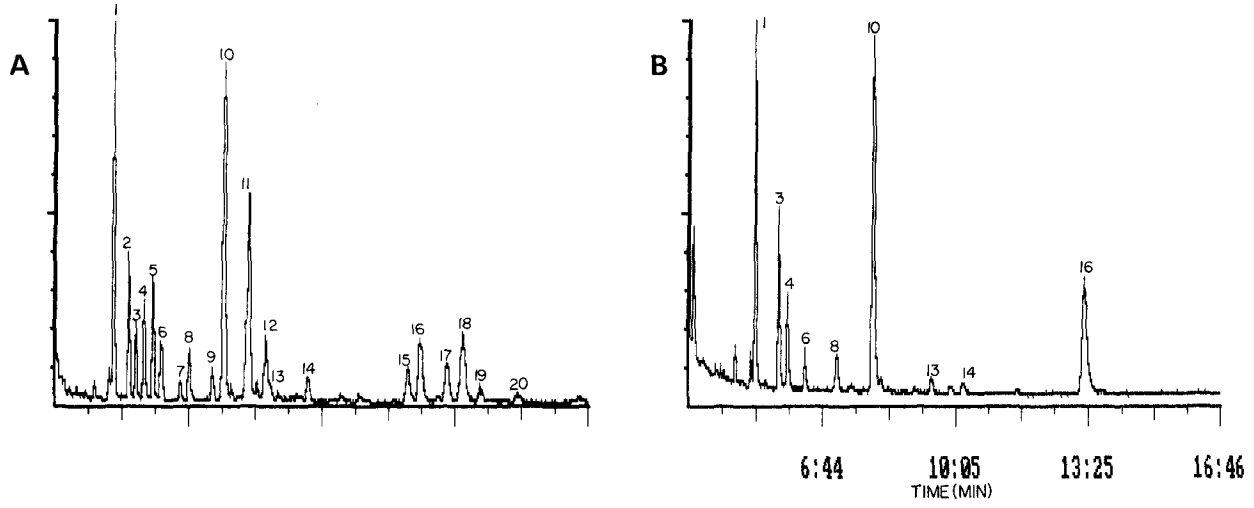

FIG. 8. Reconstituted ion chromatograph from the ITD-GC analysis of isopropylidene derivatives of deacylated glyceryl ethers from cod flesh lipids. A, unhydrogenated; B, hydrogenated. (*Note:* there is slight change in the retention time between A and B.) Peak identification: peak No. (1-0-alkyl group) 1 (14:0), 2 (14:1), 3 (iso-15:0), 4 (anteiso-15:0), 5 (iso-15:1), 6 (15:0), 7 (15:1), 8 (iso-16:0), 9 (iso-16:1), 10 (16:0), 11 (16:1), 12 (16:1), 13 (anteiso-17:0), 14 (17:0), 15 (unidentified impurity), 16 (18:0), 17 (18:1), 19 (18:1) and 20 (18:2).

357 for 1-0-alkyl 16:0 in Fig. 9). These two ions were not detected for moderate size or minor components. The complete absence or very low intensity of the molecular ion of the isopropylidene derivative of glyceryl ethers has also been noted by several other workers, even with conventional GC-MS (25-27). The presence of the M-CH_3 ions at 327 and 365 identifies the 1-0-alkyl moieties of the two peaks at 3 and 20 as iso-15 and 18:2, respectively. Hydrogenation results (Fig. 8, bottom) confirmed the identity of these components. The 1-0-alkyl 18:2 peak disappeared on hydrogenation and is converted to 1-0-alkyl 18:0. The position as well as the mass spectrum of

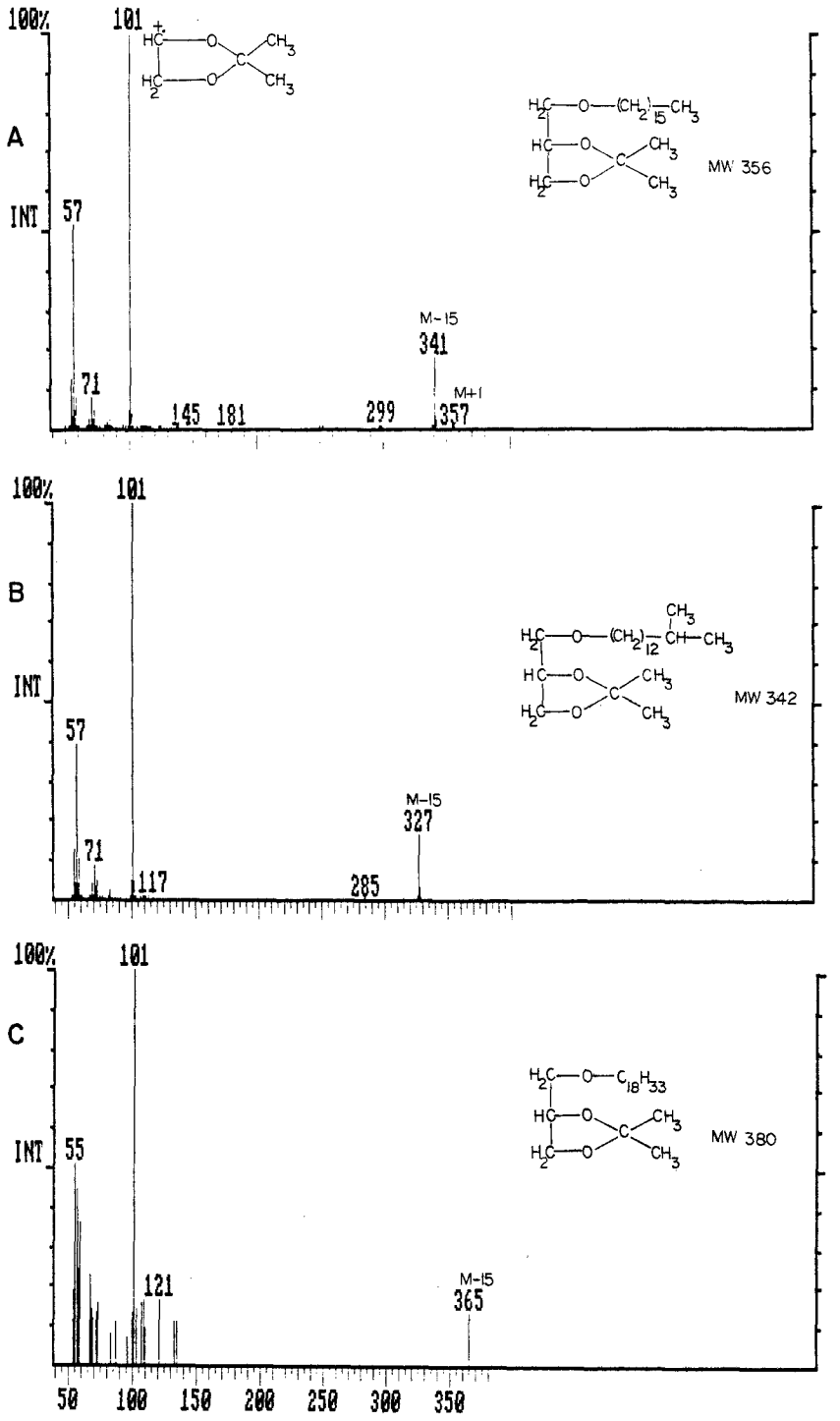

FIG. 9. Mass spectra of isopropylidene derivatives of deacylated glyceryl ethers of cod flesh lipids. A, 1-0-alkyl-16:0 compound (peak 10); B, 1-0-alkyl-iso-15:0 compound (peak 3); C, 1-0-alkyl-18:2 compound (peak 20).

peak 3 after hydrogenation remained unchanged, verifying the identity of peak 3 as 1-0-alkyl iso-15:0. As can be seen in Figure 8, the size of peak 3 increased after hydrogenation due to the contribution from 1-0-alkyl iso-15:1.

In spite of the favorable cleavage at the methyl branch, the ITD spectra do not differentiate between iso, anteiso and straight chain structures of a given chain length for the isopropylidene derivative of glyceryl ethers, as all these isomers produced identical fragmentation patterns. This is to be expected as the mass spectra are dominated by $(M-15)^+$, m/z 101 and ions of low masses. Nevertheless, the iso, anteiso and straight chain structures could easily be differentiated by their retention characteristics on both polar and nonpolar GLC columns (28). The unexpected identification of an 18:2 glyceryl ether in cod flesh illustrates that the ITD is quite sensitive and useful in characterizing trace level components present in a mixture.

The few results presented here demonstrate that the Finnigan ITD is an excellent substitute for more expensive and sophisticated GC-MS units, especially for those laboratories operating on a restricted budget. The sample size introduced into the ionization chamber should be kept as low as possible to minimize the secondary collisions of particles. Nevertheless, there is a possibility that these interactions could be utilized to advantage, especially by the protonation of the parent molecule to obtain the molecular weight, as in chemical ionization MS. Due to these collisions of particles, the ITD mass spectra should be expected to be slightly different from that of a classical mass spectrum. Our experience shows >95% correlation between the ITD spectra and that obtained with a conventional spectrometer for many lipid components. For highly unsaturated aliphatic compounds as well as aromatics, such as the phenolic acetates demonstrated above, the correlation will be expected to be much less, as the parent molecule could easily abstract a proton through collision. Very stable neutral fragments and ions have a greater tendency to acquire protons through collision, as the concentration of stable fragments is relatively higher than of unstable fragments. Ketene, obtained from phenolic acetates, is an example of such a stable neutral fragment. ITD spectra of compounds producing stable fragments will differ from the classical pattern to a moderate extent. Therefore, it should be emphasized here that the tendency for ionized molecules or fragments to become protonated varies with the type of compounds and with concentration. This may introduce uncertainty in structure assignment to fragments and in the final identification of the parent molecules, but if the fragmentation pattern of a particular class of compounds in the ITD is thoroughly understood, an unknown component of this particular class should be identifiable with little or no difficulty. However, identification of a completely unknown compound by ITD spectra alone will pose some difficulties. Nevertheless, the ITD is extraordinarily useful for confirmation of molecular structures when supporting data is available through other physical and chemical means. Another major problem with the ITD is the variation of the intensities of the ions, especially M^+ and $(M+1)^+$, with the concentration of the sample introduced into the ITD. This variability of ion concentration excludes the ITD from work with isotope-labeled metabolites and with stable isotope-labeled internal standards. Further, due to this variability of intensity of ions, the ITD is less suitable for quantitative work with either fragment ions or molecular ions.

The ITD has been in service for approximately one year in our laboratory and has been used for study of a variety of lipid classes. Further development of novel apparatus usually takes up to five years to mature and become widely known, and we expect this now familiar pattern of application and modification to develop quickly for the ITD.

ACKNOWLEDGMENT

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COMMUNICATIONS

Cyclic AMP Increases Incorporation of Exogenous Fatty Acids into Triacylglycerols in Hamster Fibroblasts

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Incorporation of various exogenous saturated or unsaturated [^{14}C]labeled fatty acids (palmitic, stearic, oleic, linoleic and arachidonic) into triacylglycerols by hamster fibroblasts was markedly enhanced (two- to fourfold) in the presence of theophylline or dibutyryl cyclic adenosine-monophosphate (dbcAMP). This effect was observed for short-term (1–6 hr) as well as long-term (15–24 hr) preincubation with dbcAMP. In the presence of sodium fluoride, a phosphoprotein phosphatase inhibitor, measurement of diacylglycerol acyltransferase (DGAT) activity in cells pretreated with dbcAMP pointed out a marked increase (3×) in specific activity. The results suggest that DGAT activity in fibroblasts could be activated by a cAMP-dependent phosphorylation process.
Lipids 21, 525–528 (1986).

Preliminary experiments have shown that cultured fibroblasts are able to store exogenous fatty acids by means of triacylglycerol (TG) synthesis. Whereas regulation of TG metabolism has been well documented in hepatocytes (1,2) and adipocytes (3,4), there is no report on the regulation of TG synthesis in cultured fibroblasts.

The only enzyme involved in TG synthesis is diacylglycerol acyltransferase (DGAT). In hepatocytes, Haagsman et al. (5) demonstrated that DGAT exists in two interconvertible states, via a phosphorylation/dephosphorylation mechanism: in vitro incubation with ATP and Mg^{2+} inactivated the enzyme, but cAMP did not influence this process. In fibroblasts, however, the regulation of DGAT by cAMP has not been studied. Pelech et al. (6) reported that in hepatocytes cAMP decreased phosphatidylcholine biosynthesis for up to 6 hr. However, prolonged incubation of hepatocytes with cAMP resulted in a reversal of inhibition of phosphatidylcholine synthesis. In view of the fact that cAMP may have different effects on a metabolic pathway depending upon the time of exposure, we investigated the time course of the effect of cAMP on TG biosynthesis.

A stimulation of exogenous fatty acid incorporation into TG was observed in the presence of dibutyryl cyclic AMP, for short-term (2–4 hr) as well as long-term (24 hr) incubations. As the short-term effect of dbcAMP leads to the hypothesis of a rapid regulation of DGAT activity by cAMP, we measured the enzyme activity on cell homogenates after pretreatment of cell cultures with dbcAMP. An increase in DGAT activity was found after dbcAMP treatment of fibroblasts.

MATERIALS AND METHODS

Materials. dbcAMP, cAMP, theophylline, epinephrine and insulin were purchased from Sigma (St. Louis, Missouri). [1-^{14}C]Stearic acid (51 mCi/mmol), [1-^{14}C]oleic acid (53 mCi/mmol), [1-^{14}C]palmitic acid (53 mCi/mmol) and [1-^{14}C]sodium acetate (55 mCi/mmol) were from C.E.A. (Saclay, France). [1-^{14}C]Linoleic acid (51 mCi/mmol), [1-^{14}C]arachidonic acid (58 mCi/mmol) and [1-^{14}C]oleyl coenzyme A (CoA) (55 mCi/mmol) were purchased from Amersham (Buckinghamshire, England).

Cell culture. Established hamster fibroblasts (EHB cells) were provided by Dr. Tournier (7). Cells were cultured in Minimum Essential Medium supplemented with 10% fetal calf serum. Cells were seeded in 60-mm Petri dishes at the density of 10^6 cells/dish. Twenty-four hr after seeding, dbcAMP and theophylline (Th) were added, and cells were treated 24 hr before incorporation of labeled precursors.

Incorporation of precursors into TG. After evaporation to dryness under nitrogen, fatty acids were resuspended in a fatty acid-free human serum albumin solution (0.2 g/l).

Cells were incubated for 2 hr with fatty acids (1 μCi/ml), except in kinetic studies, when incubations with [^{14}C]oleic acid were done for 1 hr only. After incorporation, cells were washed four times with a phosphate-buffered solution, pH 7.4, harvested with a rubber policeman and centrifuged. The pellet was resuspended in 9 g/l NaCl. Protein determination was performed on aliquots of the cell suspension by the method of Lowry et al. (8). Phospholipid and neutral lipid separation was performed by thin layer chromatography on silica gel plates in chloroform/methanol/acetic acid/water (50:30:8:4, v/v/v/v) for phospholipids and hexane/diethyl ether/acetic acid (70:30:2, v/v/v) for neutral lipids. The main radiolabeled phospholipids were phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. Aliquots of the cell suspension were directly applied to the plates before chromatography as described by Dosado et al. (9). Phospholipid and TG spots were identified by comparison with known purified standards from Sigma.

After autoradiography, phospholipid and TG spots were cut out and counted by liquid scintillation with an Intertechnique instrument. Results are expressed in pmoles of precursor incorporated/mg protein.

Diacylglycerol acyltransferase assay. After treatment with dbcAMP 10^{-4} M + Th 10^{-3} M for 24 hr, cells were washed and harvested with NaCl 9 g/l, pH 7.4, with or without NaF 5×10^{-2} M, centrifuged and resuspended in the same solution. After sonication, the enzyme activity was measured using [1-^{14}C]oleyl CoA (Amersham, 55 mCi/mmol) as substrate. The incubation mixture (final volume, 0.1 ml) contained 2×10^5 dpm of labeled substrate diluted with nonlabeled oleyl CoA (final concentration 10^{-5} M of oleyl CoA) in 0.1 M phosphate buffer (pH 7.4) 1 g/l fatty acid-free serum albumin, 5×10^{-3} M $MgCl_2$ and about 0.05 mg of cell homogenate. Incubations were carried out with 100 μg protein at 37 C for 5 min. An aliquot of the incubation mixture was then spotted on a silica gel plate, and separation of neutral lipids was achieved using hexane/diethyl ether/acetic acid (70:30:2, v/v/v) as solvent system.

RESULTS

The effect of Th alone or in the presence of dbcAMP on the incorporation of various exogenous [^{14}C]labeled fatty acids is presented in Table 1. It can be noted that 10^{-3} M Th enhanced fatty acid incorporation two- or threefold. Maximum increase was obtained with dbcAMP 10^{-3} M + Th 10^{-3} M. For internal control, we also present results concerning the incorporation of fatty acids into phospholipids, which was not affected by dbcAMP + Th. Furthermore, comparison of the relative incorporation of fatty acids into the different phospholipids (phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol) showed no significant difference between dbcAMP treated and untreated cells.

The time dependence of the effects of dbcAMP on oleic acid incorporation into TG was investigated. As shown in Table 2, a stimulation of oleic acid incorporation is observed for short-term incubations, even when dbcAMP + Th were introduced simultaneously with the precursor.

TABLE 1

Effect of Theophylline (Th) and Dibutyryl Cyclic AMP (dbcAMP) on Incorporation of Radiolabeled Fatty Acids into Phospholipids and Triacylglycerols

Precursor	Addition (M)	Phospholipids	Triacylglycerols
Stearic acid	None	3850 ± 650	400 ± 50
	Th 10^{-3}	4550 ± 750	800 ± 100
	dbcAMP 10^{-4} + Th 10^{-3}	4500 ± 650	1150 ± 150
	dbcAMP 10^{-3} + Th 10^{-3}	4050 ± 550	1550 ± 100
Oleic acid	None	9400 ± 800	500 ± 150
	Th 10^{-3}	8800 ± 1000	1500 ± 200
	dbcAMP 10^{-4} + Th 10^{-3}	8900 ± 700	1600 ± 200
	dbcAMP 10^{-3} + Th 10^{-3}	10200 ± 1000	2100 ± 300
Arachidonic acid	None	11300 ± 1100	360 ± 60
	Th 10^{-3}	10400 ± 900	950 ± 80
	dbcAMP 10^{-4} + Th 10^{-3}	11600 ± 1400	1500 ± 100
	dbcAMP 10^{-3} + Th 10^{-3}	10100 ± 1000	1600 ± 200
Linoleic acid	None	3700 ± 400	770 ± 90
	dbcAMP 10^{-4} + Th 10^{-3}	3400 ± 500	1450 ± 200
Palmitic acid	None	1500 ± 200	80 ± 20
	dbcAMP 10^{-3} + Th 10^{-4}	1550 ± 250	330 ± 40

Cells are taken in exponential growth phase one day after seeding. Incorporation of fatty acids (2 hr) was performed after a 24-hr treatment with Th or/and dbcAMP. Phospholipids and triacylglycerols are separated by chromatography on silica gel plates. Results are expressed in pmoles fatty acids incorporated/mg proteins. Means of three experiments ± s.d.

TABLE 2

Kinetics of the Effect of Dibutyryl Cyclic AMP (dbcAMP) and Theophylline (Th) on Oleic Acid Incorporation into Triacylglycerols (TG)

Addition	Preincubation time	Incorporation into TG (percentages of controls)
None		100 (± 16)
+ dbcAMP 10^{-4} M + TH 10^{-3} M	0 hr	130 ± 24
	1 hr	147 ± 22
	2 hr	172 ± 25
	3 hr	235 ± 28
	6 hr	274 ± 33
	15 hr	248 ± 26
	24 hr	282 ± 38

Cells were preincubated for the indicated time with dbcAMP 10^{-4} M + Th 10^{-3} M. [^{14}C]Oleic acid (1 μCi/ml) was then added and incorporation into TG followed during 1 hr. Results are expressed in percentages of controls. Means of three experiments ± s.d.

Under these conditions, the relative incorporation into TG was stimulated 1.3-fold. The effect of dbcAMP increased as a function of time up to 4–6 hr preincubation, when a 2.5- to 3-fold stimulation was obtained. Further incubation did not significantly increase the effect of dbcAMP.

The short-term effect of dbcAMP suggests a rapid effect of the nucleotide on DGAT activity, possibly by a phosphorylation/dephosphorylation mechanism. In this regard, we undertook the study of the enzyme activity *in vitro*. If no special care was taken during the preparation of cell extracts, only a slight increase (30%) was found in cells treated with dbcAMP + Th (Table 3). However, if sodium fluoride was introduced to inhibit phosphoprotein phosphatases, then a three-fold increase of the DGAT activity was observed in the presence of dbcAMP + Th.

In general, cAMP is known to inhibit biosynthetic pathways such as phosphatidylcholine (10) or cholesterol synthesis (11). In our system, cAMP increases TG synthesis from labeled exogenous fatty acids. To determine the effect of cAMP on TG synthesis from endogenous fatty acids, cells were labeled with [^{14}C]sodium acetate and incorporation into all lipid classes was followed. Results from Table 4 point out that cAMP decreases de novo synthesis of all lipid classes (TG, fatty acids, sterols, phospholipids). Thus, the decrease in TG synthesis is probably due to a diminution in endogenous fatty acid supply.

In the next experiment, the effect of cAMP on oleic acid incorporation into TG was comparatively studied in different cell types, namely macrophages, endothelial cells and hepatocytes. It is clear from Table 5 that, in rat hepatocytes, cAMP decreases TG synthesis from exogenous oleic acid whereas the reverse was observed in the other cell types.

DISCUSSION

From our results, it appears that exogenous [^{14}C] fatty acid incorporation into TG is markedly enhanced in the presence of cAMP. Claycomb and Kilsheimer (12) reported an elevation of acyl-CoA levels in liver slices in the presence of cAMP. In our experiments, if cAMP only exerts its action by increasing acyl-CoA levels, then a stimulation of fatty acid incorporation into phospholipids must have been observed. As such an effect did not occur, it can be assumed that in addition to acyl-CoA levels, TG formation is regulated by the activity of the DGAT itself, It must be emphasized also that phospholipids and TG have a common precursor: diacylglycerol. However, in our

TABLE 3

Activity of Diacylglycerol Acyltransferase (DGAT) from Fibroblasts Treated with Dibutyryl Cyclic AMP (dbcAMP) and Theophylline (Th) for 24 Hr

Pretreatment	Cell extract	DGAT activity
None	− NaF	19.9 ± 3.3
	+ NaF	36.4 ± 6.2
dbc AMP 10^{-4} M + Th 10^{-3} M	− NaF	26.5 ± 5.1
	+ NaF	110.7 ± 17.6

After incubation, cells were washed with NaCl 9 g/l, with or without NaF (50 mM, pH 7.4), centrifuged and resuspended in the same solution. After sonication, the enzyme activity was measured as described in Materials and Methods, and expressed in pmol/min/mg protein. Means of two experiments in triplicate ± s.d.

TABLE 4

Effect of Theophylline (Th) and Dibutyryl Cyclic AMP (dbcAMP) on Synthesis of Different Lipid Classes from Sodium Acetate

Addition (M)	Triacylglycerols	Fatty acids	Sterols	Phospholipids
None	9950 ± 1550	6850 ± 450	15100 ± 1450	48250 ± 4550
Th 10^{-3}	6800 ± 850	4950 ± 1050	13250 ± 3150	48450 ± 5950
dbcAMP 10^{-4} + Th 10^{-3}	3900 ± 450	4150 ± 650	12000 ± 12500	50500 ± 5400
dbcAMP 10^{-3} +Th 10^{-3}	2900 ± 350	2100 ± 350	6100 ± 550	35200 ± 4100

Cells on exponential growth phase were treated 24 hr with Th and/or dbcAMP before incorporation of [^{14}C]sodium acetate (20 μCi/ml). After 4 hr, cells were harvested and different lipid classes separated by thin layer chromatography. Results are expressed in pmoles of sodium acetate incorporated/mg protein. Means of three determinations ± s.d.

TABLE 5

Effect of Dibutyryl Cyclic AMP (dbcAMP) and Theophylline (Th) on Oleic Acid Incorporation into Triacylglycerols (TG) in Different Cell Types

Cell type	Addition (M)	pmoles Oleic Acid incorporated into TG
J 774	None	1940 ± 250
(Balb/c macrophages)	+dbcAMP 10^{-4} + Th 10^{-3}	4270 ± 510
CPA	None	4120 ± 660
(bovine endothelial cells)	+dbcAMP 10^{-4} + Th 10^{-3}	6590 ± 810
Rat hepatocytes	None	5710 ± 750
	+dbcAMP 10^{-4} + Th 10^{-3}	2280 ± 560

The two cell lines J774 and CPA were purchased from the American Cell Type Culture Collection. Rat hepatocytes were obtained by collagenase perfusion in the laboratory. Cells were treated 24 hr with dbcAMP and Th before incorporation of oleic acid during 4 hr (1 μCi/ml). Means of three determinations ± s.d.

experimental conditions, cAMP stimulates incorporation of fatty acids into TG, whereas incorporation into phospholipids is not modified. Thus, regulation of TG formation from exogenous fatty acids takes place after diacylglycerol synthesis, at the level of the specific enzyme DGAT.

Kinetic studies pointed out a rapid effect of dbcAMP on oleic acid incorporation into TG, thus suggesting a regulation by covalent modification of the enzyme, possibly by a phosphorylation-dephosphorylation mechanism. Fluoride is well known as an anion which inhibits phosphoprotein phosphatases, and it is evident from Table 3 that its addition to cell extract protects the enzyme against dephosphorylation and inactivation.

In vitro preincubation of cell extract with cAMP and Th gave irregular results: only a small increase (5-25%) in DGAT activity was noted. Similar results were observed after preincubation with ATP-Mg^{2+}. Thus, it appears that cellular integrity is necessary for correct activation of DGAT by cAMP.

Another question was whether the TG content of fibroblasts increases following incubation with dbcAMP and Th. Two lines of evidence indicate that the TG content is not modified. First, histochemical staining with the dye Oil-Red-O showed no modification in the number and size of lipid droplets. Second, the TG content, determined by the isotopic equilibrium method with long-term labeling in the presence of [^{14}C]glycerol, is not affected after 24 hr treatment with dbcAMP and Th. This might be accounted for by the balance between synthesis of TG from exogenous and endogenous fatty acids; it must be emphasized that if cAMP increases TG synthesis from exogenous fatty acids, it has a reverse effect on TG synthesis from endogenous substrates (Table 4).

In liver, it has been demonstrated that TG synthesis (1,5) and secretion (13) is decreased by cAMP. Our results demonstrate that, in cultured fibroblasts, the effects of cAMP are just inversed. These results have been extended to other cell types such as macrophages or endothelial cells. It should be noted that peripheral tissues may have a different regulation mechanism than liver, whose role it is to redistribute fatty acids, and our results point at tissue specificity in the regulation of TG metabolism.

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A. Lageron did histochemical studies and L. Berland typed the manuscript.

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Differences in the Long Chain (Sphingoid) Base Composition of Sphingomyelin from Rats Bearing Morris Hepatoma 7777

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The long chain bases of sphingomyelin from Morris hepatoma 7777 and host and control livers were analyzed by capillary gas liquid chromatography. Sphingosine (18:1) was the major long chain base of control livers (66.5%) and hepatomas (65.6%), but hepatomas also had a high percentage (9.3 vs 4.4) of the 16:1 homolog. Host liver had the most unusual long chain base composition, with ca. equal 16:1 (24.4%) and 18:1 (21.4%) and high amounts of 20-carbon bases (9.2% 20:0 and 15.3% 20:1). These differences may be related to the aberrant fatty acid metabolism known to occur in tumor-bearing animals. Such large perturbations in the long chain base composition of hepatic sphingomyelin are unprecedented and could have a major impact on the properties of host membranes.

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Sphingomyelin is composed of a backbone moiety, commonly referred to as a long chain or sphingoid base, to which a fatty acid and a phosphorylcholine head group are attached. The prevalent long chain base of sphingomyelin is sphingosine (*trans*-4-sphingenine), but lesser amounts of sphinganine (dihydrosphingosine), phytosphingosine (4D-hydroxysphinganine) and homologs of these compounds are sometimes found (1). It has been proposed (2) that the type of long chain base moiety of a given sphingolipid affects its physical properties.

Long chain base biosynthesis begins with the condensation of serine and a fatty acyl-CoA (3). The enzyme catalyzing this reaction is highly specific for palmitoyl-CoA (3–7), which probably explains the predominance of 18-carbon long chain bases.

In this report, we describe major differences in the long chain base composition of sphingomyelin from animals bearing Morris hepatoma 7777, whose phospholipids are already known to have an unusual fatty acid profile (8–10). Unexpectedly, the greatest differences were found with host liver, suggesting that the tumor has a major impact on host sphingolipids.

MATERIALS AND METHODS

Female Buffalo rats were used to grow Morris hepatoma 7777 as described previously (7). The rats were fed Purina rat chow ad libitum until the tumors had reached 5–10 g, after which the rats and matched controls were killed after an overnight fast.

The total lipids were extracted according to Bligh and Dyer (11) and dissolved in $CHCl_3$/methanol (2:1, v/v) and applied to preparative (0.5 mm) thin layer chromatography (TLC) plates made with Silica Gel H. After the chromatogram was developed with $CHCl_3$/methanol/acetic acid/water (50:25:9:4.5, v/v/v/v), the lipids were visualized by spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol and sphingomyelin was scraped from the plate and eluted with $CHCl_3$/methanol/acetic acid/water (50:50:1:1, v/v/v/v).

Sphingomyelin was hydrolyzed with aqueous methanolic HCl as described by Gaver and Sweeley (12). When the temperature is carefully kept at 70 C, little methyl ether formation occurs. The hydrolysis products were separated by preparative TLC on Silica Gel H developed with $CHCl_3$/methanol/water (40:10:1, v/v/v) and recovered from the silica gel as described above. Long chain bases were either converted to trimethylsilyl (TMS) derivatives (13) or oxidized to aldehydes as described by Sweeley and Moscatelli (14). The former were analyzed as previously described (15), and the aldehydes were immediately analyzed by capillary gas liquid chromatography (GLC) using a Varian 3700 chromatograph (Varian Associates, Palo Alto, California) with a flame ionization detector. The gas liquid chromatograph was equipped with a 50-m BP-10 vitreous silica capillary column (Scientific Glass Engineering, Austin, Texas) and a model CDS-111 automatic integrator. The injector and detector temperatures were maintained at 250 C, and the column was operated isothermally at 200 C with helium as the carrier gas.

RESULTS

Sphingomyelin from control livers contained mainly sphingosine and small amounts of the 16- and 20-carbon homologs (Table 1). This general distribution of long-chain bases is typical of mammalian sphingolipids (1). The results were similar with sphingomyelin from Morris hepatoma 7777, although there were somewhat higher amounts of the 16- and 20-carbon homologs.

The most striking differences were found with sphingomyelin from host livers. Sphingosine (18:1) levels were about equal to 16:1 and the chain lengths were about equally distributed among 16-, 18- and 20-carbon homologs. All of these were significantly different ($P < 0.05$) from the controls. Saturated homologs also represented a much higher proportion of the total than for the control.

All of the samples had small amounts of odd-carbon-number long chain bases. Some of these could represent phytosphingosine, which yields aldehydes with n-3 carbon atoms upon treatment with periodate. However, analyses of the TMS derivatives provided similar estimates of the relative amounts of the odd-carbon bases (and were included in the percentages given in Table 1);

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TABLE 1

Long Chain Base Composition of Sphingomyelin from Control and Host Liver and Morris Hepatoma 7777[a]

Long chain base	Percentage of total long chain bases (mean ± SD)		
	Control	Host	Hepatoma
16:0	3.1 ± 1.2	4.8 ± 3.4	1.7 ± 1.2
16:1	4.4 ± 2.0	24.4 ± 11.7	9.3 ± 5.8
Sum	7.5	29.2	11.0
17:0	1.8 ± 1.7	1.5 ± 0.7	0.9 ± 0.8
17:1	2.5 ± 1.3	1.2 ± 0.4	3.0 ± 2.0
Sum	4.3	2.7	3.9
18:0	2.6 ± 1.5	6.8 ± 3.2	2.0 ± 2.3
18:1	66.5 ± 11.5	21.4 ± 14.4	65.6 ± 9.0
Sum	69.1	28.2	67.6
19:0	2.1 ± 2.9	2.3 ± 1.9	1.1 ± 1.6
19:1	1.9 ± 1.5	1.3 ± 0.9	0.7 ± 0.9
Sum	4.0	3.6	1.8
20:0	0.7 ± 0.6	9.2 ± 5.6	2.4 ± 1.7
20:1	4.5 ± 2.1	15.3 ± 6.0	5.5 ± 4.4
Sum	5.2	24.5	7.9
Others	9.0 ± 0.9	11.6 ± 1.2	7.9 ± 2.0
Sum of saturated	10.3	24.6	8.1
Sum of unsaturated	83.4	63.6	84.1

[a]Data represent mean ± S.D. for three separate experiments.

therefore, it appears that 17- and 19-carbon species are present in small but significant percentages.

The fatty acid compositions of phospholipids from these tissues are known to be different (8–10), and this was confirmed by analyses of phosphatidylcholine (data not shown). The ratio of all 16-carbon fatty acids to 18-carbon fatty acids was lower for hepatomas (0.25) than for host liver (0.37) or controls (0.37). Myristic acid percentages were small in all groups; however, hepatomas had 3.5- to sevenfold more than control or host liver, respectively, as has been observed previously (8,9).

DISCUSSION

Relatively little is known about the factors that influence the alkyl chain length or degree of unsaturation of sphingolipid long chain bases. There are few reported examples of modification of the backbones of a particular sphingolipid in a given tissue. Brain gangliosides contain progressively greater amounts of 20-carbon long chain bases with age (16,17), and Kulmacz and Schroepfer (18) found that the nature of the exogenous fatty acids given to yeast causes a dramatic alteration in the long chain bases. This report established that Morris hepatoma 7777 causes a significant shift in the long chain base composition of host liver sphingomyelin.

Sphingomyelin long chain bases were analyzed in this study because this species constitutes a major portion of the sphingolipids of liver. Furthermore, analyses of total long chain bases could present differences due to the expression of specific glycolipids (gangliosides in hepatoma H5123 are sixfold higher than in liver, for example) (19) rather than a perturbation in long chain base biosynthesis.

It is possible that these differences are related to the aberrant fatty acid distribution in this and other hepatomas (8–10). Elevated myristic acid could, for example, increase the formation of 16-carbon homologs.

Whatever the mechanism, these findings establish that large shifts in the long chain base composition of sphingolipids can occur in neoplasia, and they appear both in the transformed tissue and in organs of the tumor-bearing animal. Since Kannagi et al. have proposed that glycolipid biosynthesis can be influenced by the types of ceramides available (20), these altered backbones might account for some of the abnormal glycolipids seen in tumor cells. Changes in the amount and type of sphingomyelin molecules could have important implications for properties of membranes throughout the organism.

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LETTER TO THE EDITOR

Effects of Purines on Human and Rat Plasma Lecithin: Cholesterol Acyltransferase Activity

Sirs:

Direct inhibition in vitro of rat plasma lecithin:cholesterol acyltransferase (LCAT) (EC.2.3.1.43.) by hypoxanthine, xanthine, guanine and uric acid has been described by Solera et al. (1). Since this publication, there apparently have been no further reports concerning the effects of purines on LCAT activity in plasma of rat or other species, except for a study by Gillett et al. (2) which showed that none of the above-mentioned purines were inhibitory in human plasma at concentrations similar to those used in rat plasma. However, this later study made exclusive use of the Stokke and Norum (3) method for LCAT determination and, therefore, does not allow strict comparison with the earlier results for rat plasma, which were obtained using different methods. Solera et al. have interpreted their results with rat plasma to suggest that the well-known association between human hyperuricemia and hypertriglyceridemia (4) may result from the interaction between raised levels of uric acid and its precursors and LCAT. This could impair the metabolism of surface cholesterol from triglyceride-rich lipoproteins and lead to an accumulation of these lipoproteins in the plasma. Because no obvious metabolic link has been uncovered between purine and triglyceride metabolism, the results obtained by Solera et al. would appear to be important, together with the question of whether or not purines inhibit the activity of LCAT in human plasma.

We accordingly have repeated the experimental work of Solera et al. (1) with similar methods and using both human and rat plasma. Basically two radiochemical methods were used by these authors to test for effects of purines on LCAT activity. In the first, the esterification of ^{14}C-labeled cholesterol added to native plasma was measured after an incubation of 24 hr. In the second method, heated homologous plasma mixed with an albumin-stabilized emulsion of labeled cholesterol was used as substrate for LCAT during 4-hr incubations of native plasma as described by Glomset and Wright (5).

Some detailed changes from the procedures outlined by Solera et al. were found necessary. These included the testing of all purines at a final concentration of 1.4 mM (not 1.4 μM) because it is recognized that known potent inhibitors of LCAT, such as 5,5′-dithiobisnitrobenzoic acid, are only effective at close to mM final concentrations (3,6). In both methods, strict observance of a pH of 7.0 for all incubation mixtures was stipulated, although many published methods use a pH of 7.4 (3,7). Solubility problems for some purines in the solution of tris-0.025 M and NaOH-0.5 M, and the high pH of this solution, made it impossible to add sufficient purine to reach a final concentration of 1.4 mM at pH 7.0, without also adding a buffer in the acid range. Finally, antibiotics were excluded from the incubation mixture, as several of these substances, including benzylpenicillin, have been shown to be inhibitors of LCAT (8).

In the present study, the albumin-stabilized emulsion of [4-^{14}C]cholesterol was made up in 0.2 M sodium phosphate buffer pH 6.2 and not pH 7.4 as in the original description (3). For the 24-hr incubations, the assay mixtures contained 150 μl native plasma, 50 μl labeled cholesterol-albumin emulsion and 2.5 μl of the solution containing tris (0.025 M)-NaOH (0.5 M) or this solution containing purines (113.4) mM); final pH was 7.0. The assay mixtures for the Glomset and Wright method contained 180 μl of heat-inactivated (56 C, 30 min) plasma-labeled cholesterol substrate (eight vol plasma, one vol cholesterol-albumin emulsion), 20 μl native plasma and 2.5 μl purine solution or plain tris-NaOH; final pH was 7.4.

The results of Solera et al. were presented as means ± standard errors, with very wide variation being apparent in both assays, with and without purines, but especially in the 24-hr incubations (e.g., control percentage esterification during 24 hr was 15.93 ± a recalculated standard deviation of 31.3% for 18 rats and 18.32 ± 9.3% in 4 hr for the Glomset and Wright method). Besides demonstrating remarkable variations for animals of the same species kept under the same conditions, there is reason to question the absolute values of the means for these two control experiments. Values of the order of 60% for 24-hr cholesterol esterification in rat plasma have been reported (9,10), whereas in the original Glomset and Wright assay for human LCAT (5) incubation conditions were chosen which allowed less than 2% esterification of the original free cholesterol. The much higher values reported by Solera et al. (1) may indicate that LCAT activity in the substrate plasma was not completely destroyed by the heating procedure used.

Nevertheless, the results of the present study (Table 1) do validate the results obtained by Solera et al. for the inhibition by some purines of LCAT activity in rat plasma, while also being consistent with the report (2) that they do not inhibit LCAT activity in human plasma, as measured by Stokke and Norum method (3).

In both rat and human plasma, values for cholesterol esterification in the absence of purines obtained with both methods were consistent with previously published values (5,8,9). Hypoxanthine, xanthine, guanine and adenine all significantly reduced cholesterol esterification in rat plasma in both types of incubation, but neither were inhibitory in human plasma (Table 1). Uric acid had no apparent effect on esterification in either plasma. Of the inhibitory purines, hypoxanthine was the most potent, while xanthine and guanine had lesser and apparently equal effects and adenine was least effective. However, all four purines showed enhanced inhibitory activity in rat plasma incubated according to the Glomset and Wright method. These results with rat plasma are generally similar to those reported by Solera et al., except that in the present study, significant inhibition of cholesterol esterification was observed in the presence of adenine, while uric acid was totally inert and the inhibitory effects of the other purines were greater during the 4-hr rather than the 24-hr incubations.

TABLE 1

Influence of Purines on LCAT Activity[a]

	Percentage radioactivity incorporated into EC[b]					
	Assay without purine	Assay + uric acid (1.4 mM)	Assay + adenine (1.4 mM)	Assay + guanine (1.4 mM)	Assay + xanthine (1.4 mM)	Assay + hypoxanthine (1.4 mM)
Rat plasma						
Method 1, 24 hr	69.3 ± 2.1	67.6 ± 2.5	57.9 ± 3.1	28.6 ± 4.0	28.1 ± 3.9	18.0 ± 2.8
Method 2, 4 hr	2.97 ± 0.37	3.23 ± 0.66	1.20 ± 0.04	0.79 ± 0.05	0.78 ± 0.06	0.55 ± 0.04
Significance						
Method 1		NS[c]	$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Method 2		NS	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Inhibition percentage						
Method 1		—	16	59	59	74
Method 2		—	60	73	74	81
Human plasma						
Method 1, 24 hr	39.2 ± 2.5	39.4 ± 2.6	38.9 ± 2.2	38.6 ± 2.3	38.2 ± 2.2	37.9 ± 2.1
Method 2, 4 hr	2.46 ± 0.17	2.41 ± 0.14	2.31 ± 0.14	2.78 ± 0.29	2.44 ± 0.15	2.51 ± 0.13
Significance						
Method 1		NS	NS	NS	NS	NS
Method 2		NS	NS	NS	NS	NS

[a]Results represent mean values ± standard errors for plasma from 7 rats and 6 humans.
[b]EC, esterified cholesterol.
[c]NS, nonsignificant.

Thus the present study, while confirming already published results, does not permit conclusions on the possible influence of raised purine levels on LCAT activity and the development of hypertriglyceridemia in human hyperuricemia, because none of the purines tested was inhibitory for cholesterol esterification in human plasma. Nevertheless, the differences between purine effects on the rat plasma LCAT reaction and that of human plasma are of interest and are potentially important, since further studies may reveal new details of the action of this enzyme at the level of lipoprotein metabolism.

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Reaction of Thiobarbituric Acid with Saturated Aldehydes

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The reaction of thiobarbituric acid (TBA) with saturated aldehydes, i.e., 1-butanal, 1-hexanal and 1-heptanal, produced a 455-nm yellow and a 532-nm red pigment. Formation of the pigments depended on the reaction conditions. The yellow pigment was unstable in the presence of excess amounts of the saturated aldehydes. The red pigment was formed only when the reaction was performed at a TBA/aldehyde ratio of 1:1 in aqueous acetic acid. Formation of the yellow and red pigments required molecular oxygen. The colorless adducts, intermediates for the yellow and the red pigments, were isolated from the reaction mixtures. Aldol condensation and dehydration of 2 mol of the saturated aldehydes initially gave the α,β-unsaturated aldehydes, which in turn reacted with TBA to form the colorless adducts, pyranopyrimidine derivatives. The adducts were then converted into the yellow and red pigments under aerobic conditions.

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The thiobarbituric acid (TBA) test is commonly used for measurement of peroxidation of unsaturated fatty acids or lipids (1). The peroxidation of lipids has been shown to give malonaldehyde (2) and monofunctional aldehydes (3). TBA produces a red pigment with a maximum absorption at 532 nm due to malonaldehyde (4,5). TBA also reacts with the saturated and unsaturated monofunctional aldehydes (6-11), giving, respectively, an unstable yellow pigment with a maximum absorption at 452 nm and a red pigment with a maximum absorption at 532 nm.

The reaction of 2 mol of TBA with 1 mol of malonaldehyde gives the red crystalline 2:1 adduct whose structure has been unambiguously established (12,13). The mechanisms of the reaction of TBA with the saturated and unsaturated monofunctional aldehydes, however, remain obscure. We investigated the reaction of TBA with the saturated aldehydes and found that a yellow and a red pigment were formed from an intermediate colorless adduct in the presence of molecular oxygen. We describe here the structure of the intermediate colorless adducts and their relevance to the formation of the yellow and red pigments.

MATERIALS AND METHODS

Materials. TBA, 1-butanal and 1-hexanal were the products of Wako Pure Chemical Industries Ltd. (Osaka, Japan). 1-Heptanal, 2-ethyl-2-hexenal and malonaldehyde bis(dimethylacetal) were the products of Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Thin layer chromatography (TLC) was performed with Wakogel B-5F (Wako Pure Chemical Industries Ltd.). Silica gel column chromatography was performed by use of silica gel for column chromatography (above 100 mesh) from Kanto Chemical Co. Ltd. (Tokyo, Japan).

Analysis. Absorption spectra were measured with a Shimadzu UV-240 UV-visible recording spectrophotometer. Mass spectra were obtained with a Hitachi M-80 double focusing mass spectrometer. ^{1}H-Nuclear magnetic resonance (NMR) spectra were taken in d_6-dimethylsulfoxide on a Bruker AM-400 NMR spectrometer with tetramethylsilane (TMS) as an internal standard. ^{13}C-NMR spectra were taken in d_6-dimethylsulfoxide on a JEOL JNM-FX-100 Fourier-Transform NMR spectrometer with TMS as an internal standard, using both noise and off-resonance decoupling techniques. High performance liquid chromatography (HPLC) was carried out with a Shimadzu LC-5A liquid chromatograph equipped with a stainless steel column (4.6 mm × 25 cm) of Zorbax ODS. The chromatograph was operated by elution with a solvent mixture of MeOH/H_2O (8:2, v/v) or MeOH/0.04 M acetate buffer (pH 5.5) (4:6, v/v) at a flow rate of 0.8 ml/min. The peak was detected by use of a Shimadzu SPD-2A spectrophotometer.

Formation of a yellow and a red pigment. TBA and each aldehyde at the indicated concentration were dissolved in 5.0 ml of glacial acetic acid or 15% acetic acid in a test tube with a screw cap. The mixture was kept or heated either in air or under nitrogen gas. A solution of each colorless adduct at the indicated concentration in 5.0 ml of 15% acetic acid was similarly treated. After cooling, the absorption spectrum of the mixture was measured. HPLC of the reaction mixture was performed.

Colorless adducts of TBA and the saturated aldehydes. (i) Adduct W_B from 1-butanal. A mixture of 1.44 g (10 mmol) of TBA and 1.16 g (16 mmol) of 1-butanal in 170 ml of 15% acetic acid was heated for 15 min under reflux. The pale yellow mixture was extracted with chloroform several times. The chloroform extracts were washed with water and dried over anhydrous sodium sulfate. The solvent was removed in vacuo, and the residue was crystallized from ethyl alcohol to afford colorless needles of W_B in a yield of 15% (384 mg). Recrystallization from ethyl alcohol gave pure sample of W_B, mp 179-182 C (dec). UV: λ_{max}^{EtOH} 293 nm (ε: 13600), 336 nm (16800). ^{13}C-NMR and ^{1}H-NMR spectra are shown in Tables 1 and 2, respectively. Mass spectrum: m/e (rel int); M^+ 252 (62), M^+-15 ($CH_3\cdot$) 237 (6), M^+-29 ($C_2H_5\cdot$) 223 (22), M^+-43 ($C_3H_7\cdot$) 209 (100). TLC: Rf 0.50 (solvent: $CHCl_3$/MeOH, 9:1, v/v). Anal: calcd for $C_{12}H_{16}N_2O_2S$: C, 57.11; H, 6.40; N, 11.10. Found: C, 57.06; H, 6.36; N, 11.06.

(ii) Adduct W_B from 2-ethyl-2-hexenal. A mixture of 5.0 g (35 mmol) of TBA and 5.0 g (40 mmol) of 2-ethyl-2-hexenal in 170 ml of 15% acetic acid was heated for 15 min under reflux. The pale yellow reaction mixture was extracted with chloroform as described. White needles of W_B were obtained from ethyl alcohol in a yield of 42% (3.70 g). Recrystallization from ethyl alcohol gave pure specimen, mp 181-184 C (dec). UV: λ_{max}^{EtOH} 293 nm (ε: 13700), 337 nm (17100). ^{1}H-NMR spectrum taken in d_6-dimethylsulfoxide was identical to that of the adduct obtained from 1-butanal. Mass spectrum: m/e (rel int); 252 (40), 237 (5), 223 (10), 209 (100). Mixed fusion test of this specimen and the adduct obtained from 1-butanal showed that they were identical. Anal: calcd for $C_{12}H_{16}N_2O_2S$: C, 57.11; H, 6.40;

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N, 11.10. Found: C, 57.14; H, 6.32; N, 11.14.

(iii) Adduct W_{Hx}. A mixture of 5.0 g (35 mmol) of TBA and 5.0 g (50 mmol) of 1-hexanal in 170 ml of 15% acetic acid was heated for 45 min under reflux. To a pale yellow reaction mixture was added an equal amount of chloroform. Insoluble yellow precipitate was removed by filtration. The chloroform layer was separated from the aqueous layer and dried over anhydrous sodium sulfate. It was evaporated to dryness, and the residue was applied to a column of silica gel (3 × 21 cm). Compound W_{Hx} was eluted with chloroform, and it was crystallized from ethyl alcohol-water as colorless granules in a yield of 1.9% (198 mg), mp 132–138 C (dec). Recrystallization from the same solvent gave pure sample, mp 142–146 C. UV: λ_{max}^{EtOH} 293 nm (ε: 12600), 340 nm (16000). ^{1}H-NMR (d_6-dimethylsulfoxide): ppm; 13.08 (1H, s, NH), 12.21 (1H, s, NH), 5.99 (1H, s, =CH-), 4.98 (1H, d, =CH-), 2.01 (2H, m, $-CH_2-$), 1.71 (1H, m), 1.2-1.6 (11H, bm), 0.89 (3H, t, $-CH_3$), 0.87 (3H, t, $-CH_3$). Mass spectrum: m/e (rel int); M^+ 308 (40), M^+-43 ($C_3H_7\cdot$) 265 (10), M^+-57 ($C_4H_9\cdot$) 251 (12), M^+-71 ($C_5H_{11}\cdot$) 237 (100). TLC: Rf 0.60 (solvent: $CHCl_3$/MeOH, 9:1, v/v). Anal: calcd for $C_{16}H_{24}N_2O_2S$: C, 62.29; H, 7.86; N, 9.08. Found: C, 62.15; H, 7.88; N, 9.12.

(iv) Adduct W_{Hp}. A mixture of 5.0 g (35 mmol) of TBA and 5.0 g (44 mmol) of 1-heptanal was treated and purified as in the case of the reaction of TBA and 1-hexanal. A small amount of colorless needles of W_{Hp} was obtained (7 mg) from ethyl acetate-*n*-hexane, mp 122–124 C (dec). UV: λ_{max}^{EtOH} 290 and 340 nm. Mass spectrum: m/e (rel int); M^+ 336 (80), M^+-43 ($C_3H_7\cdot$) 293 (10), M^+-57 ($C_4H_9\cdot$) 279 (20), M^+-71 ($C_5H_{11}\cdot$) 265 (95), M^+-85 ($C_6H_{13}\cdot$) 251 (100). TLC: Rf 0.62 (solvent: $CHCl_3$/MeOH, 9:1,v/v).

RESULTS

Formation of a yellow and a red pigment in the reaction of TBA with saturated aldehydes. Formation of a yellow and a red pigment in the reaction of TBA with 1-butanal, 1-hexanal and 1-heptanal was investigated. TBA was reacted with the 0.2–8 equivalents of 1-butanal. When 0.5 mM TBA was reacted with an equivalent amount of 1-butanal in glacial acetic acid at 100 C for 15 min, absorbance at 455 nm of the reaction mixture increased from 0.05 to 2.4 during standing at room temperature for 21 hr (Fig. 1). When the reaction mixture was heated for a longer period (6 hr), the absorbance decreased to 0.5. Thus, the yellow pigment formed in the reaction mixture was degraded. This instability of the yellow pigment may be due to the amount of 1-butanal. This possibility was supported by the fact that treatment of TBA with 4 and 8 equivalents of 1-butanal produced less yellow pigment after 15-min heating followed by 21-hr standing and also after 6-hr heating. In these reactions in glacial acetic acid, no significant amount of red pigment was produced.

TBA (0.5 mM) was reacted with an equivalent amount of 1-butanal in 15% acetic acid at 25 C. A yellow pigment with a maximum at 455 nm was produced gradually, but it subsequently degraded due to its instability in the aqueous solution (6–9,11) (Fig. 2). When the same reaction mixture was heated at 100 C for 6 hr, a yellow pigment was completely degraded and a

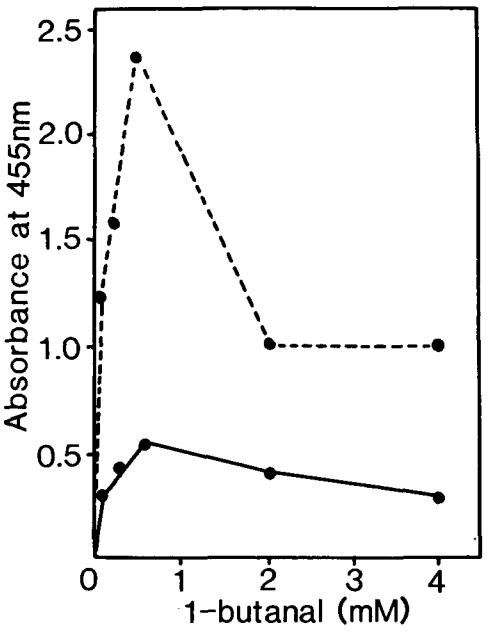


FIG. 1. Relationship between the amount of 1-butanal and absorbance at 455 nm of the reaction mixture in glacial acetic acid containing 0.5 mM TBA. The mixture was heated at 100 C for 15 min followed by standing at room temperature for 21 hr (- - -) or was heated at 100 C for 6 hr (——).

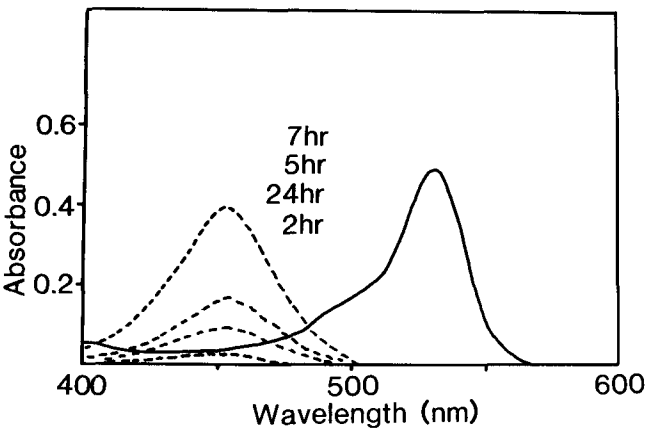


FIG. 2. Absorption spectra of the reaction mixture of 0.5 mM TBA and 0.5 mM 1-butanal in 15% acetic acid. The mixture was kept at 25 C for indicated periods (- - -) or was heated at 100 C for 6 hr (——).

red pigment with a maximum at 532 nm was formed (Fig. 2). The absorption spectrum of the red pigment was quite similar to that of the 2:1 adduct of TBA and malonaldehyde (12). It is likely that the red pigment was produced via the yellow pigment. Formation of the yellow and red pigments was suppressed completely when the reaction mixture was substituted with nitrogen gas, indicating that molecular oxygen is involved in the reaction.

TBA was reacted with various amounts of 1-butanal in 15% acetic acid at 100 C for 15 min or 6 hr (Fig. 3). Formation of the yellow pigment increased as the concentration of 1-butanal increased. It is, however,

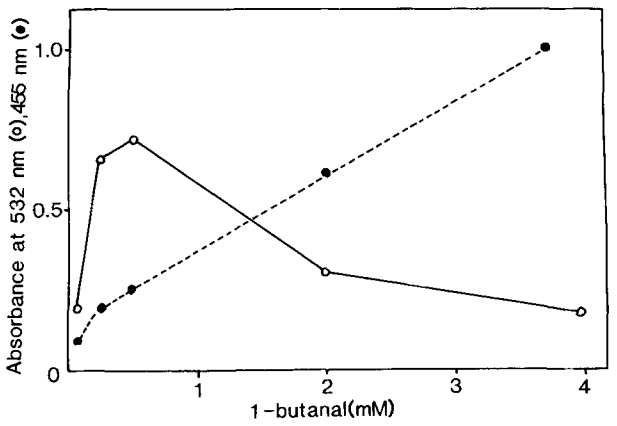


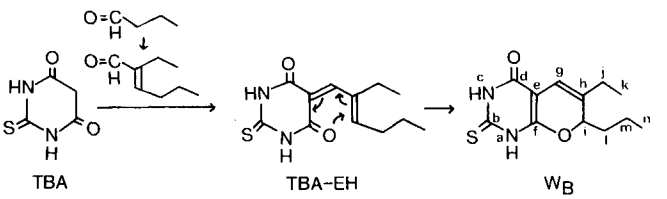
FIG. 3. Relationship between the amount of 1-butanal and absorbance at 455 and 532 nm of the reaction mixture in 15% acetic acid containing 0.5 mM TBA. The mixture was heated at 100 C for 15 min (- - -) or 6 hr (——), and the absorbance was measured immediately.

impossible to measure the amount of the yellow pigment, since the pigment is unstable. The maximal yield of the stable red pigment was obtained at about equimolar concentrations of 1-butanal. It is interesting that there is an optimal concentration of 1-butanal for the red pigment formation.

Reaction of TBA with 1-hexanal in 15% acetic acid gave the same results. Thus, the reaction of 0.5 mM TBA and 0.5 mM 1-hexanal at 25 C gradually produced an unstable yellow pigment and at 100 C for 6 hr produced a stable red pigment. As the concentration of 1-hexanal increased, formation of the yellow pigment increased, and the highest formation of the red pigment was observed in the reaction mixture containing nearly equal amounts of TBA and 1-hexanal. The reaction of TBA with 1-heptanal gave similar results.

Formation of colorless adducts in reaction of TBA and saturated aldehydes. We found that the reaction of TBA with the saturated aldehydes gave colorless adducts besides the yellow and red pigments. A mixture of TBA and a slight excess amount of 1-butanal in 15% acetic acid was heated at 100 C for 15 min under reflux. Colorless adduct W_B was isolated in a crystalline form from the chloroform extract of the yellow-colored reaction mixture. Absorption spectrum of W_B exhibited maxima at 293 and 336 nm. Elemental analysis and the mass spectrum of W_B revealed an empirical formula of $C_{12}H_{16}N_2O_2S$, suggesting that it was produced by dehydration of 1 mol TBA and 2 mol 1-butanal. Adduct W_B was produced in a much higher yield by reaction of TBA with 2-ethyl-2-hexenal, a compound produced by aldol condensation and dehydration of 2 mol 1-butanal, under the same reaction conditions. 2-Ethyl-2-hexenal could not, however, be produced from 1-butanal alone in 15% acetic acid heated at 100 C for 30 min. The unsaturated aldehyde may be produced in the presence of TBA as a catalyst.

Condensation of TBA with 2-ethyl-2-hexenal would give the 1:1 adduct (TBA-EH) shown in Scheme 1, as suggested in the reaction of TBA and aromatic aldehydes (14). While the empirical formula of adduct W_B supported the structure TBA-EH, mass spectral fragmentations, ^{1}H-NMR spectrum and ^{13}C-NMR spectrum did not support the structure. The compound in structure TBA-EH must be colored due to its high conjugation. The compound TBA-EH can be readily cyclized to its isomeric pyranopyrimidine, which may be colorless. Noise and off-resonance decoupling ^{13}C-NMR spectrum of W_B revealed the presence of five different primary, two different secondary, three different tertiary and two different quaternary carbons. All the carbon signals of W_B can be reasonably assigned as the pyranopyrimidine structure (Table 1). Two amide carbons in TBA-EH are symmetrically oriented and cannot be distinguished in ^{13}C-NMR spectrum, and the spectrum of TBA-EH would give only four different primary carbons. ^{1}H-NMR of W_B revealed 16 protons (Table 2). Two protons at 13.08 and 12.21 ppm were exchangeable and can be ascribed to the NH protons. Twelve alkyl protons due to ethyl and propyl groups appeared at the ppm values lower than 2.05, and they were assigned as listed in Table 2 by decoupling techniques. Characteristic signals, which appeared at 5.98 (singlet) and 5.01 ppm (doublet), can be assigned as the protons at the *g* position and the *i* position forming a pyran ring, respectively. The latter proton may be coupled with one of the adjacent hindered protons at the *l* position. If W_B had the noncyclized structure as TBA-EH, the proton signal at 5.01 ppm must be split into a triplet coupled with two adjacent protons. Mass spectral fragmentations of W_B gave an intense peak at m/e 209, indicating the loss of a propyl radical to leave a stable pyran radical. Thus, the structure of colorless adduct W_B was determined as 2-thio-4-oxo-6-ethyl-7-



SCHEME 1

TABLE 1

Noise and Off-Resonance Decoupling of ^{13}C-NMR Data of Adduct W_B (d_6-Dimethylsulfoxide)

Tetramethylsilane (ppm)		Assignment[a]
173.0	Singlet (C)	*d*
159.0	Singlet (C)	*b*
155.3	Singlet (C)	*f*
132.0	Singlet (C)	*e*
109.0	Doublet (CH)	*g*
92.2	Singlet (C)	*h*
81.9	Doublet (CH)	*i*
34.5	Triplet (CH_2)	
24.5	Triplet (CH_2)	*i,l,m*
17.4	Triplet (CH_2)	
13.6	Quartet (CH_3)	*k,n*
11.4	Quartet (CH_3)	

[a]See Scheme 1.

TABLE 2

^{1}H-NMR Data of Adduct W_B (d_6-Dimethylsulfoxide)

Tetramethylsilane (ppm)		Assignment[a]
13.08	1H Singlet	a,c
12.21	1H Singlet	
5.98	1H Singlet	g
5.01	1H Doublet (J=7.3 Hz)	i
2.05	2H Multiplet	j
1.73	1H Multiplet	l
1.51	2H Multiplet	l
1.39	1H Multiplet	m
1.04	3H Triplet	k
0.91	3H Triplet	n

[a]See Scheme 1.

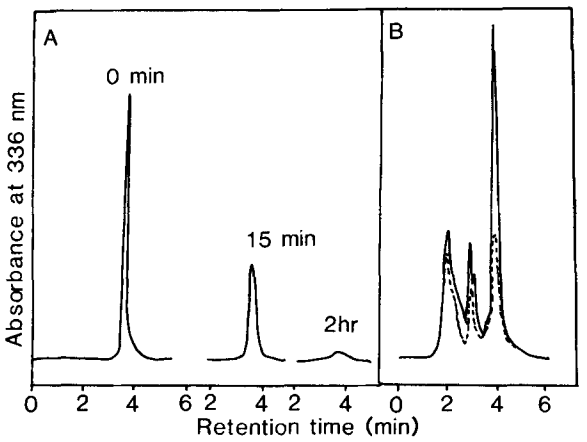


FIG. 4. HPLC of adduct W_B. Samples were analyzed by a Zorbax ODS column with an elution solvent, MeOH/H_2O (8:2, v/v), at a flow rate of 0.8 ml/min. (A) A solution of 2.4 mM W_B in 15% acetic acid was heated at 100 C for 15 min and 2 hr. The peaks due to the yellow and red pigments could not be detected under HPLC conditions. (B) A mixture of 2.5 mM TBA and 5 mM 1-butanal in 15% acetic acid was heated at 100 C for 15 min (——) and 6 hr (- - -). Peaks that eluted faster than that of W_B may be due to the yellow and red pigments.

n-propyl-1H,2H,3H,4H,7H pyrano[2,3-*d*]pyrimidine as shown in Scheme 1.

Colorless adducts W_{Hx} and W_{Hp} were produced by reaction of TBA with 1-hexanal or 1-heptanal, respectively. These adducts were found to be of the same types derived from 1 mol TBA and 2 mol of the corresponding aldehydes.

Formation and degradation of W_B in 15% acetic acid was monitored by use of HPLC (Fig. 4). When W_B was heated at 100 C in 15% acetic acid, it was readily degraded. Thus, the peak with a retention time of 3.6 min corresponding toW_B was reduced to 40% by 15-min heating and to 5% by 2-hr heating (Fig. 4A). When a mixture of 2.5 mM TBA and 5 mM 1-butanal in 15% acetic acid was heated, the peak corresponding to W_B appeared. Formation of W_B was higher at 15-min than at 6-hr heating (Fig. 4B). Formation of W_B was not affected by substituting the reaction mixture with nitrogen gas, indicating that the reaction does not require molecular oxygen.

Formation of the yellow and red pigments from the colorless adducts. Colorless adduct W_B was heated in 15% acetic acid at 100 C for 15 min and subsequently kept at room temperature. An unstable yellow pigment gradually formed in the solution (Fig. 5). When W_B was heated in 15% acetic acid at 100 C for 6 hr, a stable red pigment was produced (Fig. 5). It is likely that the red pigment was produced via the yellow pigment. Formation of the yellow and red pigments was suppressed completely when the solution was saturated with nitrogen gas before the reaction. Treatment of adduct W_{Hx} in 15% acetic acid gave similar results. Thus, the yellow pigment was produced with short heating and the red pigment with long heating. The results of the color development from the colorless adducts were in good agreement with those of the reaction of TBA and the saturated aldehydes described above. These observations support the idea that the formation of the yellow and red pigments in the reaction of TBA with saturated aldehydes proceeded via the intermediary colorless 1:2 adducts, i.e., W_B and W_{Hx}, under aerobic conditions. The red pigment may be formed via the yellow pigment (Scheme 2).

Comparison of the yellow and red pigments by HPLC. To obtain information on the identity of the yellow and

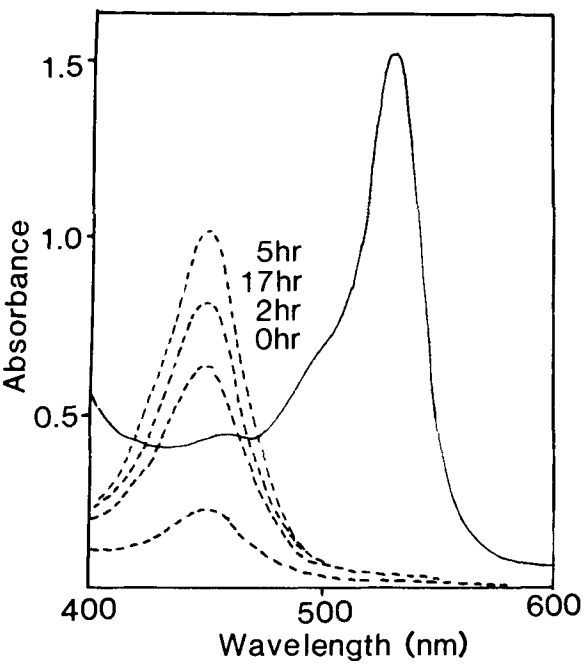


FIG. 5. Spectral changes of adduct W_B heated in 15% acetic acid. A mixture of 2.4 mM W_B in 15% acetic acid was heated at 100 C for 15 min followed by standing at room temperature for the indicated periods (- - -), or was heated at 100 C for 6 hr (——).

red pigments produced from 1-butanal, W_B and malonaldehyde, HPLC of the pigments was carried out (Fig. 6). The yellow pigment produced in the reaction of equimolar amounts of TBA with 1-butanal in 15% acetic acid (Fig. 6A-1) and that produced from W_B in 15% acetic acid (Fig. 6A-2) showed the same retention time at about 3.1 min, indicating that the pigments were identical. Chromatography of the red pigment derived

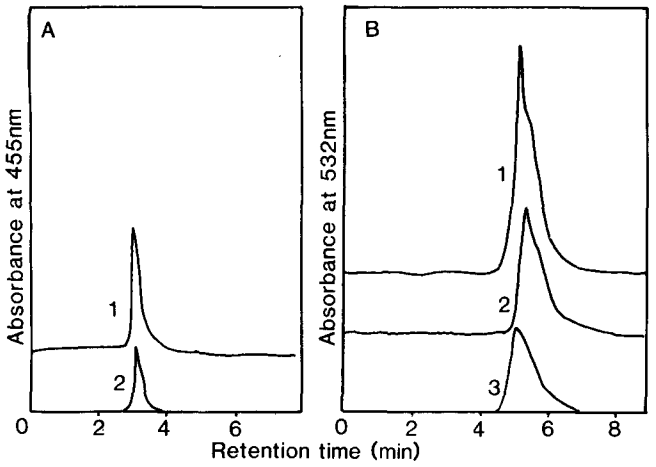
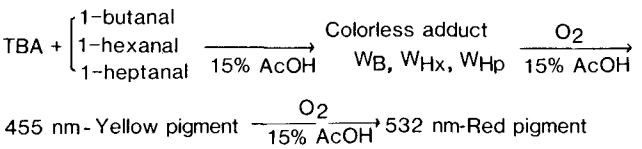


FIG. 6. HPLC of the yellow (A) and red (B) pigments. Samples were analyzed by a Zorbaz ODS column with an elution solvent, MeOH/0.04 M acetate buffer (pH 5.5) (4:6, v/v), at a flow rate of 0.8 ml/min. (A-1) A mixture of 1 mM TBA and 1 mM 1-butanal in 15% acetic acid was heated at 100 C for 15 min. (A-2) A mixture of 2.4 mM W_B in 15% acetic acid was heated at 100 C for 15 min. (B-1) A mixture of 8.7 mM TBA and 2.6 mM malonaldehyde bis(dimethyl-acetal) in 45% acetic acid was heated at 100 C for 20 min. (B-2) A mixture of 1 mM TBA and 1 mM 1-butanal in 15% acetic acid was heated at 100 C for 6 hr. (B-3) A mixture of 2.4 mM W_B in 15% acetic acid was heated at 100 C for 6 hr.

from the reaction of TBA and 1-butanal (Fig. 6B-2) and from W_B (Fig. 6B-3) revealed a broad peak with a retention time at about 5.1 min, again indicating identical pigments. The retention time was close to that of the red pigment derived from the reaction of TBA and malonaldehyde (Fig. 6B-1). Cochromatography of the red pigments derived from W_B and malonaldehyde revealed that the pigments were similar. Several attempts to differentiate the red pigments derived from these reactions in HPLC analysis under various conditions failed. It is interesting to note that the red pigments derived from 1-butanal, W_B and malonaldehyde showed the same absorption spectra with a maximum at 532 nm and the same retention times in HPLC analysis.

DISCUSSION

While the TBA reaction with malonaldehyde produces the red 2:1 adduct whose structure is unambiguously established (12,13), the reaction mechanisms of TBA with other aldehydes have not yet been elucidated. With respect to the reaction with aromatic aldehydes, Dox and Plaisance (14) obtained the yellow colored 1:1 adducts. The yellow pigment formed in the reaction mixture of TBA and the saturated aldehydes could not be isolated owing to its instability in aqueous media (6,7). Pryor (10) studied the reaction of TBA with 1-propanal. The yellow 450-nm pigment was produced first, and the red 532-nm pigment was subsequently produced in the presence of air. From the results, they tentatively suggested a mechanism. An initial 1:1 adduct at the 5 position of TBA is formed that undergoes autoxidation to give a 2-propenal-TBA adduct. This adduct then undergoes Michael addition of



SCHEME 2

a second molecule of TBA to give the red malonaldehyde adduct.

Our experiments demonstrated that the reaction of TBA with the saturated aldehydes progressed diversely. Formation of the yellow and red pigments depended on the reaction conditions. The yellow pigment was degraded in the presence of a large excess of saturated aldehydes in glacial acetic acid, probably due to subsequent reaction with the saturated aldehydes. Red pigment formation was observed only in the aqueous acetic acid and depended on the ratio of the reactants. The optimal ratio of TBA and the saturated aldehydes was about 1:1. The formation of the yellow and red pigments required molecular oxygen, indicating that some oxidative mechanisms are involved in the formation of these pigments.

The 1:1 reaction of TBA and the saturated aldehydes provided unexpected colorless adducts in the absence of molecular oxygen. Structural analysis of the colorless adducts suggested the following reaction pathway. Aldol condensation and dehydration of 2 mol of the saturated aldehydes initially gave the α,β-unsaturated aldehydes by the catalytic effect of TBA. The unsaturated aldehydes reacted with TBA to form the 1:1 adducts at the 5 position of TBA, which were in turn cyclized to colorless adducts with pyranopyrimidine structure (Scheme 1). The colorless adducts were converted into the yellow and red pigments in the presence of molecular oxygen (Scheme 2).

Patton and Kurtz (15) reported that α,β-unsaturated aldehydes give the yellow pigment and subsequently the red pigment in the presence of cupric ion or after standing exposed to air for several days, suggesting an autoxidation mechanism for the conversion. From the present results, the reaction of TBA with α,β-unsaturated aldehydes may proceed as illustrated in Schemes 1 and 2.

The absorption spectrum and the retention time in HPLC analysis of the red pigment produced in the reaction of TBA and 1-butanal or from colorless adduct W_B were close to those of malonaldehyde-TBA adduct. It appears unlikely, however, that the structure of the red pigment derived from W_B is the same as that of the malonaldehyde-TBA adduct. The structure of the red pigment derived from W_B must be closely related to that of the malonaldehyde-TBA adduct.

The reaction of TBA with the saturated aldehydes is of particular interest, since the red 532-nm color of the TBA reaction has been generally ascribed to malonaldehyde. Oxidized lipids contain a variety of saturated aldehydes (3), which may be involved in the development of the red color under the restricted TBA reaction conditions described in this paper.

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Distribution of Hexadecenoic, Octadecenoic and Octadecadienoic Acid Isomers in Human Tissue Lipids

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The *trans* 16:1, 18:1 and 18:2 fatty acid composition of various human organ lipids was studied to determine if isomers accumulated in specific tissues. "*Trans*" isomers are defined as those fatty acids containing one or more *trans* double bonds. Adipose, kidney, brain, heart and liver tissue lipids were analyzed. Gas chromatography with a 100-SP2560 capillary column was used to characterize the various positional and/or geometrical isomers. The distribution of *trans* 16:1 and 18:1 isomers ranged from 0.3% in the brain to 4.0% in adipose tissue, while *trans* 18:2 isomers ranged from 0.0% in the brain to 0.4% in adipose tissue. No *trans* 18:3 isomers were detected. Positional isomer ratios for *cis* 16:1 (Δ9 vs Δ7) and *cis* 18:1 (Δ11 vs Δ9) were also determined. Since these ratios are reproducible from one individual to the next, they might be useful for diagnosis of human metabolic disorders.
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Fats constitute about 40% of total calories in the American diet (1). Vegetable fats are industrially hydrogenated to harden them and to enhance odor and flavor stability by reduction of the number of double bonds. However, this procedure also produces geometrical and positional isomers. Geometrical isomers are defined as those isomers containing *trans* double bonds in addition to or instead of the *cis* double bonds usually found in fats. Positional isomers are formed by movement of the double bond along the chain. Hydrogenated vegetable oils are composed of 5–50% *trans* isomers (2). Although vegetable sources account for only 40–45% of the total fat in the food supply (vs 55–60% from animal sources) (3), they account for ca. 75% of the *trans* isomers available in the diet (4).

The large range of published values for *trans* fatty acid intake (4.5–12.1 g/capita/day) (4) indicates the difficulty of accurately estimating the quantity of *trans* 18:2 fatty acids ingested daily in a typical Western diet. Emken (5) estimated that hydrogenated vegetable oil contributes ca. 6.8 g of *trans* monoene isomers per day to the diet based on an average daily consumption of 34 g of hydrogenated vegetable oil. Using these assumptions and the fact that Enig et al. (6) found the *trans* fatty acids of hydrogenated salad and cooking oils to be composed of ca. 2% *trans* 18:2, we can calculate that ca. 0.7 g of *trans* 18:2 fatty acid isomers is ingested daily. We also calculated a value of 0.7 g/capita/day based on the assumption that adipose fatty acid composition adequately reflects the fatty acid composition of the diet (7). The latter calculation is based on an availability of 166 g fat/capita/day (4) and an adipose tissue composition (gas chromatography [GC] data) of 0.4% *trans* 18:2.

Trans fatty acids in human tissues were first reported in 1957 and were analyzed by infrared spectroscopy (IR) (8,9). More recently, workers in the field have relied on either capillary GC (SP 2340 [10], OV275 [11]) or a combination of GC/micro ozonolysis (12) to determine the isomer distribution in tissue lipids. These procedures have better sensitivity than IR for low levels (<2%) of *trans* isomers (10).

Several groups of investigators have analyzed vegetable oils and margarines (6,11,13), and others have studied the incorporation of *trans* isomers in tissue lipids (7,12,14–16). The double bond distribution pattern of *trans* monoenes in adipose tissue was found to be similar to the pattern observed in commercially hydrogenated vegetable oil products and indicates vegetable oil rather than ruminant fat is the major source of *trans* isomers in Western diets (10,12).

While the distribution of monoenoic *trans* fats in human tissue lipids has been well documented (see ref. 10 for a review), few investigators have studied the *trans* diene isomer composition of human tissues. "*Trans* dienes" refers to those fats containing two double bonds, of which one or both have a *trans* geometry. Heckers et al. (17) found no trace of 9t,12t-, 9c-12t or 9t,12c-18:2 in the myocardium, jejunum and aorta of German men with varying degrees of atherosclerosis, while Enig et al. (18) found 0.2–0.8% *trans* diene isomers in adipose tissue. GC analysis of blood samples indicated 0.7–0.8% *trans* diene isomers (19). None of these workers reported the percentage of individual *trans,trans-*, *cis,trans-* or *trans,cis*-18:2 fatty acids in individual lipid classes or the presence of 18:3 isomers.

Since *trans* dienoic and trienoic fatty acid isomers may be involved in prostaglandin synthesis (20), blood clotting and other biological functions (1), a study was undertaken to determine the *trans* dienoic and trienoic fatty acid composition of various human tissues and tissue lipid classes and to determine if any accumulation of these isomers had occurred.

EXPERIMENTAL

Tissue samples were obtained from autopsies performed on six males, aged 33, 34, 35, 42, 57 and 60 years. Causes of death included suicide, car accident, heart attack, ruptured aneurysm and cerebral hemorrhage. The tissues were frozen within 4 hr of death and stored at -70 C. Samples (ca. 20 g) were removed, thawed and extracted with chloroform/methanol (21), and the extracted lipids were stabilized with tertiary butylhydroxyquinone. A known amount of heptadecanoic acid (17:0) was added as an internal standard to check the completeness and reproducibility of the total lipid extractions.

Preparative thin layer chromatography (TLC) plates (Silica Gel 60, 2 mm thickness; E. Merck, Darmstadt, Federal Republic of Germany) were used to isolate the various lipid classes from the total lipid (TL) extracts.

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Phospholipids (PL) were isolated using chloroform/methanol/acetic acid (65:25:4, v/v/v) as solvent, while triglycerides (TG) and cholesteryl esters (CE) were separated using petroleum ether/ether/acetic acid (80:20:1, v/v/v) (22,23). The purified lipids were esterified using benzene/10% HCl in methanol (12). Ester composition was determined on a Packard 428 gas chromatograph equipped with a 100 m × 0.25 mm (0.2 μ coating) SP 2560 fused silica capillary column (Supelco Inc., Bellefonte, PA). GC standards (fatty acid methyl esters) were obtained from a wide variety of sources for all configurational and positional isomers discussed in this manuscript. Helium carrier gas and a flame ionization detector were utilized. The oven temperature was held isothermally at 190 C for 30 min, and then was programmed from 190–215 C at a rate of 10 C/min.

RESULTS AND DISCUSSION

The SP 2560 fused silica GC column provided excellent separation of the positional and geometrical isomers. As shown in Figure 1, the 9t-16:1, 7c-16:1 and 9c-16:1 isomers are well separated, as are the 9t,12t-, 9c,12t- and 9t,12c-18:2 isomers. A further advantage of the SP 2560 column is that most of the 18:1 positional and geometrical isomers are also separated. Even at 190 C, an excellent separation was achieved between the *trans* and *cis* 18:1 isomers, the latter being further separated into the Δ9/Δ10, Δ11, Δ12 and Δ13 positional isomers. A further separation of the Δ8, Δ9, Δ10 and Δ11 *trans* 18:1 positional isomers occurred at 175 C (see Fig. 1, insert A).

The *trans* fatty acid contents of the various tissue lipids are tabulated in Table 1. Van Der Vusse et al. (24) found that storage of tissue samples at room temperature for 60 min and even -20 C for two weeks resulted in significant tissue CE losses and a corresponding rise in the amount of free fatty acids (FFA). We encountered similar problems. They also reported that "No effect on TG and PL was seen." Since storage times and conditions before and after the autopsies could not be controlled, CE and FFA results were not tabulated. Ohlrogge (10) provides a review of *trans* compositions that can be used for comparison. While our results tended to be at the low end of the scale, the data for adipose, brain and liver TL are consistent with previous results, as are the heart and liver individual lipid classes. Kidney and heart TL values tended to be lower than the levels established by the IR work of Kaufmann et al. (25). The *trans* 16:1 composition was found to be 0.0–0.3%; *trans* 18:1 was the predominant *trans* monoene (0.2–4.0%). Except for brain tissue, the *trans* dienes were present in all tissues studied and have their highest concentration in the adipose tissue. The total *trans* 18:2 isomer concentration was found to be 0.0–0.4%.

As noted in Table 1, adipose tissue had the highest concentration of *trans* dienes (0.4% total), while the concentration of brain TL isomers was too small to be measured. The amount of *trans* diene we found in adipose TL was similar to that reported by Enig et al. (18) in the subcutaneous fat of Israeli men. It can be assumed, therefore, that the *trans* fatty acid levels in these samples are representative of the general population. Of the individual lipid classes analyzed, the highest concentration of *trans* diene was found in the heart and liver TG (both 0.3%). While hydrogenated vegetable oil does con-

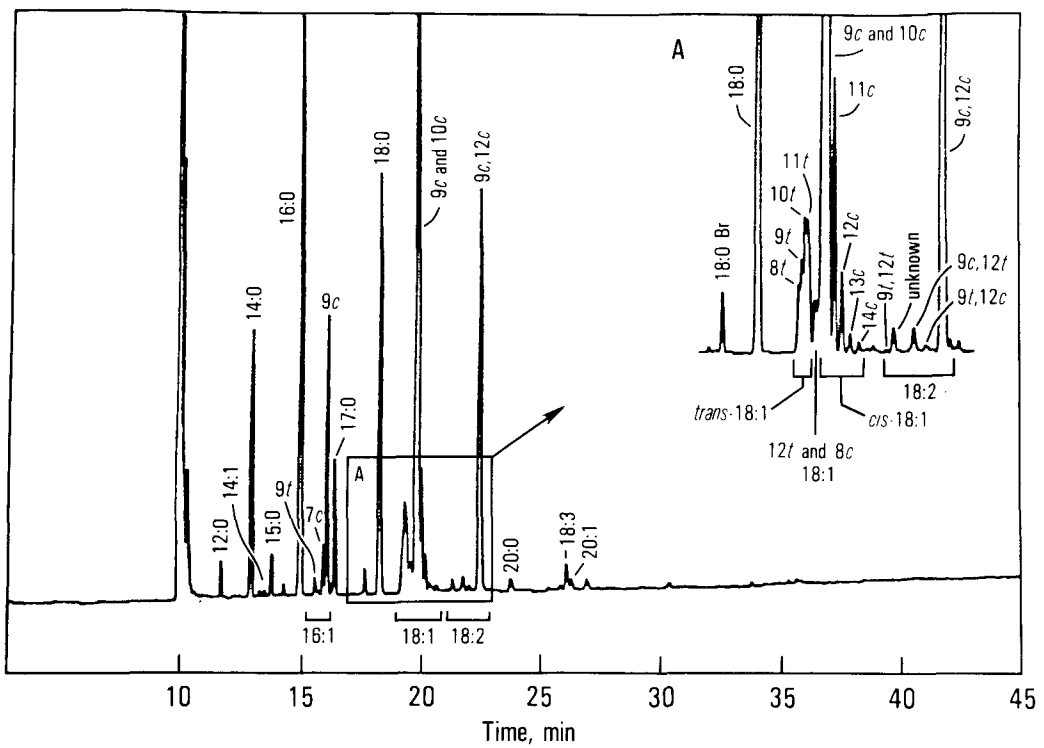

FIG. 1. Adipose tissue: total lipids, 100 m × 0.25 mm SP 2560; 190–215 C at 10 C/min; 30 min initial pause. Insert A: Adipose tissue—total lipids, 175 C isothermal.

TABLE 1

Tissue Lipid Composition: *trans* 16:1, 18:1 and 18:2 Isomers

Tissue	Fraction[a]	Σ% *trans* (16:1 [range])[b]	Σ% *trans* (18:1 [range])	Σ% *trans* (18:2 [range])	Σ% *trans* 18:2/ Σ% *trans* 18:1
Adipose	TL (5)	0.3 (0.2–0.4)	4.0 (2.8–5.3)	0.4 (0.4–0.5)	0.10
Kidney	TL (5)	0.1 (0.0–0.1)	1.2 (0.5–3.3)	0.2 (0.1–0.3)	0.17
Brain	TL (4)	0.1 (0.0–0.1)	0.2 (0.0–0.3)	N.D. (—)[c]	—
Heart	TL (3)	0.1 (—)	1.7 (0.9–2.4)	0.2 (—)	0.12
	TG (3)	0.1 (0.1–0.2)	1.5 (1.2–1.9)	0.3 (0.1–0.4)	0.20
	PL (3)	0.1 (0.0–0.1)	0.6 (0.3–1.1)	0.2 (—)	0.33
Liver	TL (6)	0.2 (0.0–0.4)	1.0 (0.5–1.7)	0.3 (0.2–0.4)	0.33
	TG (3)	0.3 (0.2–0.4)	1.4 (0.5–2.4)	0.3 (0.2–0.5)	0.21
	PL (3)	0.1 (—)	1.9 (0.7–2.6)	0.2 (0.2–0.3)	0.11

[a]TL, total lipid; TG, triglyceride; PL, phospholipid. Number of subjects given in parentheses.
[b]Range of values obtained.
[c]N.D., not detectable (<0.05%).

TABLE 2

Tissue Lipid Composition: Individual 18:2 Isomer Percentages

Tissue	Fraction[a]	9c,12c-18:2, Average (range)	9c,12t-18:2 Average (range)	9t,12c-18:2 Average (range)
Adipose	TL (5)	12.9 (8.3–17.3)	0.3 (—)	0.1 (—)
Kidney	TL (5)	18.2 (13.2–20.4)	0.1 (0.1–0.2)	0.1 (—)
Brain	TL (4)	0.8 (0.6–1.0)	N.D.[c]	N.D.
Heart	TL (3)	15.0 (14.7–15.3)	0.1 (—)	0.1 (—)
	TG (3)	5.5 (3.2–7.2)	0.2 (0.1–0.2)	0.1 (—)
	PL (3)	16.2 (14.9–18.7)	0.1 (—)	0.1 (—)
Liver	TL (6)	15.3 (13.2–18.5)	0.2 (0.1–0.2)	0.1 (0.1–0.2)
	TG (3)	10.7 (6.3–15.9)	0.2 (0.2–0.3)	0.1 (0.0–0.2)
	PL (3)	18.0 (16.1–19.4)	0.1 (0.1–0.2)	0.1 (—)

[a]See Table 1 for definitions. Number of subjects given in parentheses.
[b]N.D., not detectable (<0.05%).

tain other positional isomers (9,15- and 12,15-18:2), we could not detect them at the 0.05% level. We used 0.05% as our lower limit, since accuracy at this level is questionable.

The level of *trans* 9,12-18:2 isomers relative to *trans* 18:1 isomers was also calculated for the various tissue lipids (Table 1). The ratios for adipose TL (0.10), heart TL (0.12) and liver PL (0.11) were lower than values calculated from the data of Slover et al. for hydrogenated vegetable oils (ca. 0.18)(11). Higher values were obtained for kidney TL (0.17), heart TG (0.20) and liver TG (0.21), while the highest values were calculated for liver TL and heart PL fractions (both 0.33). Whether this is due to a preferential incorporation of *trans* 18:2 or a preferential exclusion or removal of *trans* 18:1 isomers is unknown.

Concentrations of the individual 18:2 isomers are summarized in Table 2. The 9c,12t-18:2 isomer content was consistently greater than or equal to the 9t,12c-18:2 content. Only traces (<0.05%) of the 9t,12t-18:2 isomer were found (see Fig. 1, insert A). Unknown A was originally assumed to be the 9t,12t-18:2 isomer, but coinjection of a 9t,12t-18:2 standard resulted in two peaks. Unknown A has not been identified but appears to be the same unidentified diene isomer reported by Slover et al. for hydrogenated oils (11). Problems such as this illustrate the difficulties encountered when reporting tissue lipid results obtained by GC.

In an attempt to ascertain reproducible patterns in the fatty acid distribution of tissue lipids, the ratios of *cis* 16:1 (Δ9 vs Δ9 + Δ7) isomers were compared for the various tissue fractions as were the *cis* 18:1 isomers (Δ9 vs Δ9 + Δ11) (see Table 3). The concentration of *cis* 16:1 was greatest in adipose TL (4.3%) and liver TG (4.1%). The Δ9 vs total 16:1 (Δ9 plus Δ7) ratio was consistently 0.84–0.88. The only exceptions were noted in brain TL (0.59) and heart PL (0.73).

The ratio of the *cis* 18:1 (Δ9 vs Δ9 + Δ11) positional isomers was also calculated. While the SP 2560 capillary

TABLE 3

Ratios of *cis* Positional Isomers

Tissue	Fraction[a]	Total 16:1 (%), average (range)	16:1 Ratio[b] average (range)	Total 18:1 (%), average (range)	18:1 Ratio[c] average (range)
Kidney	TL (5)	1.9 (1.2–3.0)	0.87 (0.83–0.90)	21.7 (15.5–26.2)	0.89 (0.84–0.93)
Adipose	TL (5)	4.3 (2.6–5.1)	0.86 (0.81–0.90)	43.7 (39.9–46.7)	0.93 (0.90–0.95)
Brain	TL (4)	0.9 (0.8–1.0)	0.59 (0.57–0.65)	24.1 (22.9–24.6)	0.80 (0.78–0.82)
Heart	TL (3)	2.0 (—)	0.88 (0.86–0.91)	21.0 (20.7–21.1)	0.91 (0.89–0.94)
	TG (3)	1.9 (1.7–2.0)	0.87 (0.82–0.91)	17.9 (15.2–20.8)	0.93 (0.93–0.95)
	PL (3)	0.7 (0.4–0.9)	0.73 (0.65–0.79)	7.2 (6.5–8.0)	0.83 (0.82–0.83)
Liver	TL (6)	3.0 (1.8–4.1)	0.85 (0.82–0.88)	27.1 (21.9–30.4)	0.90 (0.86–0.93)
	TG (3)	4.1 (3.2–5.5)	0.84 (0.78–0.88)	38.1 (37.4–38.4)	0.92 (0.92–0.93)
	PL (3)	1.8 (1.0–2.8)	0.86 (0.79–0.90)	13.4 (11.7–16.0)	0.81 (0.80–0.84)

[a]See Table 1 for definitions. Number of subjects given in parentheses.
[b]16:1 Ratio = Δ9/(Δ9 + Δ7).
[c]18:1 Ratio = Δ9/(Δ9 + Δ11).

column could not separate the Δ9 and Δ10 *cis* 18:1 isomers, no correction factor was used, because previous researchers (12) had found very little (<1%) Δ10 *cis* 18:1 in tissue samples. The percentage of *cis* 18:1 in kidney, brain, heart and liver TL (21–27%) was approximately half that of adipose TL (44%). The lowest percentage of *cis* 18:1 was found in heart and liver PL (7.2 and 13.4%). The ratios of Δ9 vs Δ9 plus Δ11 *cis* 18:1 for most of the tissue fractions were 0.89–0.93. These values agree with those reported by Muskiet et al. (26) for the cholesteryl ester composition of a frozen erythrocyte fraction (0.93), amniotic fluid TL (0.88) and cerebrospinal fluid TL (0.88) and by Wood (27) for the neutral lipids (0.92), but not for PL (0.81) isolated from beef and several processed meats (total of 15 samples). The lower PL value for meats is consistent with the results we obtained for the brain TL (0.80) and the heart and liver PL fractions (0.83 and 0.81). These values would seem to indicate selective metabolism. The ratios vary among tissues but are very reproducible from subject to subject. Wood et al. (28,29) have examined both the *cis*-16:1 Δ9 vs *cis*-16:1-Δ7 and the *cis*-18:1-Δ9 vs *cis*-18:1-Δ11 ratios of various rat tissues and tissue lipid classes. They have also documented changes in these ratios when rat liver and hepatoma lipids were compared (30,31). Thus the possibility exists that these ratios could be used as an indicator for human lipid metabolism disorders.

If one assumes that the *trans* diene concentration in adipose tissue (0.2–0.4%) adequately reflects the dietary intake of fats, then no significant accumulation of *trans* dienoic fatty acids was found in human tissue total lipids or individual lipid classes. Considering the low levels of *trans* dienoic acids present and the detectability limits of our GC method, it is not surprising that there was no evidence for the presence of *trans* trienoic or tetraenoic fatty acids. The implication of these data is that the *trans* 18:2 isomers would not be expected to have significant physiological impact.

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Accumulation of Surfactant Phospholipids in Lipid Pneumonia Induced with Methylnaphthalene

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Lipid analyses were carried out on the lungs of female $B_6C_3F_1$ mice treated with methylnaphthalene. Cholesteryl ester, which could not be detected in lungs of control animals, was present in lungs of treated animals. Cholesterol and dipalmitoylglycerophosphocholine (DPPC) content was increased about five times in lungs of treated mice compared with control mice, and the content of a minor phospholipid was increased six times. The latter phospholipid was purified by high performance liquid chromatography and identified as phosphatidylglycerol by thin layer chromatography and by fast atom bombardment-mass spectrometry. Both DPPC and phosphatidylglycerol are known to be pneumonal surfactants produced from type II pneumocytes. Therefore, the accumulation of these lipids in lung tissue was assumed to be caused by the proliferation of type II cells induced by the administration of methylnaphthalene. The results provide important information concerning the underlying mechanism of endogenous lipid pneumonia in mice.

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It is generally accepted that lipid pneumonia in humans can be pathologically divided into exogenous and endogenous forms. Exogenous lipid pneumonia is generally caused by oil entering the trachea and being aspirated. Endogenous lipid pneumonia may occur alone, behind a bronchial obstruction, in association with other inflammatory lesions or in the absence of apparent cause in the lung (1).

Methylnaphthalene is a complex of α-isomer and β-isomer and is widely used as a carrier in dyeing. Recently, we found that lipid pneumonia was induced by painting methylnaphthalene on the shaved skin of the backs of female $B_6C_3F_1$ mice (2). The lung lesion is histologically characterized by the appearance of foam cells and cholesterol crystals in the alveoli and by multinucleated giant cell reaction. The alveolar walls are slightly thickened, and type II pneumocytes show hypertrophy and are increased in number.

In the present study, we determined the lipid composition of the lungs of mice with lipid pneumonia induced by methylnaphthalene administration to elucidate the biochemical changes underlying the histological appearance. We report the details of the accumulation of cholesterol as well as dipalmitoyllecithin and phosphatidylglycerol.

MATERIALS AND METHODS

Animals and treatments. Inbred strain $B_6C_3F_1$ female mice (Charles River Co. Ltd., Osaka, Japan) weighing ca 20 g each were used. Methylnaphthalene (Yamakawa Kogyo Yakuhin Co., Tokyo, Japan) dissolved in acetone was painted on the shaved skin of the backs of mice twice a week. Mice were divided into three groups: the control group received 0.2 m of acetone; the low dose and high dose groups received 118.8 and 237.6 mg/kg body weight of methylnaphthalene, respectively. Mice were decapitated 50 wk after the beginning of the experiment.

Lipid extraction. Three samples of lung tissue from the control animals, eight samples from the low dose group and seven samples from the high dose group were used for lipid analysis. Each tissue was homogenized with 20 ml of chloroform/methanol (2:1, v/v). The extraction was repeated twice by the same procedure. The combined lipid extracts of each tissue were washed with 0.9% NaCl solution (8 ml). The resulting lower layer was taken and adjusted to 10 ml with chloroform/methanol (2:1, v/v) after removing the solvent under a nitrogen stream.

Thin layer chromatography (TLC). Precoated Silica Gel 60 thin layer plates (Merck, Darmstadt, Federal Republic of Germany) were used. As the developing solvent mixture for neutral lipid separation, petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v) was used. For the separation of phospholipids, solvent mixtures of chloroform/methanol/water (65:25:3, v/v/v), chloroform/methanol/2.5 N NH_4OH (70:30:5, v/v/v) and methyl acetate/n-propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, v/v/v/v/v [3]) were used. For detection of all lipids, the thin layer plate was exposed to iodine vapor. Cholesterol and cholesteryl ester were visualized by heating the plate after spraying with 60% sulfuric acid. Phospholipids were visualized by spraying molybdenum blue reagent.

Triglyceride determination. Triglyceride was assayed by enzymatic determination. An aliquot from each sample was transferred to an incubation tube, and the solvent was removed under a nitrogen stream. The dried sample was suspended in 0.3 ml of 1% Triton X-100 solution in a water bath at 80 C for one min. Then assay reagent (2.7 ml) was added and the mixture was incubated at 37 C for 1 hr. The assay reagent contained 0.2 M Tris-HCl buffer (pH 8.0; 0.6 ml), 0.3% 4-aminoantipyrine (0.3 ml), 0.2% phenol (0.3 ml), 0.1 M $MgCl_2$ (0.3 ml), 10 mM ATP (0.3 ml), 5 units of peroxidase (0.2 ml), 3 units of glycerol-3-phosphate oxidase (20 μl), 400 units of lipoprotein lipase (20 μl), 0.5 units of glycerol kinase (50 μl) and 0.71 ml of water. After the reaction, the developed color was measured at 500 nm. Tripalmitin was used as standard.

Determination of cholesterol and cholesteryl ester. Determination of cholesterol and cholesteryl ester was performed by the methods of Allain et al. (4). An aliquot of the sample was transferred to an incubation tube, and the solvent was removed under a nitrogen stream. The dried sample was suspended in 0.3 ml of 2% Triton X-100 solution in a water bath at 80 C for 1 min. The

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assay reagent was added and assay was carried out according to Allain et al. (4).

Cholesteryl ester was separated by TLC from other lipids. The area containing cholesteryl ester was scraped off and transferred to an incubation tube. Then the cholesteryl ester was determined by using a reagent containing cholesterol esterase according to the method of Allain et al. (4). All assay reagents for cholesterol and for triglyceride were obtained from Toyo Jozo Inc. (Shizuoka, Japan).

Phospholipid determination. Phospholipid determination was performed by using a chromatoscanner (Shimadzu CS 910) as described previously (5). Sphingomyelin was used as a standard.

High performance liquid chromatography (HPLC). For purification of phospholipid X, phospholipids from experimental animals were subjected to liquid chromatography (Shimadzu LC-4A). A solution of phospholipids (several mg) in 1 ml of chloroform/methanol/water (74:25:1, v/v/v) was applied to a silica beads column (6 mm × 150 mm, packed with Iatrobeads 6RS-6010 [10μm], Iatron Lab. Inc., Tokyo, Japan) and eluted with 20 ml of the first solvent mixture, chloroform/methanol/water (74:25:1, v/v/v). Then phospholipids were eluted with a gradient of a mixture of chloroform/methanol/water (74:25:1 to 46.5:51.8:1.7, v/v/v) at a flow rate of 1 ml/min for 20 min and with a mixture of chloroform/methanol/water (19:78:3, v/v/v) for 20 min to complete the elution of all other phospholipids. Eluates were collected every minute with a fraction collector, and aliquots were monitored by TLC.

Fatty acid analysis by gas chromatography (GC). Phospholipids were methanolyzed with 3% anhydrous methanolic HCl (by weight) for 3 hr at 100 C in sealed tubes. Fatty acid methyl esters were extracted with hexane three times. The fatty acid methyl esters thus obtained were analyzed by GC on a 10% Silar 10 C coated on a Gas-Chrom Q (100–200 mesh) column (2 m) programmed from 160–220 C at a rate of 2 C/min. Fatty acids used as standards were obtained from NuChek-Prep Inc. (Elysian, Minnesota).

Mass spectrometry (MS). Phospholipid X purified by HPLC was analyzed by fast atom bombardment-mass spectrometry (FAB-MS). A solution of phospholipid (several μg) in chloroform/methanol (1:1, v/v) was mixed with 2 μl of triethanolamine or glycerol on a sample holder and analyzed with an FAB-MS instrument (JMS-HX, JEOL Ltd., Tokyo, Japan) equipped with an FAB ion source and a DA 5000 data system (JEOL Ltd., Tokyo, Japan). Xenon (Xe) was used as a reaction gas. The neutral Xe beam (energy, 6 Kv) was collided with the sample. Mass range was 100–1000 m/z.

RESULTS

Neutral lipid from lungs of mice with lipid pneumonia. Compositions of neutral lipid from the lungs of control and experimental animals were determined by TLC. Triglyceride and cholesterol were increased in lungs from experimental animals. Cholesteryl ester was not detected in control animals, but appeared in every experimental lung. Triglycerides, cholesterol and cholesteryl esters were analyzed, and the results are summarized in Figure 1. In low dose experiments, the

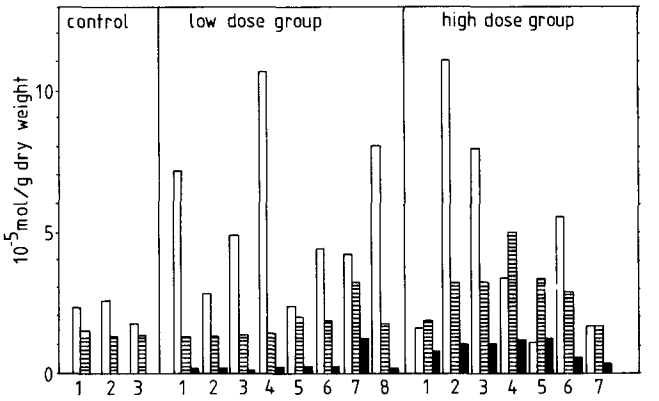

FIG. 1. Contents of cholesterol, triglycerides and cholesteryl ester from control and experimental lungs. □, Triglyceride; ▤, cholesterol; ■, cholesteryl ester.

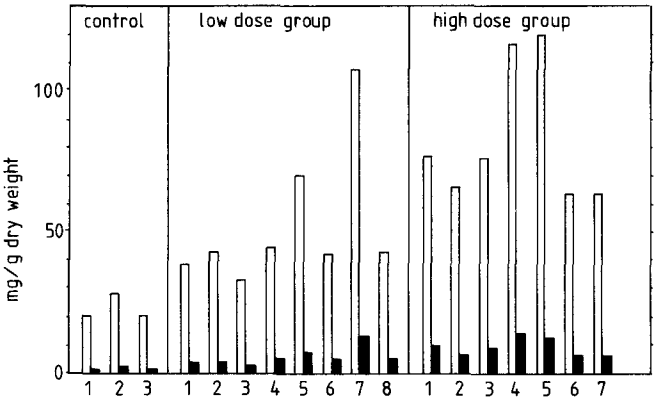

FIG. 2. Contents of phosphatidylcholine and phospholipid X in control and experimental lungs. □, Phosphatidylcholine; ■, phospholipid X. Numbers are the same as in Fig. 1.

TABLE 1

Phospholipid Composition of Control Lung

Phospholipid	Dry weight (mg/g)	Percentage
Phosphatidylethanolamine	4.19	11.7
Phospholipid X	2.33	6.5
Phosphatidylinositol	1.63	4.5
Phosphatidylserine	3.49	9.7
Phosphatidylcholine	21.20	59.0
Sphingomyelin	3.10	8.6

TABLE 2

Fatty Acid Composition of Phosphatidylcholine

Fatty acid	Control (%)	Low dose (%)	High dose (%)
14:0	1.5	2.6	2.1
16:0	71.0	70.6	76.6
16:1	9.0	10.3	12.4
18:0	7.2	4.3	2.6
18:1	6.1	6.2	3.0
18:2	4.1	3.9	2.6
Other	1.1	2.2	0.6

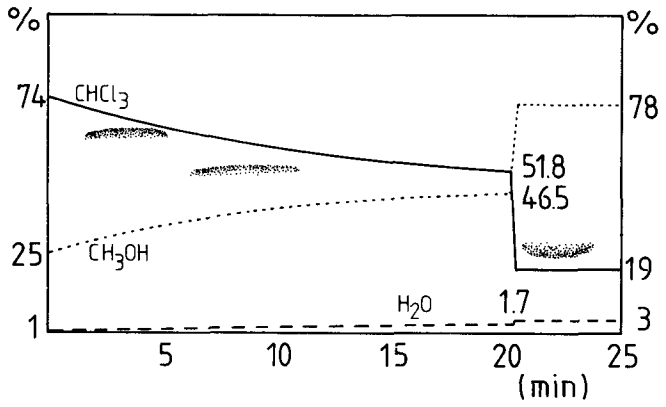

FIG. 3. Separation of phospholipid X from phosphatidylethanolamine by HPLC. The figure shows the gradient and the chromatographic profile on a thin layer plate. An aliquot from each tube was applied to the plate and analyzed by TLC. The solvent system was a mixture of chloroform/methanol/water (60:35:8, v/v/v).

level of triglyceride was increased 1-5 times. However, in the high dose group, three of the experimental animals showed triglyceride contents similar to or lower than those of control animals. The other animals of the high dose group showed marked accumulation of trigylceride.

Cholesterol levels in the low dose group were 1.2-2.3 times higher than those of the control. In the high dose group, cholesterol contents were 1.3-3.7 times those of the control. Cholesteryl ester amounted to 1.5-12.5 μmol/g dry weight in the low dose group and 4-13 μmol/g weight in the high dose group.

Phospholipids from lung of mice with lipid pneumonia. Phospholipid analyses of control animals are listed in Table 1. Phosphatidylcholine was predominant. As shown in Figure 2, the increase of phospholipid content in experimental animals was largely due to phosphatidylcholine and a phospholipid (named phospholipid X) which moved between phosphatidylcholine and phosphatidylethanolamine in TLC. Phosphatidylcholine content was 1.5-5 times higher in the low dose group and

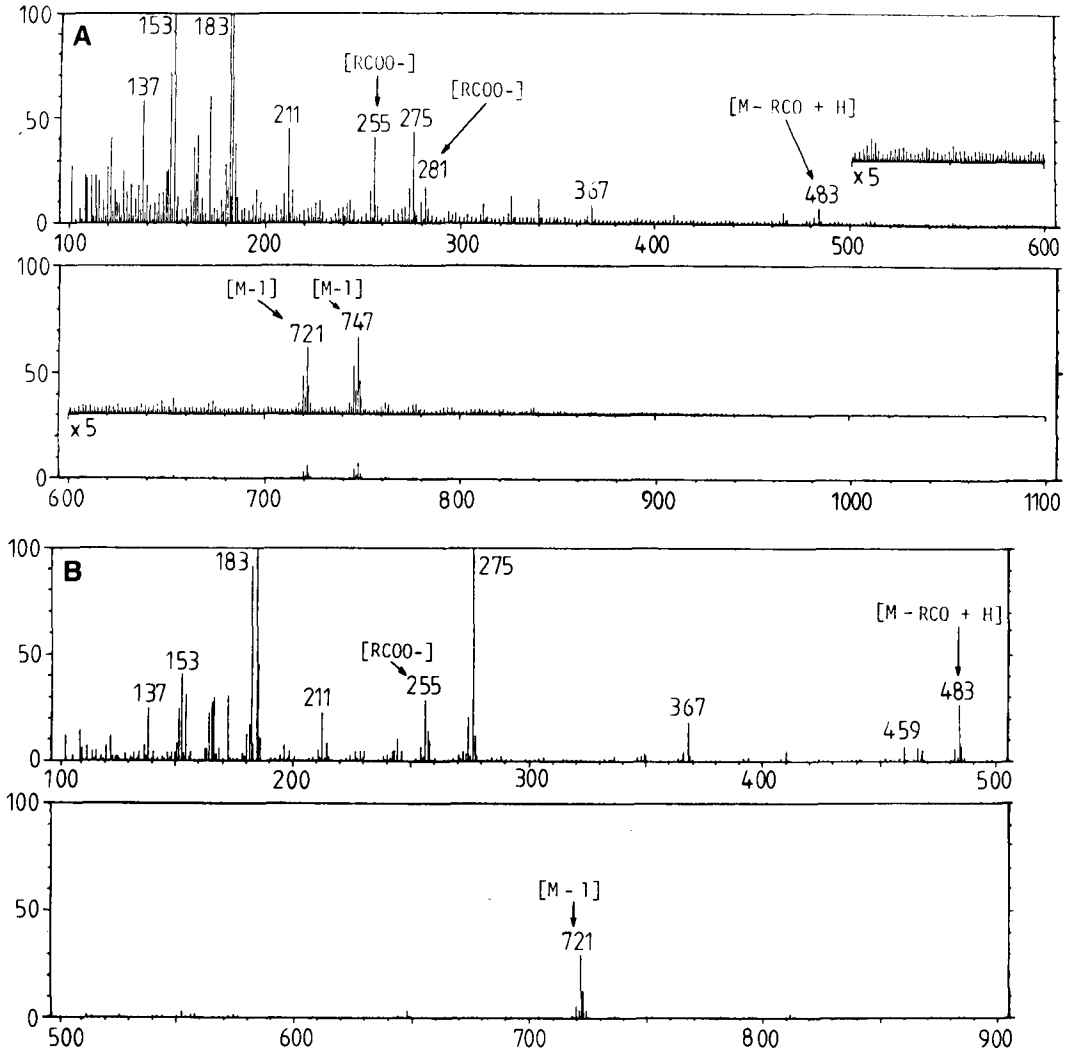

FIG. 4. (A) FAB-MS of phospholipid X; (B) FAB-MS of dipalmitoylphosphatidylglycerol. Analytical conditions are given in Materials and Methods.

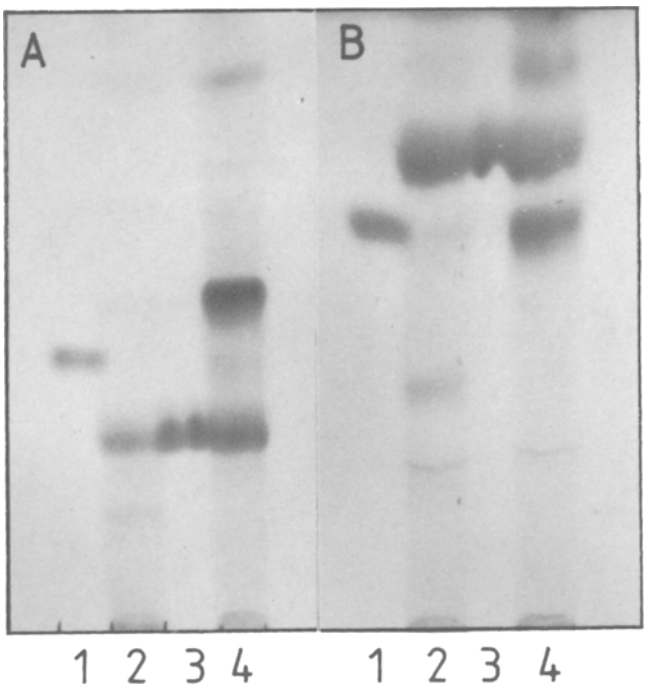

FIG. 5. TLC of phospholipid X. Lane 1, N,N-dimethylphosphatidylethanolamine; lane 2, phospholipid X isolated by HPLC; lane 3, standard phosphatidylethanolamine and phospholipid X from lungs of experimental animals. (A) Solvent system, chloroform/methanol/water (65:25:3, v/v/v), (B) solvent system, chloroform/methanol/2.5N NH_4OH (70:30:5, v/v/v). Lipids were made visible with iodine vapor.

3-5.7 times higher in the high dose group than in control lungs. Content of phospholipid X was 1.5-5.8 times higher in the low dose group and 3-5.8 times higher in the high dose group than in control animals (Fig. 2).

The fatty acid compositions of phosphatidylcholine obtained from each group are shown in Table 2. The most abundant fatty acid was palmitic, which accounted for more than 70% in each case. The fatty acid compositions of phosphatidylcholine were similar in all three groups.

Identification of phospholipid X. To identify phospholipid X, total lipids of experimental lungs were collected and subjected to HPLC. Figure 3 shows the chromatographic separation of phospholipid X from phosphatidylethanolamine. The purified phospholipid X was readily hydrolyzed with 0.5 N NaOH in methanol at 37 C for 30 min. The major fatty acids of lipids were 16:0 (43.7%), 18:1 (27.6%) and 18:0 (14.4%).

On the basis of these properties, we subjected phospholipid X to FAB-MS. The mass fragment pattern is shown in Figure 4A. The molecular mass ions (M-1), 721 and 747 (m/z), corresponded well to those of dipalmitoylphosphatidylglycerol and palmitoyloleoylphosphatidylglycerol, respectively. Other fragment ions are indicated in the spectrum. The mass pattern of standard dipalmitoylphosphatidylglycerol was taken and compared with that of phospholipid X. As shown in Figures 4A and 4B, the mass pattern of phospholipid X is identical to that of dipalmitoylphosphatidylglycerol. Further, as shown in Figure 5, the chromatographic behavior of phospholipid X on TLC was identical with that of standard phosphatidylglycerol in two different solvent systems. Thus phospholipid X was concluded to be phosphatidylglycerol.

DISCUSSION

Lipid analysis of the lungs of mice with lipid pneumonia induced by administration of methylnaphthalene on the skin confirmed the accumulation of cholesterol crystal observed in a histological study (2). In addition, the accumulation of cholesteryl ester was found in the present study. The appearance of cholesteryl ester may reflect a mechanism that seems intended to avoid cholesterol accumulation in the lung tissue. The marked increase of phospholipids, especially phosphatidylcholine and phosphatidylglycerol, is noteworthy. Analysis of the fatty acid composition of phosphatidylcholine showed that more than 70% of fatty acids consisted of palmitic acid. Phosphatidylglycerol was identified by FAB-MS and by TLC. Since DPPC and phosphatidylglycerol are known to be pulmonary surfactant lipids (6-9), and they are produced by pulmonary type II cells, the increase of these two phospholipids in the lungs of mice treated with methylnaphthalene was assumed to be caused by type II cell proliferation. These observations, based on the chemical analysis of phospholipids, are consistent with the results of a histological study (2), which showed not only the proliferation and swelling of type II cells, but also the desquamation of the cells into alveoli.

It may be generally considered that type II cell hyperplasia is a typical response of the lung to various injuries. Kikkawa et al. (10) reported the stimulation of lecithin synthesis in type II cells in bleomycin-treated animals. Bleomycin induces pulmonary fibrosis (10), and fibrosis of the lung is accompanied by alveolar type II cell hyperplasia. However, in the present case, pulmonary fibrosis was not observed. It is not clear how methylnaphthalene painted on the skin induced the proliferation and swelling of the pulmonary type II cells. Further investigation of the toxicity of methylnaphthalene to lung tissue seems necessary in view of the fact that this agent is widely used as a carrier in dyeing. This experimental model of lipid pneumonia should also be useful for the investigation of phospholipid metabolism in type II cells.

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Prostaglandin-Like Substances Formed during Autoxidation of Methyl Linolenate[1]

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Prostaglandin-like substances other than 3-(2-ethyl-5-hydroxy-3-oxo)-cyclopentanyl-2-propenal (compound I), which upon reacting with amino acids form reddish pigments, were purified, and their chemical structures were determined. Three red pigment-forming substances (RPS) in autoxidized methyl linolenate were purified successively by gel chromatography on Sephadex LH-20, column chromatography and thin layer chromatography on Silica Gel 60, and by high performance liquid chromatography on μ-Porasil. IR spectra of the RPS showed the absorption of an ester group (νC=O 1730 cm^{-1} in $CHCl_3$) and of a longer branched chain (νC-H 2800-3100 cm^{-1}), in addition to the absorption bands observed in compound I. Electron impact mass spectra of the trimethylsilyl ethers obtained after reduction with $NaBH_4$ and $NaBD_4$ were analyzed in detail and compared with those of compound I. The structures of the RPS were identified as stereoisomeric methyl 8-[2-(2-formylvinyl)-3-hydroxy-5-oxo-cyclopentanyl]-octanoates derived from methyl 12-hydroperoxy-linolenate.
Lipids 21, 553–557 (1986).

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Autoxidation of lipids containing polyunsaturated fatty acids with more than three double bonds produce compounds which react with amino acids and form reddish pigments (λmax 510-520 nm) (1). In a previous paper (2), a group of compounds produced by autoxidation of linolenic acid in linseed oil was identified as stereoisomers of 3-(2-ethyl-5-hydroxy-3-oxo)-cyclopentanyl-2-propenal (compound I; see Fig. 1).The mechanism, deduced from the biosynthesis (3,4) and from the nonenzymic formation (5–7) of prostaglandin (PG), indicated that compound I was produced via 13-hydroperoxyradical (or 13-hydroperoxide), followed by formation of the bicyclic endoperoxide analogue. If this mechanism is valid, then the formation of compound II via 12-hydroperoxyradical should occur.

This report concerns the isolation and the characterization of compound II produced during autoxidation of methyl linolenate.

MATERIALS AND METHODS

Preparation of red pigment-forming substances (RPS). (i) Autoxidation. Methyl linolenate (99%, Sigma Chemical Co., St. Louis, Missouri) was oxidized for 72 hr at 40 C in the dark with stirring (peroxide value 2000–2500 meq/kg).

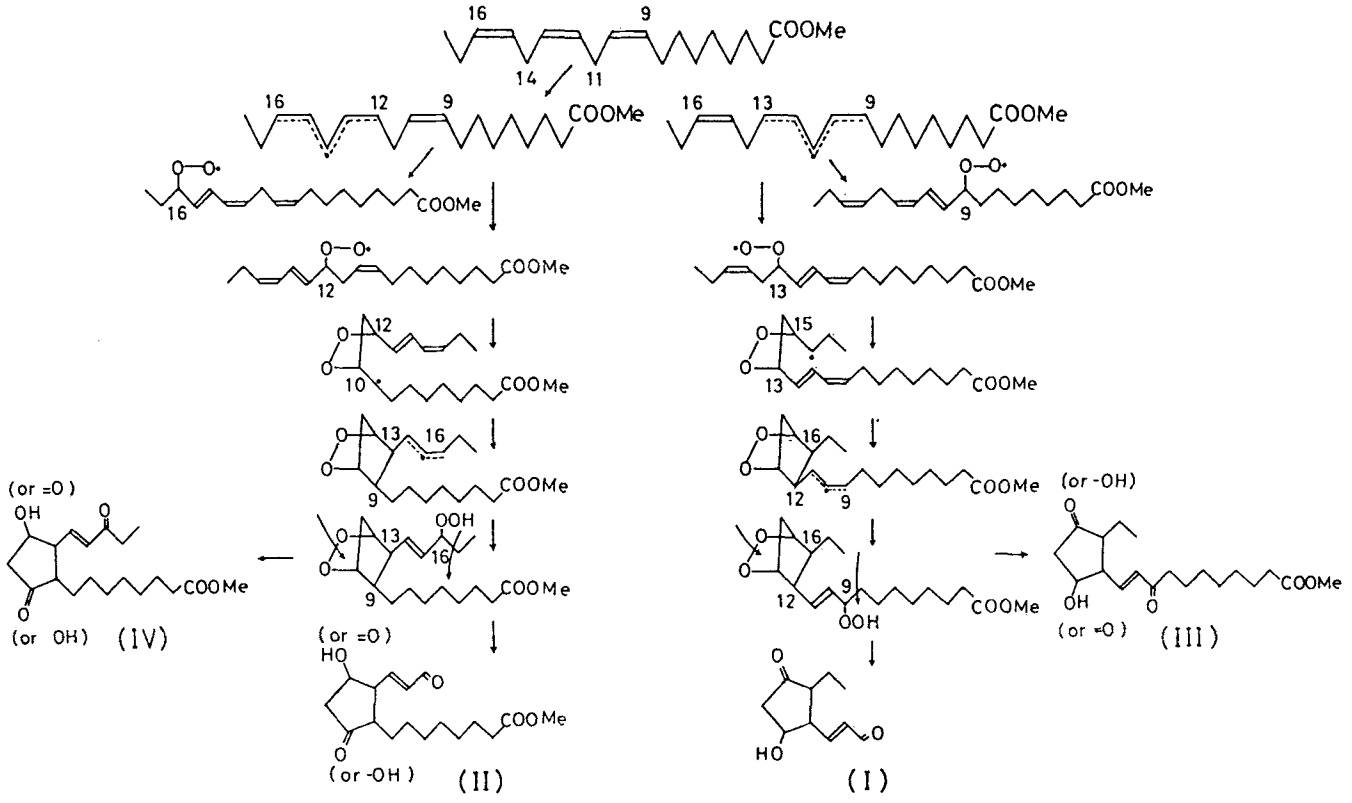

FIG. 1. Mechanism for the formation of red pigment-forming substances (RPS) from methyl linolenate.

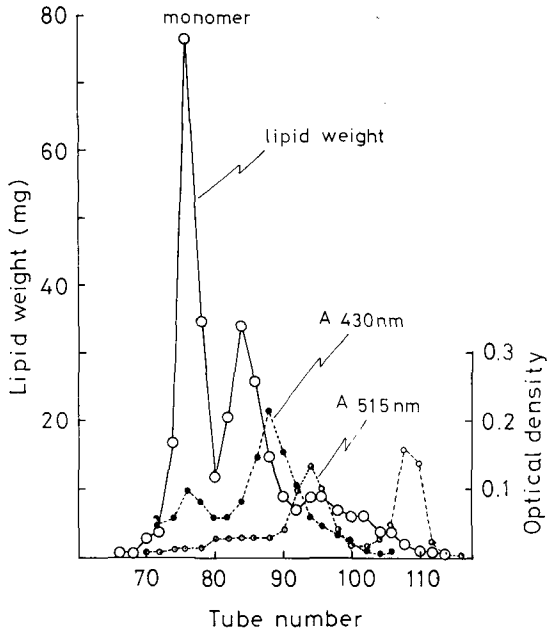

FIG. 2. Separation of oxidation products of methyl linolenate on Sephadex LH-20 with acetone as solvent. Load: 580 mg; column size: 270 ml, 138 cm long; fraction volume: 120 drops (ca. 2 ml)/tube. O——O, Lipid weight; ●- - -●, $A_{430\ nm}$ of lipophilic part; O- - -O, $A_{515\ nm}$ of hydrophilic part. Both parts were prepared after the coloring reaction with glycine (1).

(ii) Gel chromatography. The autoxidized methyl linolenate was separated on a Sephadex LH-20 column (1080 ml, 125 cm long) with $CHCl_3$/MeOH (1:1, v/v) as solvent. The degradation-product fraction containing RPS was pooled and again separated on a Sephadex LH-20 column (270 ml, 138 cm long) using acetone. The RPS eluted were monitored by the color reaction with glycine (1).

(iii) Silicic acid chromatography. The RPS fraction obtained by the gel chromatography was purified on a Silica Gel 60 prepacked column (24 × 1 cm, E. Merck, Darmstadt, Federal Republic of Germany) with $CHCl_3$/MeOH (97:3, v/v) as the eluent.

(iv) High performance liquid chromatography (HPLC). The crude RPS fraction from silicic acid chromatography was further purified by HPLC on a μ-Porasil column (30 × 0.78 cm; Waters Assoc. Inc., Framingham, Massachusetts), which was eluted with n-hexane/2-propanol (9:1, v/v) at a flow rate of 4.0 ml/min.

(v) Thin layer chromatography (TLC). Preparative and analytical TLC were carried out on Silica Gel 60 plates containing a fluorescent indicator (E. Merck) using benzene/acetone/EtOH (70:30:2, v/v/v) as solvent. Reddish spots were obtained after spraying with a glycine solution (0.5 M, pH 7.0) and subsequent heating in an oven at 45 C for 0.5–2 hr. After prolonged heating, the color faded.

Characterization of RPS. (i) IR spectrometry. IR spectra were measured in $CHCl_3$ with an EPI-G Hitachi IR spectrometer.

(ii) Gas liquid chromatography (GLC). Trimethylsilyl (TMS) ethers of the RPS were prepared after reduction with $NaBH_4$ or $NaBD_4$ (98%, E. Merck) and were analyzed using a Shimadzu GC-4BPF gas chromatograph equipped with glass columns (2 m × 3 mm) packed with 1.5% Silicone GE SE-30 (60-80 mesh, Shimadzu Co., Kyoto, Japan). The flow rate of N_2 carrier gas was 40 ml/min, and the column temperature was 220 C or 160 C. Preparation of the derivatives used was as described (2). The equivalent chain length (ECL) of RPS derivatives was determined using saturated methyl esters of known fatty acids as standards (8).

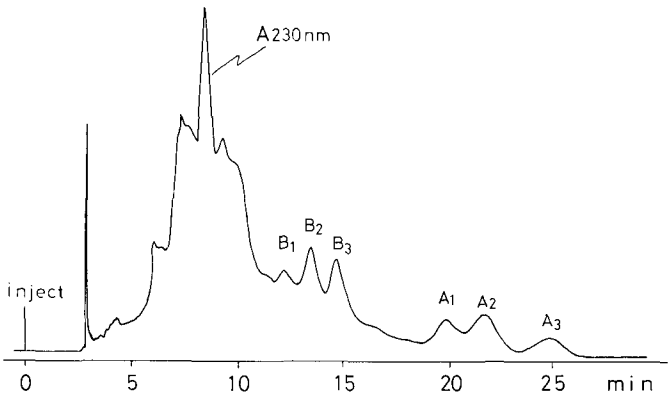

FIG. 3. HPLC of an RPS fraction corresponding to tubes 90–100 in Fig. 2.

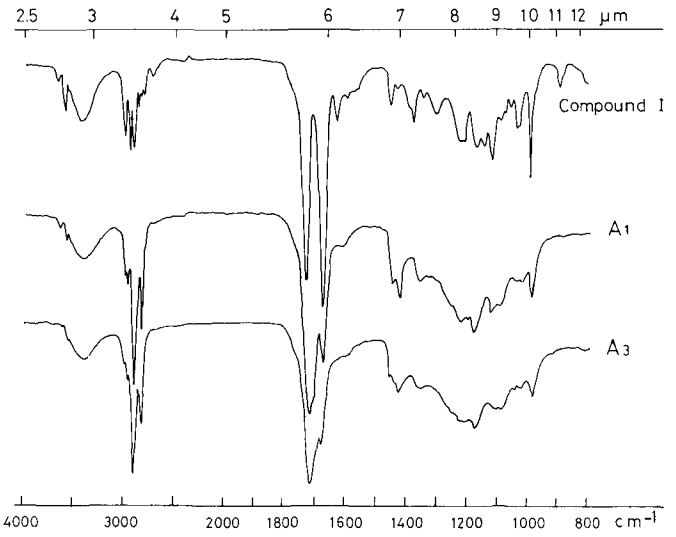

FIG. 4. IR spectra of RPS. Spectra were measured in chloroform solution (0.1 mm-thick cell).

(iii) Gas chromatography-mass spectrometry (GC-MS). Electron impact (EI)-mass spectra were obtained using a Hitachi RM-50 GC equipped with a 1.5% Silicone GE SE-30 glass column. Ionizing voltage was 20 eV.

RESULTS

Preparation of RPS. The pooled degradation products, the latter half of the monomer fraction (1) obtained by gel chromatography with $CHCl_3$/MeOH (1:1) as solvent, were further separated using acetone. The coloration reaction of the eluted fraction with glycine showed the presence of two groups each of brown pigment-forming substances ($A_{430\ nm}$) and of RPS ($A_{515\ nm}$), as shown in

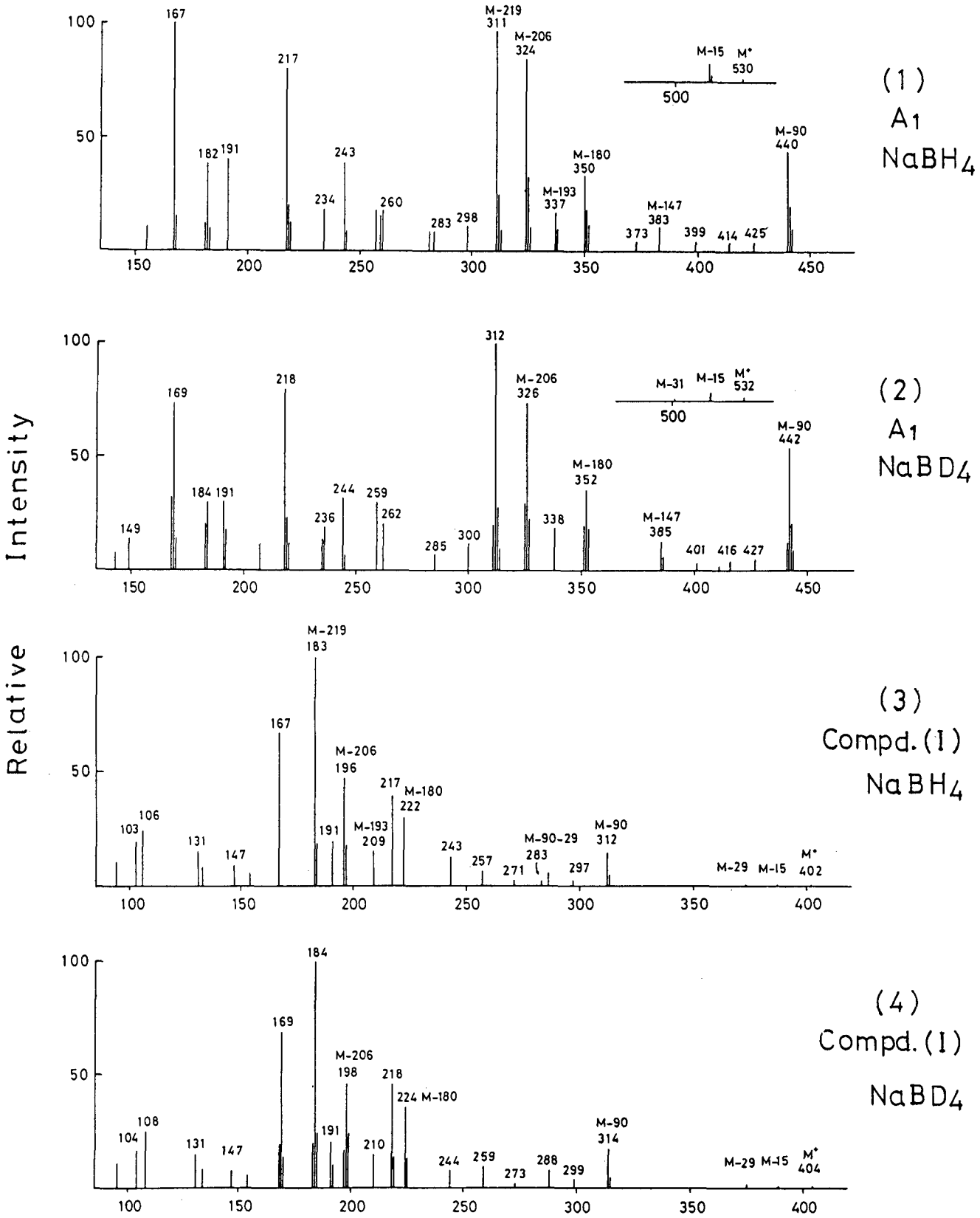

FIG. 5. Mass spectra of TMS ethers prepared after reduction with $NaBH_4$ or $NaBD_4$.

Figure 2. RPS eluted in tubes 104 to 112 were determined to be stereoisomers of compound I (2). The other fraction eluted in tubes 90 to 100 was pooled, and a triplet of RPS (A_1, A_2, A_3) was successively purified by silicic acid chromatography, TLC and HPLC. The HPLC and TLC-pure RPS, A_1 and A_3, shown in Figure 3, were prepared. A_2 was unstable and its yield decreased remarkably during purification, especially by TLC; the contamination with A_1 and A_3 also could not be excluded. Ca. 2–4 mg of RPS was prepared from 30 g of methyl linolenate.

Identification of compound II. Although clear differences were observed in the IR spectra between the known compound I and A_1 and A_3 (Fig. 4), there were no significant differences between the spectra of A_1 and A_3. The absorption band at 1730 cm^{-1} of A_1 and A_3 indicates ester $\nu C{=}O$, and wide absorption bands in this region indicate the overlap of ketone $\nu C{=}O$ (1740 cm^{-1}) located on the five-member ring and conjugated aldehyde $\nu C{=}O$ (1688 cm^{-1}) (2). Relatively stronger νC-H absorption bands at 2700–3100 cm^{-1} in the RPS spectra indicate the presence of longer carbon chains in A_1 and A_3 compared to compound I.

TMS ethers prepared after reduction with $NaBH_4$ and $NaBD_4$ were analyzed using GLC. One main peak (ECL 22.5) and two splitting peaks (ECL 22.2 and 22.6) were observed for A_1 and A_3, respectively. No difference in retention times was observed between the derivatives obtained after $NaBH_4$ and $NaBD_4$ reduction. EI-mass spectra at 20 eV of the three peaks showed a close resemblance, but there were slight differences in the relative intensities of the fragment ions. A mass spectrum of A_1-TMS, as the representative, was compared with that of compound I (Fig. 5). Although the molecular ions of TMS ether of A_1 (ECL 22.5) and compound I (ECL 15.0 corresponding to R-1 [2]) obtained after $NaBH_4$ reduction were, respectively, M^+530 and M^+402 (usually very small), the same mass fragment ions {m/e 167 ($C_6H_6O^+$TMS), 191 (SMTO$^+$CHOTMS), 217 (SMTO$^+C_3H_3$OTMS), 243 (SMTO$^+C_5H_5$OTMS), 283 M-[HOTMS, branched chain of C_2H_5 or $(CH_2)_7COOCH_3$]} and the same type ions [M-90 (-HOTMS), M-105 (-HOTMS, $-CH_3$), M-131 ($-C_2H_3$OTMS, $-CH_3$), M-206 (-HOTMS, $-C_2H_3$OTMS), M-219 (-SMTOC_3H_5OTMS)] were observed in both compounds (Figs. 5-1 and 5-3). The close resemblance of fragmentation patterns indicates a similarity of the basic structure, and the ion m/e 373 (M-157) in A_1-TMS indicates the presence of a branched chain of methyl octanoate instead of an ethyl group (M-29) in compound I. Thus, the structures of A_1 and A_3 are tentatively assigned to be compound II in Figure 1. TMS ethers of the deuterium-labeled one (Figs. 5-2 and 5-4) indicate the position of carbonyl groups and support the validity of the estimated structure. For instance, the fragment ions m/e 167 and M-206 in Figure 5-1 and their corresponding ions m/e 169 and M-206 in the deuterium-labeled A_1 (Fig. 5-2) indicate the position of the carbonyl groups. Namely, a keto group seems to locate on C-10 of the original methyl linolenate. In conclusion, A_1 and A_3 and also A_2 are stereoisomers of methyl 8-[2-(2-formylvinyl)-3-hydroxy-5-oxo-cyclopentanyl]-octanoate.

In the course of separation of compound II by HPLC, the presence of another type of RPS, B_1, B_2 and B_3 in Figure 3, was noted. The substances were peroxide-negative, and the color development was much slower than the RPS A_1, A_2 and A_3. These compounds were purified by recycling HPLC on the μ-Porasil column, using the same method as for the A-series. Mass spectra of TMS ethers obtained after $NaBH_4$ reduction indicated that their molecular weight was M^+558. The mass spectrum of TMS ether of derived B_2 (ECL 23.4) is shown in Figure 6 as an example. The findings are similar to those of the PGF analogues (9,10). Although a classification based only on fragmentation patterns cannot be made, they appear to be the stereoisomers of compounds III or IV in Figure 1.

DISCUSSION

While quantitative determinations of RPS in autoxidized lipids have not been made, the production of RPS monitored by coloration with glycine was found to increase with progressing autoxidation. In the case of methyl linolenate, this increase was made apparent by gel chromatography after half of the linolenate had been oxidized. The separation of compound II from compound I could well be achieved using the Sephadex

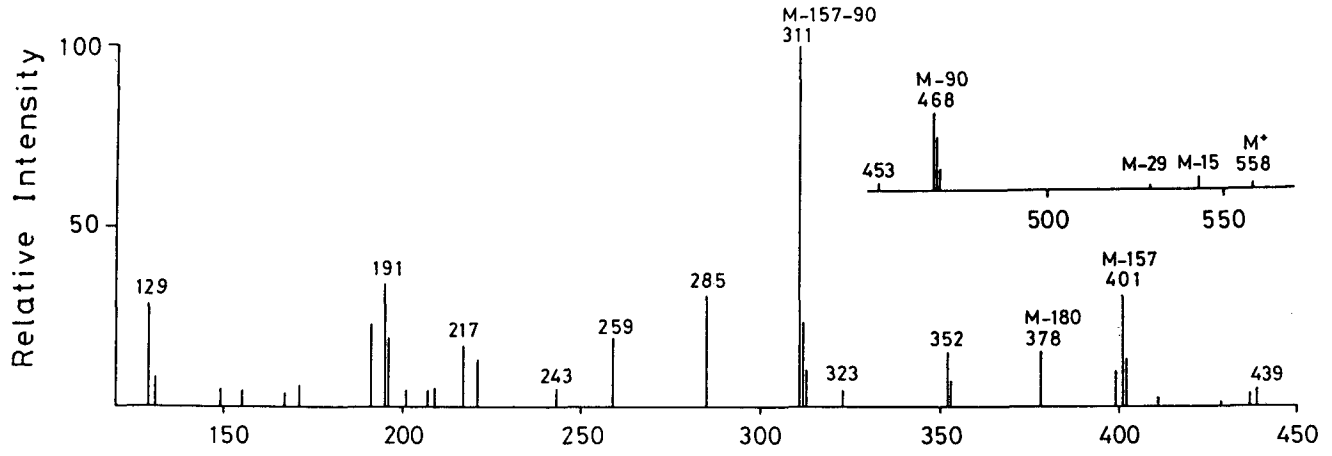

FIG. 6. Mass spectrum of TMS ether of $NaBH_4$ -reduced B_2.

LH-20 acetone system, which made full use of the substantial difference in molecular weight. The properties and behavior of the triplet (A_1, A_2, A_3) of compound II closely resembled the corresponding triplet (R-1, R-2, R-3) of compound I (2): unstable property of A_2 and R-2, appearance of splitting peaks of A_3 and R-3 on the GLC analysis. All observations were compatible with the proposed structure.

Because of a lack of reference materials, the stereoconfigurations of RPS remain to be elucidated. Little work has been done on the stereochemistry of PG analogues produced during autoxidation of polyunsaturated lipids. O'Connor et al. (10,11) have isolated and characterized PG-analogues produced by oxidation of ethyl 13-hydroperoxy-*cis*-9,*trans*-11, *cis*-15-octadecatrienoate and ethyl 9-hydroperoxy-*cis*-6,*trans*-10,*cis*-12-octadecatrienoate. They also studied the stereochemistry of the compounds and concluded that the formation of endoperoxides possessing *trans* ring substituents of natural PG does not appear to be favored, although minor compounds, PGF_1 and 11-*epi*-PGF_2 (natural PG), were found after reduction to triols with $SnCl_2$. Although the stereoconfiguration and significance of compounds I and II remain to be determined, their coloration ability and high reactivity with amino groups suggest participation not only in discoloration of fatty foods but possible hazardous lipid peroxidation in biological systems.

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Fatty Acids in Echinoidea: Unusual *cis*-5-Olefinic Acids as Distinctive Lipid Components in Sea Urchins

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Open tubular gas liquid chromatographic (GLC) analyses of fatty acids from total lipids of 12 species of Echinoidea collected at several locations along the Pacific coast of Japan showed the same unusual *cis*-5-olefinic acids in all species, i.e., *cis*-5-octadecenoic acid (5-18:1), *cis*-5-eicosenoic acid (5-20:1), all-*cis*-5,11- and 5,13-eicosadienoic acids (5,11- and 5,13-20:2), all *cis*-5,11,14-eicosatrienoic acid (5,11,14-20:3) and all-*cis*-5,11,14,17-eicosatetraenoic acid (5,11,14,17-20:4). The structural analysis of partially purified 5,11,14,17-20:4 was undertaken by reductive ozonolysis with GLC and gas chromatographic-mass spectrometric analyses of the products. ^{13}C-Nuclear magnetic resonance analyses of the totals and fractions of fatty acid methyl esters from the sea urchin lipids did not show any occurrence of fatty acids having an isolated olefinic bond in the 2, 3 or 4 positions. The 5-olefinic acids were concentrated on the polar lipids rather than neutral lipids. The branched and odd chain fatty acid contents of mud-feeding sea urchins were found to be relatively greater proportions of total fatty acids than in algae feeders.
Lipids 21, **558–565 (1986).**

The fatty acid details of sea urchin *Strongylocentrotus droebachiensis* harvested near Halifax, Nova Scotia, Canada, have been reported previously (1). The occurrence of unusual 5-olefinic acids was noticeable in all samples, amounting to as much as 10–21% of fatty acids from the total lipids of whole animals. The major components found were 5-18:1, 5-20:1, 5,11- and 5,13-20:2. In a subsequent study, fatty acids from six species of sea urchins collected in Japan were investigated, and the occurrence of the 5-olefinic acids was similar to that in Atlantic sea urchins (2,3).

In this study, fatty acids of lipids from 12 species of Echinoidea obtained in Japan have been investigated in greater detail. The 5-olefinic acids were found in the lipids of all of the samples, and their occurrence is thus established as a common and characteristic feature of Echinoidea lipids. The 5,11,14,17-20:4 and 5,11,14-20:3 were found to be minor ($\leq$0.5%) components of the total fatty acids. The former has been found in seed lipids of some gymnospermae (4), but has not been reported as a fatty acid component of animals.

MATERIALS AND METHODS

Materials. The species of Echinoidea studied are listed in Table 1. Samples 1, 5, 6, 8, 9 and 11 were obtained in Kanagawa prefecture; 3, 4, 7, 10 and 12 at Hokkaido; and 2 in Okinawa prefecture. Samples were collected from shallow subtidal water, except samples 3, 4 and 7 (50 m off Hakodate), 10 (50 m off Sarufutsu), and 9 (740 m in Sagami Bay).

Salted gonads of sea urchins *S. intermedius* and *S. nudus* were obtained at a food market in Hakodate.

All samples were kept frozen at -20 C in MeOH for a few months until used.

Preparation of methyl esters. The total lipids of sea urchins and gonads were extracted by the method of Bligh and Dyer (5). Fractionation of total lipids (TL) into neutral (NL) and polar (PL) lipids was carried out by column chromatography using silicic acid (Kiesel Gel 60, Merck, Darmstadt, Federal Republic of Germany) with chloroform and methanol as the developing solvents. The lipids were converted to fatty acid methyl esters by direct transesterification with 5% HCl solution in methanol by heating at 80 C in screw-cap test tubes for 3 hr under nitrogen. The methyl esters were separated from other products by thin layer chromatography (TLC) with Silica Gel G plates of 0.5 mm thickness by developing with n-hexane/ether (85:15, v/v).

Gas liquid chromatography (GLC). Open tubular GLC of the methyl esters was done with Shimadzu GC 6AM and 7A instruments (Shimadzu Seisakusho Co., Kyoto, Japan), with an FID detector, on wall-coated open-tubular glass columns coated with SP 2300 (50 m $\times$ 0.3 mm id). The column temperatures were 175 or 180 C and the injector and detector were held at 230 C. Peak area percentages were obtained with Shimadzu integrators C-R1A and C-R2AX.

^{13}C-Nuclear magnetic resonance (^{13}C- NMR). A JEOL FX-90Q spectrometer (Nippon Denshi Co., Tokyo, Japan) in the Fourier transform mode at 22.5 MHz for ^{13}C was used to obtain the ^{13}C-NMR spectrum of methyl esters in $CDCl_3$.

Argentation chromatography. The trienoates and tetraenoates were concentrated by argentation column chromatography using 20% $AgNO_3$-silica gel as the packing and ether/n-hexane mixtures as the eluting solvents. The percentage of ether in the mixtures was progressively increased from 2 to 100%.

The separation of the trienoates and tetraenoates was carried out by preparative argentation-TLC ($AgNO_3$-TLC) on $AgNO_3$-impregnated layers of Silica Gel G by developing with ethyl acetate/n-hexane (1:9, v/v) for the trienoates and (3:7, v/v) for the tetraenoates.

Reductive ozonolysis. Reductive ozonolysis of 1–5 mg methyl ester samples was carried out in methylene chloride at -70 C using the procedure of Kleiman et al. (6). The ozonides were reduced by addition of a few crystals of triphenylphosphine. Aldehydic products were analyzed by GLC using a 2 m $\times$ 3 mm id glass column packed with 3% Silar 10C on Gas Chrom Q (100–120 mesh). The column temperature was programmed from 60 to 280 C at 2 C/min. The weight percentages were calculated from the peak area percentages using C-factor (7). The detector and injector temperatures were kept at 300 C. The carrier gas was nitrogen at 30 ml/min.

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Gas chromatography-mass spectrometry (GC-MS). GC-MS of the ozonolysis products was carried out with a Hitachi M-60 GC-MS system containing a 2 m × 3 mm id glass column packed with 3% Silar 10C on Gas Chrom Q (100–120 mesh), using helium gas as the carrier gas. All mass spectra were obtained at 20 eV ionization electron energy and a source temperature of 180 C.

RESULTS AND DISCUSSION

Unusual fatty acids. In our previous paper (1), three unusual 5-olefinic acids, 5-20:1, 5,11- and 5,13-20:2, were identified as major and unusual components of the Atlantic sea urchin *S. droebachiensis*. Their structures were determined by GLC of the ozonolysis products, mass spectroscopic analysis of the pyrrolidides and other procedures. A small component was tentatively identified as 5,11,14-20:3 by comparisons of GLC retention data.

A typical gas chromatogram of the fatty acid methyl esters (C_{20} fraction) obtained in this study is shown in Figure 1A. All peaks found in the previous paper (1) were also observed in the chromatograms obtained in this study. An additional peak "b" for an unknown minor component was noted between the peaks of 20:3(n-3) and 20:4(n-3).

Large-scale fractionation of polyenoic acids. To facilitate identification of peak "b," which was common to all samples, a large-scale fractionation of fatty acids from a readily available sea urchin material was undertaken. Total lipids (9.78 g) were extracted from 100 g of salted gonads of *S. intermedius* (sample 3) and *S. nudus* (sample 4). The fatty acids (7.27 g) obtained from these lipids were fractionated by urea adduct methods using urea (20 g) and methanol (100 ml). The polyenoic acid fraction (3.94 g) recovered from the filtrate was converted to methyl esters. The methyl esters of polyenoic acids separated by argentation column chromatography, and the trienoate and tetraenoate fractions were further fractionated by $AgNO_3$-TLC. The gas chromatograms of the trienoate and tetraenoate fractions obtained are shown in Figures 1B and 1C. Peaks "a" and "b" in each figure were identified as 5,11,14-20:3 and 5,11,14,17-20:4 on the basis of the agreement of the retention data with those of the reference methyl esters of those acids obtained from *Podocarpus nagi* seed and *Juniperus chinensis* seed (4), respectively. These results were confirmed by open tubular GLC analyses on another liquid phase SP-2340 (50 m × 0.3 mm id; column temperature 160 C; injector and detector temperature 230 C).

The tetraenoate fraction could be separated into two fractions by $AgNO_3$-TLC, using ethyl acetate/n-hexane (3:7, v/v) as the developing solvent. Fraction 1 (Rf = 0.44) contained 46.3% 20:4(n-6) and 53.7% 20:4(n-3). Fraction 2 (Rf = 0.34) contained 1.3% 20:4(n-6), 53.8% 20:4(n-3) and 44.9% "b" (5,11,14,17-20:4). The compositions of these two fractions indicate the order of mobility from the top to the bottom to be 20:4(n-6), 20:4(n-3) and "b" (5,11,14,17-20:4).

Products from reductive ozonolysis. Since complete separation of individual 20:3 and 20:4 acids was impossible, fractions 1 and 2 were subjected to ozonolysis followed by GLC of the products. Some component peaks were identified on the basis of the agreement of the retention times with those of authentic reference esters and through oxidative fission products. Thus, reductive ozonolysis products from 5,11,14-20:3 contained n-hexanal (I), 1,6-hexanedial (II) and methyl 5-oxopentanoate (III); those from 5,11,14,17-20:4 contained II and III; those from 20:4(n-3) contained methyl 8-oxooctanoate; and those from cyclohexene contained II. The compositions of the ozonolysis products from fractions 1 and 2 are shown in Table 2. The fair agreement of the proportions of mole percentages (II/III) between fraction 2 and reference acid 5,11,14,17-20:4 (Table 2) characterized peak "b" as 5,11,14,17-20:4.

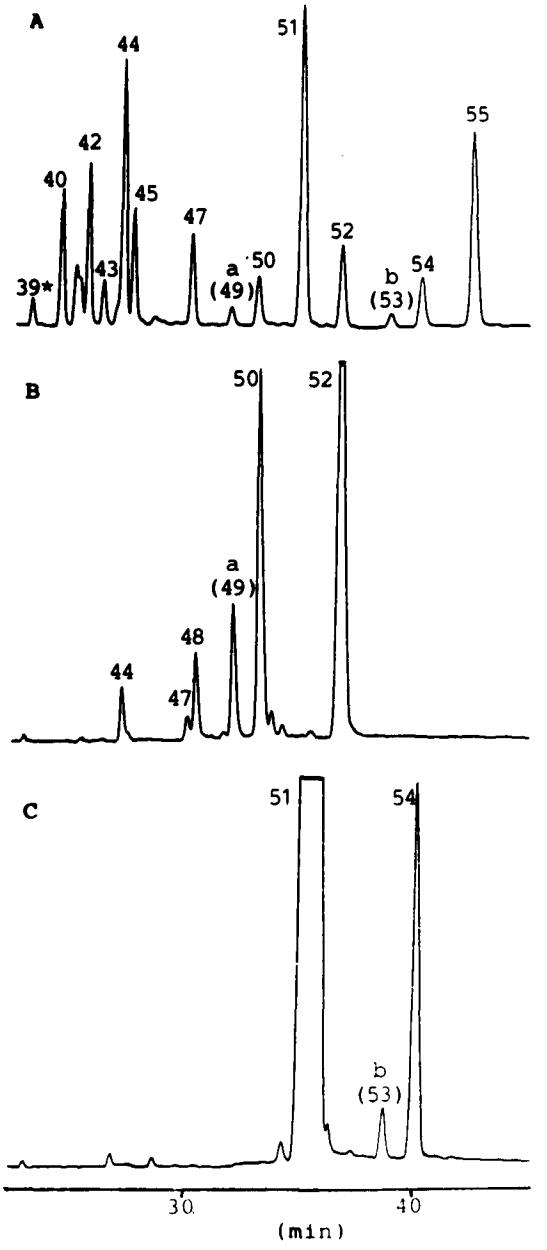

FIG. 1. Parts of gas chromatogram of fatty acid methyl esters of the total lipids from *H. pulcherrimus*. A, Total; B, trienoate fraction; C, tetraenoate fraction. *, See Table 4.

GC-MS of ozonolysis products. The mass spectra of the hexanedial fraction from the ozonolysis products of the 5,11,14,17-20:4 concentrates were taken by the GC-MS

TABLE 1

Echinoidea Samples and Their Contents of Lipids and 5-Olefinic Acids

No.	Order	Genus and species	Lipids (%)[a]	5-Olefinic acids (%)[b]	Date obtained
1	Echinoida	*Anthocidaris crassispina*	5.20	11.13	July 80
2	Echinoida	*Echinometra mathaei*	0.50	5.90	August 83
3	Echinoida	*Strongylocentrotus intermedius*	0.56	19.18	March 84
4	Echinoida	*Strongylocentrotus nudus*	0.58	21.14	March 84
5	Echinoida	*Hemicentrotus pulcherrimus*	1.84	11.39	July 80
6	Echinoida	*Pseudocentrotus depressus*	5.30	9.88	August 80
7	Arbacioida	*Glyptocidaris crenularis*	1.12	10.88	Feb. 84
8	Diadematoida	*Diadema setosum*	2.10	9.07	August 83
9	Cassiduloida	*Echinolampas sternopetala*	1.00	14.49	August 80
10	Spatangoida	*Echinocardium cordatum*	0.30	12.44	August 83
11	Clypeasteroida	*Clypeaster japonicus*	0.64	8.38	July 81
12	Clypeasteroids	*Scaphechinus mirabilis*	0.60	12.54	August 79

[a]Wt %: total lipids to a wet sample.
[b]Wt %: all 5-olefinic acids in total fatty acids.

instrument. The mass to charge ratio of the fragment ions and their intensities are shown in comparison with those of an authentic reference sample in Table 3. The agreement further supported the occurrence of 5,11,14,17-20:4 in the sea urchin lipids. In the assignment of the ions in Table 3, the mass numbers of the fragment ions were attributed to the loss of the following fragments: 18 ($H_6O^{\bullet}$), 29 ($HCO^{\bullet}$), 44 ($CH_2=CHOH^{\bullet}$), 57 ($OHCCH_2CH_2^{\bullet}$) and 70 ($CH_2=CH\text{-}CH_2\text{-}CHO^{\bullet}$). The (M-70) and (M-57) ions were formed by the fragmentation process, including McLafferty rearrangement, and the scission of the central C_3-C_4 bond, respectively.

^{13}C-NMR, infrared (IR) and ultraviolet (UV) analyses. Previously, Kochi reported occurrence of 3,11-20:2 as a component of roe lipids of sea urchins obtained in Japan and Korea (8,9). To investigate the occurrence of 3-olefinic acids and any other acids having an olefinic bond near the ester group, the ^{13}C-NMR spectra of the original acid methyl esters and their fractions were examined. No signals were found at the chemical shifts of *cis*- and *trans*-2- to 4-olefinic acids expected from literature data (10) as shown in Figure 2. The characteristic signals of the *cis*-5-olefinic acids shown in Figure 2 were in accord with those reported in previous papers (4,10). This result also supported the occurrence of the 5-olefinic acids in the sea urchins. IR spectra showed no absorbance near 971 cm^{-1}, and UV spectra showed no absorbance near 232 nm, 270 nm and 302 nm, respectively. These results showed the absence of *trans* and conjugated unsaturated compounds in methyl esters of the sea urchins (11).

Unusual 5-olefinic acids. The compositions of the fatty acids from the total lipids of Echinoidea are shown in Table 4, and the gas chromatogram of fatty acid methyl esters of total lipids from *Pseudocentrotus depressus* is shown in Figure 3. All the samples contained about 6–20% of the 5-olefinic acids. The results are similar to those of the Atlantic sea urchin *S. droebachiensis* (1), which showed somewhat higher percentages of total 5-olefinic acids (10–20%).

It is noteworthy that all samples of Echinoidea in Table 4 show very significant contents of the 5-monoenoic acids (2.3–9.3% to the total fatty acids). The 5-monoenoic acids have been reported as minor components of certain marine invertebrates, such as periwinkle *Littorina littorea*, moon snail *Lunatia triseriata* and sand shrimp *Crangon septemspinosus*, but at levels of 0.2% or less (12).

TABLE 2

Compositions of Ozonolysis Products from the 20:4 Fraction and a Sample of Authentic 5,11,14,17-20:4 from *Juniperus chinensis* Seed

	Products[a]			
	6A	5AE	6AA	8AE
RT[b] (min)	3.65	28.03	34.87	43.93
Fraction 1[c]				
Wt %	23.85	29.17	0	46.97
Mol %	32.41	30.49	0	37.11
Fraction 2[c]				
Wt %	0	47.98	6.33	45.70
Mol %	0	53.47[d]	8.04	38.49
Authentic 5,11,14,17-20:4				
Wt %	0	86.97	13.58	0
Mol %	0	84.81	15.19	0

[a]6A, n-hexanal; 5AE, methyl 5-oxo-pentanoate; 6AA, 1,6-hexanedial; 8AE, methyl 8-oxo-octanoate.
[b]Retention time on Silar 10C.
[c]Compositions given in text.
[d]Calculated percentages of the ozonolysis products from 5,11,14,17-20:4 in this fraction: 5AE 52.17 and 6AA 9.34.

The totals of 5,11- and 5,13-20:2 fatty acid methyl esters of acids from the total lipids of Echinoidea were as much as 2.6–10.0% in this study. These contents were relatively higher than in some other marine animals (12–14), where starfish *Asterias vulgaris* had the highest content at 5.6%, and most of the totals were less than 1%. However, the totals of 5,11- and 5,13-20:2 found in this study were rather lower than those reported for the Atlantic sea urchins (5-10%) (1). In all of the samples studied, the proportion of 5,11-20:2 was always higher than that of 5,13-20:2, similar to the results of the previ-

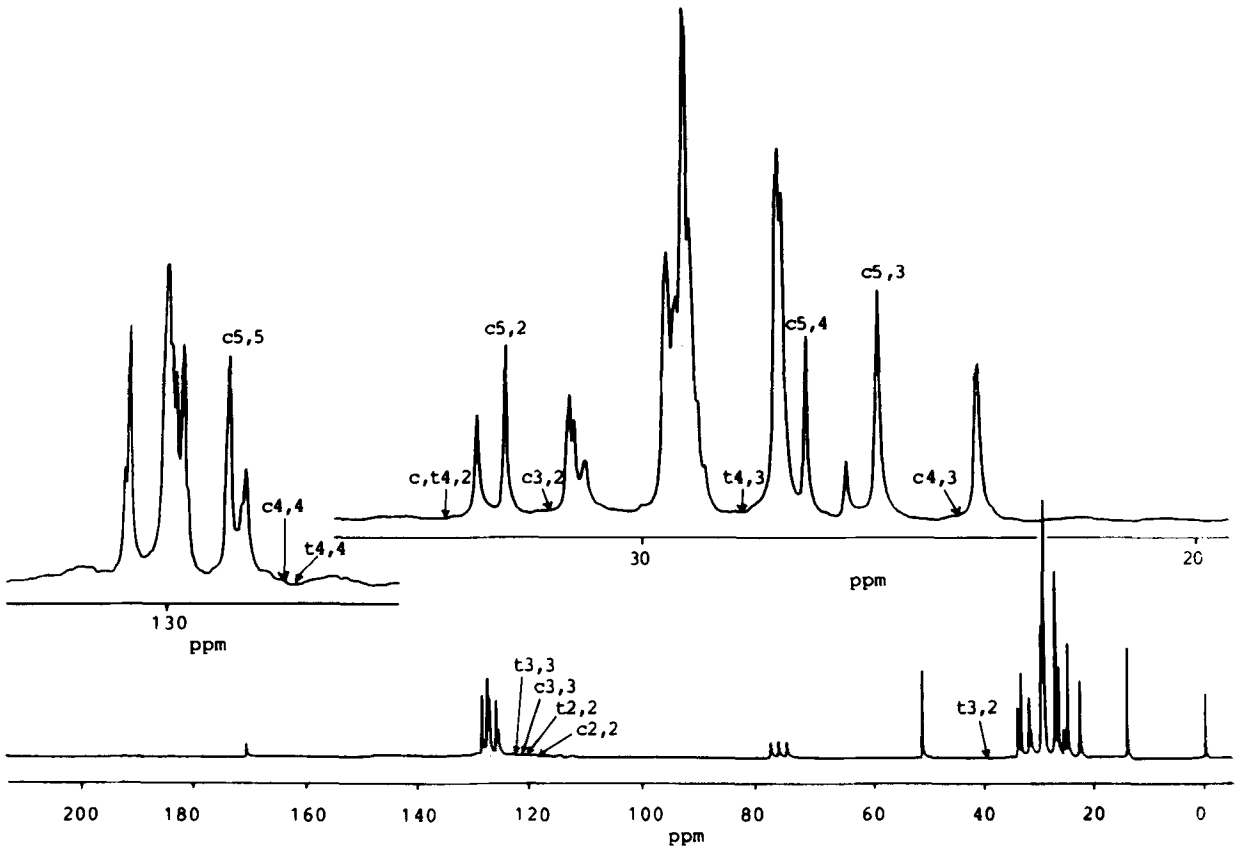

FIG. 2. ^{13}C-NMR spectrum of dienoate fraction from the lipids of salted gonads of sea urchins. c5,3: C_3 carbon of *cis*-5-olefinic acid; t3,2: C_2 carbon of *trans*-3-olefinic acid.

ous study (1).

The distribution of the 5-olefinic acids in triacylglycerols (TG) of *S. intermedius* was elucidated by Grignard hydrolysis and following GLC analysis in our previous study (15). These 5-olefinic acids distributed mainly in the 1(3)-position of TG.

Fatty acid compositions of TL. Samples 1–6 in Table 1 belong to the same animal order. Fatty acid compositions were similar in samples 1, 5 and 6, and in 3 and 4, in the contents of 5-20:1, 5,11- and 5,13-20:2, 20:4(n-6) and 20:5(n-3), respectively. Sample 2 showed a rather different composition. These samples were obtained from Hokkaido (3 and 4), Kanagawa (1,5 and 6) and Okinawa (2), respectively. The differences among the fatty acid compositions of these samples may be attributed to the environments and ecosystems of the respective habitats.

Samples 7–12 belong to different orders, and it is surprising that the fatty acid compositions of 7–12 showed individually different patterns. Samples 9 and 10 contained higher contents of 22:6(n-3). Since the proportions of 22:6(n-3) are usually very low among the fatty acids of marine algal lipids (16,17), the high content of this fatty acid in 9 and 10 suggests that it came from their diets, e.g., the carcasses of fish and other marine animals. Sample 9 was obtained at 740 m depth, where algae are not directly available to the sea urchins as part of the diet. In such a case, it is known that the diet includes the carcasses of marine animals (18). The animals belonging to the order Clypeasteroida (samples 11 and 12) live on sand bottoms, and their diets are generally detritus from marine animals and plants. Their specific fatty acid features (Table 4), notably the low (<3%) contents of 20:4(n-6), could be attributed to diets different from those of other sea urchins.

Fatty acid compositions of NL and PL. Fatty acid compositions of NL and PL are shown in Table 5. Fatty acids having a nonmethylene-interrupted (NMI) 5-olefinic bond [5-20:1 and 5,11-20:2] and those having a methylene-interrupted 5-olefinic bond with other olefinic bonds [20:4(n-6) and 20:5(n-3)] were generally rich in fatty acids from PL. On the contrary, monoenoic acids such as 18:1(n-9) and 18:1(n-7) and minor polyenoic components such as 18:2(n-6), 18:3(n-3), 18:4(n-3) and 22:6(n-3) were rich in fatty acids from NL. The total 5-olefinic acids in PL described above will have important roles as the constituents of lipids in membranes, as well as the source of supply for physiologically active components and biological energy. The NMI 5-olefinic bond increases the stability of the nearby ester linkage toward lipolytic hydrolysis, and the effect would increase stability of the membrane against the microbial lipases (12).

Branched and odd chain fatty acids. The fatty acids from samples 3, 4, 6, 7, 9, 10 and 11 in Table 1 contained high levels of iso and anteiso branched and odd chain fatty acids, totaling more than 4.7%, as shown by the data in Table 4. The high contents of the branched and odd chain acids in the neutral lipids of the gonads and visceras from a mud-feeding sea urchin, *S. franciscanus*,

were reported in a previous paper (19). High contents of the branched and odd chain acids were also found in other mud-feeding marine animals, such as the mullet *Mugil cephalus* (20,21), the smelt *Osmerus mordax* from a specific location (22) and the holothurian *Scotoplanes theeli* (23). The origin of these branched and odd chain acids in the mud-feeding marine animals is known to be bottom material taken into their diets. Both branched and odd chain acids in sediment and bottom material have been reported to be formed by the action of microorganisms (24). In this study, it is thought that the origin of the branched and odd chain acids in the lipids from the Echinoidea samples is also basically from their diets.

Origin of the unusual 5-olefinic fatty acids. Several explanations can be put forward to account for the 5-olefinic bond in the fatty acids of Echinoidea. These are in situ production by a 5-desaturase on preexisting fatty acids, absorption from lipids of food such as algae and sediment, assimilation from lipids of microorganisms normal in Echinoidea digestive system or absorption through the skin from seawater or epiflora. However, only trace amounts of 5-olefinic acids were found in most algae (16,17), with exceptions being *Cladophora rupestris* and *Ascophyllum nodosum* (25,26). Particularly, 5-20:1 in the major 5-olefinic acids of Echinoidea has not been reported among the constituents of algal lipids. The occurrence of the 5-olefinic acids has not been reported in marine microorganisms or in seawater. Therefore, the most plausible explanation for the origin of the 5-olefinic acids is that Echinoidea have a 5-desaturase. It is suggested that the biochemical formation of 5-20:1 from 20:0, 5,11-20:2 from 11-20:1, 5,13-20:2 from 13-20:1, 5,11,14-20:3 from 11,14-20:2 and 5,11,14,17-20:4 from 11,14,17-20:3 is by a 5-desaturase, as suggested for the sea urchin *S. droebachiensis* (1). The acyl group in seed oil of meadowfoam *Limnanthes alba* contains about 60% 5-20:1 with small amounts of 5-22:1, 5-18:1 and 5,13-22:1 as 5-olefinic acids, and 5-desaturation of fatty acids from oilseeds was demonstrated by incubation of ^{14}C-labeled substrates with developing seed slices and with a cell-free homogenate of meadowfoam (27,28). The unusual 5-olefinic acids in the Echinoidea can be formed in a similar manner by a 5-desaturase. An analogous desaturation has been suggested for the formation of the unusual 5-olefinic acids and the acids derived from C_2 elongation (7-olefinic acids) in gymnospermae seeds (4), and some molluscs (12, 29 and 30). The longer chain 5-olefinic fatty acids have also been found in some sponges, and formation by 5-desaturase has also been suggested (31,32) in these cases. However, the occurrence of the 5-monoenoic fatty acids in appreciable amounts has not yet been reported in lipids of other marine organisms. The facts suggest that the remarkable features of the 5-

TABLE 3

Comparison of Mass Spectra of 1,6-Hexanedial from Ozonolysis with a Reference Sample

m/e	Intensity percent[a]		Relation with M[b]
	Ozonolysis products	Reference	
113	3.0	1.3	M-1
96	57.7	42.7	M-18
95	22.8	18.6	M-18-1
85	2.6	2.8	M-29
84	1.8	3.7	M-29-1
78	1.6	0.5	M-18-18
70	65.5	63.7	M-44
69	5.2	4.0	M-44-1
68	18.3	18.9	M-46
67	100.0	100.0	M-18-29
57	45.8	36.5	M-57
56	2.7	1.3	M-29-29
44	4.3	2.7	M-70

[a]Percentages relative to the intensity of the base peak m/e 67.
[b]See text for the assignment. M = m/e of molecular ion 114.

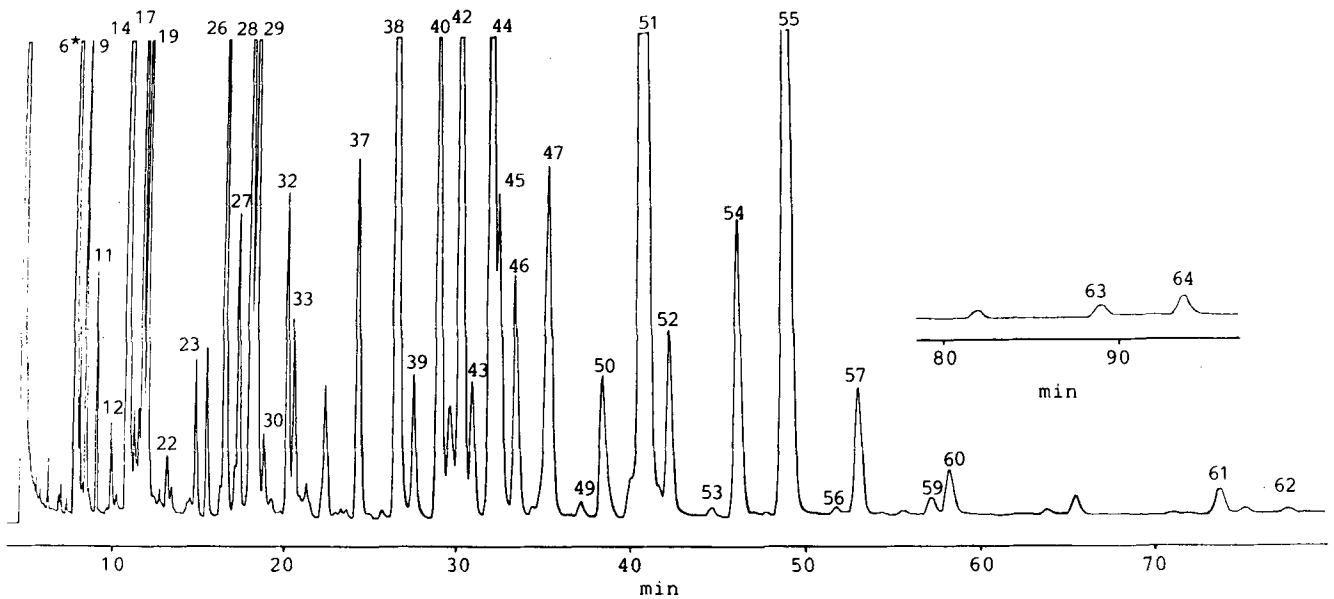

FIG. 3. Gas chromatogram of fatty acid methyl esters of total lipids from *Pseudocentrotus depressus*. *, See Table 4.

TABLE 4

Fatty Acid Compositions of the Total Lipids from Echinoidea (wt%)

No.	Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12
1	*iso*-13:0	0.01	–	0.01	0.01	–	–	0.01	–	0.11	–	0.13	–
2	*anteiso*-13:0	–	–	0.01	–	–	–	0.01	–	–	–	–	–
3	13:0	0.31	0.28	0.03	0.01	0.37	0.02	0.08	0.06	1.58	0.09	5.52	–
4	*iso*-14:0	0.03	0.24	0.23	–	0.04	0.02	0.10	0.13	0.26	0.60	0.24	–
5	*anteiso*-14:0	–	–	–	0.03	–	–	0.02	–	–	–	–	–
6	14:0	15.15	9.31	4.61	4.25	8.40	19.69	8.17	15.75	3.90	4.43	2.78	2.62
7	14:1 (n-7)	0.05	0.26	–	0.13	0.04	0.05	–	–	0.09	–	0.46	0.01
8	14:1 (n-5)	1.14	0.83	–	–	0.64	–	–	0.70	1.56	2.23	2.42	–
9	*iso*-15:0	0.02	–	0.47	0.55	0.01	0.93	0.71	–	–	–	0.30	–
10	*anteiso*-15:0	0.07	–	0.20	0.37	0.09	0.05	0.88	–	0.80	–	0.70	0.21
11	15:0	0.38	1.73	0.33	0.96	0.45	0.32	1.01	1.07	1.13	2.19	1.96	0.44
12	*iso*-16:0	0.28	0.21	0.12	0.52	0.36	0.16	0.78	0.73	0.76	1.42	0.44	0.07
13	*anteiso*-16:0	–	–	0.12	0.42	–	–	0.47	–	–	–	–	–
14	16:0	16.42	18.39	14.45	9.84	16.30	16.42	12.25	20.81	10.81	15.53	18.87	14.96
15	16:1 5	0.20	0.18	0.17	1.73	0.36	0.18	1.74	0.13	0.81	0.53	–	0.15
16	16:1 (n-9)	0.20	0.70	0.47	0.48	0.26	0.16	–	0.15	0.98	0.67	2.57	14.11
17	16:1 (n-7)	1.60	3.92	6.04	1.99	2.35	2.21	5.06	3.25	4.47	9.05	3.60	–
18	16:1 (n-5)	5.02	0.60	–	–	1.39	–	–	0.77	1.97	2.08	0.33	0.05
19	*iso*-17:0	0.25	–	2.28	2.59	0.21	2.78	0.83	–	0.58	–	0.37	–
20	*anteiso*-17:0	0.04	–	0.06	0.41	0.09	0.01	1.00	–	0.48	–	0.64	–
21	16:2 (n-6)	–	0.46	0.23	0.23	0.02	–	0.08	0.13	0.16	0.34	0.11	0.29
22	17:0	0.27	0.43	0.30	1.18	0.31	0.11	1.05	1.29	1.19	1.03	1.98	0.37
23	*iso*-18:0	–	–	0.12	0.40	0.26	0.37	1.18	–	0.07	–	0.17	–
24	*anteiso*-18:0	–	–	–	0.17	–	–	0.06	–	–	–	–	–
25	16:4 (n-3)	3.69	2.16	0.59	0.43	2.27	0.06	–	0.22	1.10	0.11	1.33	1.30
26	18:0	3.57	4.24	2.16	2.61	3.14	2.09	3.22	3.43	4.46	3.35	10.22	3.72
27	18:1 5	0.50	0.16	1.01	1.00	0.58	0.77	0.98	1.20	1.79	1.27	0.83	0.45
28	18:1 (n-9)	0.65	0.59	2.85	0.98	2.03	2.53	6.23	6.26	2.59	1.41	6.27	1.64
29	18:1 (n-7)	4.43	2.75	3.01	3.28	3.56	2.77	6.89	3.27	5.43	6.11	4.68	3.88
30	18:1 (n-5)	0.36	–	0.29	–	0.12	0.23	0.37	0.26	0.49	0.64	0.16	0.04
31	*anteiso*-19:0	–	–	0.10	0.17	–	–	0.36	–	–	–	–	–
32	18:2 (n-9)	1.07	0.51	1.03	0.32	0.58	0.90	0.84	0.17	0.16	0.60	–	–
33	18:2 (n-6)	0.37	1.75	1.27	0.44	0.82	0.58	1.09	0.84	1.00	0.79	1.18	0.83
34	19:0	0.38	0.14	0.34	0.95	0.23	0.16	0.38	0.31	0.89	0.74	0.61	0.60
35	18:3 (n-6)	0.34	0.95	–	–	0.28	–	0.34	0.28	1.37	–	0.77	0.49
36	*iso*-20:0	–	–	–	–	–	–	0.35	–	–	–	–	–
37	18:3 (n-3)	0.43	3.47	0.82	0.30	1.90	1.23	1.15	0.78	0.08	0.16	0.19	0.36
38	18:4 (n-3)	1.09	3.83	0.93	0.33	4.18	4.27	0.82	1.54	0.26	0.29	0.38	1.26
39	20:0	0.85	0.19	0.46	0.59	0.78	0.60	0.89	0.77	0.75	0.67	1.34	0.74
40	20:1 5	3.75	2.10	7.76	8.25	3.84	2.57	4.05	3.67	6.85	5.32	4.53	5.22
41	20:1 (n-11)	2.14	0.29	0.94	1.37	0.96	0.58	2.09	1.77	0.65	0.58	1.60	0.10
42	20:1 (n-9)	2.17	0.51	2.53	2.21	2.98	3.76	1.52	2.76	0.52	0.69	1.62	4.17
43	20:1 (n-7)	0.83	0.34	0.81	1.28	0.81	0.57	0.98	1.00	0.39	0.49	0.86	3.60
44	20:2 5,11	5.17	2.31	6.69	7.23	4.59	4.97	3.04	3.32	3.10	4.14	2.17	3.72
45	20:2 5,13	1.34	0.27	3.27	2.47	1.56	1.25	0.80	0.67	1.45	0.91	0.63	2.80
46	20:2 (n-9)	0.20	–	0.06	–	0.05	1.14	0.33	0.12	–	0.08	–	0.04
47	20:2 (n-6)	2.67	1.15	2.83	1.98	1.46	2.17	0.82	1.01	0.94	0.34	0.40	1.10
48	20:3 (n-9)	–	1.50	–	–	–	–	0.87	0.88	–	0.50	–	–
49	20:3 5,11,14	0.08	0.35	0.27	0.40	0.25	0.08	0.26	0.06	0.43	0.24	0.15	0.18
50	20:3 (n-6)	0.79	0.85	0.80	0.25	1.03	0.85	0.59	0.41	0.19	0.20	0.89	0.28
51	20:4 (n-6)	10.53	15.22	15.44	19.21	10.80	10.76	8.50	7.77	15.12	8.00	3.16	2.93
52	20:3 (n-3)	0.79	–	0.56	0.73	2.01	1.08	0.48	0.07	0.16	0.04	–	1.91
53	20:4 5,11,14,17	0.09	0.53	0.01	0.06	0.21	0.06	0.01	0.02	0.06	0.03	0.08	0.02
54	20:4 (n-3)	0.80	1.80	–	–	1.95	1.79	0.25	0.24	–	0.24	0.11	0.10
55	20:5 (n-3)	6.21	12.65	6.54	7.83	9.91	6.54	11.86	6.53	10.31	13.82	2.29	18.26
56	22:1 (n-11)	–	–	–	–	0.19	0.06	0.47	0.19	0.34	0.21	0.66	0.86
57	22:1 (n-9)	1.87	0.27	2.01	1.19	1.77	0.87	1.55	0.96	0.30	0.41	2.18	2.99
58	22:1 (n-7)	–	–	–	0.02	0.09	0.02	0.17	0.44	0.28	0.07	0.93	0.47
59	22:2 7,13	0.13	–	0.46	0.51	0.14	0.13	0.23	0.29	0.12	0.09	0.45	0.01
60	22:2 7,15	0.25	–	2.13	2.39	0.71	0.34	1.14	0.14	0.99	0.49	0.21	0.38
61	22:4 (n-6)	0.40	0.20	0.23	0.39	0.51	0.24	–	0.29	0.58	0.27	1.73	0.75
62	22:5 (n-6)	–	–	0.11	0.39	0.29	0.05	0.07	0.36	1.06	0.38	0.36	–
63	22:5 (n-3)	–	0.83	0.75	0.56	0.26	0.15	0.07	0.29	0.55	0.58	0.25	0.06
64	22:6 (n-3)	0.64	0.55	0.69	2.59	1.20	0.26	0.46	2.45	3.55	5.58	1.96	1.23
65	24:1 (n-9)	–	–	–	0.03	–	–	–	0.02	–	–	1.25	0.22

TABLE 5

Fatty Acid Compositions of the Neutral and Polar Lipids from Echinoidea (wt%)

No.	Fatty acid	2 NL	2 PL	3 NL	3 PL	4 NL	4 PL	7 NL	7 PL	8 NL	8 PL	10 NL	10 PL
1	*iso*-13:0	–	–	0.01	–	0.03	0.03	0.01	0.01	–	–	–	–
2	*anteiso*-13:0	–	–	0.01	0.01	0.02	0.06	0.02	0.02	–	–	–	–
3	13:0	0.30	0.14	–	–	–	0.11	0.11	0.02	0.08	–	–	0.07
4	*iso*-14:0	0.32	0.07	0.09	0.09	0.16	0.13	0.14	0.06	0.19	0.08	0.18	0.46
5	*anteiso*-14:0	–	–	0.01	0.02	0.03	0.03	0.02	–	–	–	–	–
6	14:0	8.84	8.88	6.39	3.15	4.23	4.11	11.93	0.95	20.60	4.59	5.08	3.85
7	14:1(n-7)	0.36	0.06	0.19	0.04	0.27	0.06	–	–	–	–	–	–
8	14:1(n-5)	0.91	0.29	–	–	–	–	–	–	0.91	0.31	0.83	2.19
9	*iso*-15:0	–	–	1.14	0.19	0.92	0.36	0.98	0.13	–	–	–	–
10	*anteiso*-15:0	–	–	0.24	0.17	0.51	0.27	1.21	0.12	–	–	–	–
11	15:0	2.11	1.26	0.44	0.24	1.90	0.67	1.36	0.25	1.54	0.66	1.24	2.19
12	*iso*-16:0	0.28	0.04	0.21	0.08	1.01	0.27	0.67	0.87	0.69	0.16	0.50	1.29
13	*anteiso*-16:0	–	–	0.17	0.01	0.35	0.06	0.46	0.45	–	–	–	–
14	16:0	15.52	24.43	19.04	10.09	11.95	9.01	14.50	9.42	18.71	29.99	18.18	12.67
15	16:1 5	0.10	0.10	0.19	0.09	1.49	0.42	1.83	1.55	0.34	0.04	0.50	0.59
16	16:1(n-9)	1.22	0.12	0.59	0.33	0.55	0.41	–	–	0.20	0.14	0.30	0.73
17	16:1(n-7)	4.87	0.65	11.64	2.51	2.53	1.50	6.63	0.93	4.37	1.94	4.98	9.76
18	16:1(n-5)	0.68	0.17	–	–	–	–	–	–	1.00	0.32	1.23	2.13
19	*iso*-17:0	–	–	3.27	1.10	2.69	1.51	0.86	0.84	–	–	–	–
20	*anteiso*-17:0	–	–	0.11	0.07	0.51	0.16	0.95	0.63	–	–	–	–
21	16:2(n-6)	0.78	0.03	0.26	0.04	0.29	0.04	0.05	–	0.16	0.06	0.06	0.40
22	17:0	0.53	0.52	0.31	0.47	1.28	1.84	1.27	0.93	1.38	1.91	1.13	1.07
23	*iso*-18:0	–	–	0.07	0.14	0.89	0.18	0.59	3.02	–	–	–	–
24	*anteiso*-18:0	–	–	0.01	0.01	0.12	0.04	0.04	0.09	–	–	–	–
25	16:4(n-3)	2.91	0.26	0.54	0.06	0.28	0.10	–	–	0.14	0.17	0.88	0.06
26	18:0	3.01	6.70	1.72	2.20	2.89	1.51	3.05	5.15	2.95	5.11	3.40	3.87
27	18:1 5	0.15	0.11	0.71	1.33	0.71	1.09	0.72	1.01	1.13	0.66	1.21	1.57
28	18:1(n-9)	0.91	0.29	5.37	1.15	1.46	0.76	7.75	1.76	7.03	1.86	3.56	1.48
29	18:1(n-7)	3.64	1.05	4.08	2.43	4.00	2.88	7.69	3.50	3.93	1.14	7.26	4.76
30	18:1(n-5)	0.37	0.05	0.30	0.25	0.51	0.34	0.37	0.02	0.02	0.20	0.70	0.32
31	*anteiso*-19:0	–	–	0.03	0.03	0.19	0.08	0.31	0.11	–	–	–	–
32	18:2(n-9)	0.61	0.07	1.71	0.69	0.35	0.20	0.90	0.16	0.10	–	0.06	0.26
33	18:2(n-6)	2.04	0.40	2.15	0.47	0.61	0.24	1.35	0.27	0.95	0.33	0.84	0.57
34	19:0	0.32	0.73	0.26	0.57	0.82	0.67	0.35	0.47	0.28	0.66	0.27	0.64
35	18:3(n-6)	1.12	0.16	–	–	–	–	0.44	–	0.36	0.10	–	–
36	*iso*-20:0	–	–	–	0.02	–	0.10	0.40	0.09	–	–	–	–
37	18:3(n-3)	3.86	0.82	1.27	0.29	0.67	0.16	1.41	0.43	0.97	0.37	0.33	0.16
38	18:4(n-3)	4.78	0.36	0.26	0.04	0.61	0.12	1.20	0.18	1.81	0.36	0.26	0.32
39	20:0	0.41	0.64	0.55	0.54	0.98	0.40	1.08	0.70	0.62	1.04	0.91	0.77
40	20:1 5	1.73	2.90	3.38	13.26	10.80	7.99	2.87	8.21	2.59	6.13	5.75	5.33
41	20:1(n-11)	0.43	0.75	0.93	–	2.51	1.05	2.33	1.47	1.36	3.76	1.24	0.64
42	20:1(n-9)	0.88	0.77	3.26	2.24	3.48	1.75	1.64	0.91	3.03	2.11	1.45	0.81
43	20:1(n-7)	0.55	0.39	1.07	0.67	2.12	0.93	1.25	0.76	0.95	0.68	0.77	0.57
44	20:2 5,11	2.04	2.92	5.64	8.86	4.78	9.33	1.88	4.93	3.23	3.91	3.06	5.05
45	20:2 5,13	0.37	0.31	2.98	3.30	2.13	2.89	0.76	0.96	0.48	0.31	0.71	1.12
46	20:2(n-9)	0.06	0.05	0.13	0.10	0.11	0.05	0.25	0.07	0.12	–	0.04	0.08
47	20:2(n-6)	1.00	1.08	2.07	3.92	2.29	1.75	0.91	1.39	0.80	1.02	–	0.44
48	20:3(n-9)	1.39	3.26	–	–	–	–	–	1.18	0.72	1.79	3.23	0.55
49	20:3 5,11,14	0.33	0.25	0.24	0.43	0.30	0.51	0.03	0.15	0.04	0.06	0.11	0.25
50	20:3(n-6)	0.94	0.65	1.11	–	0.51	0.18	0.58	0.23	0.31	0.56	0.15	0.21
51	20:4(n-6)	14.73	16.70	7.76	21.06	7.10	24.86	2.11	17.32	5.66	14.16	4.71	9.55
52	20:3(n-3)	–	0.04	0.59	0.91	1.01	0.73	0.26	0.62	0.04	0.06	0.04	0.07
53	20:4 5,11,14,17	0.95	0.19	0.08	0.09	0.12	0.06	0.01	0.01	0.01	0.01	0.02	0.05
54	20:4(n-3)	1.65	0.53	–	–	0.02	0.24	0.45	0.07	0.21	0.08	0.14	0.32
55	20:5(n-3)	9.99	18.12	4.14	8.43	3.82	9.38	5.53	21.03	4.93	7.18	12.78	16.07
56	22:1(n-11)	–	0.09	0.02	0.04	0.27	0.08	0.27	0.27	0.14	0.15	0.95	0.30
57	22:1(n-9)	0.51	0.79	0.06	2.14	2.04	1.41	1.94	1.77	0.91	1.00	0.92	0.55
58	22:1(n-7)	–	0.04	0.13	0.06	0.20	0.06	0.22	0.21	0.46	1.45	0.16	0.08
59	22:2 7,13	–	0.10	1.00	0.55	0.91	0.48	0.32	0.20	0.31	0.29	0.21	0.15
60	22:2 7,15	–	0.13	0.03	3.18	6.49	2.49	1.19	1.76	0.12	0.18	1.04	0.63
61	22:4(n-6)	0.25	0.41	0.02	0.24	0.16	0.32	0.21	0.12	0.34	0.35	0.18	0.32
62	22:5(n-6)	0.19	0.18	0.17	0.14	0.45	0.37	0.44	0.33	0.28	0.44	0.39	0.55
63	22:5(n-3)	0.64	0.50	0.99	0.71	0.66	0.57	0.70	0.30	0.34	0.30	0.67	0.84
64	22:6(n-3)	0.45	0.46	0.79	0.70	1.92	2.60	3.19	1.54	2.15	1.84	7.20	3.18
65	24:1(n-9)	–	–	0.06	–	–	–	–	–	–	–	0.23	0.09

desaturase in Echinoidea are a high activity which desaturates saturated fatty acids to the corresponding 5-monoenoic acids similarly to the meadowfoam seeds. ^{13}C-NMR analyses of fatty acid methyl esters from the sea urchin lipids did not show any occurrence of the 3-olefinic acids. Thus, it is hardly thought that the 5-olefinic acids are derived from C_2 elongation of the 3-olefinic acids formed by 3-desaturase.

Recently, fatty acid compositions for sea urchins and other marine invertebrates from the North Pacific have been reported (33). In that study, unusual 5- and 7-olefinic acids were not reported. It is believed that this is due to the low resolution of the packed column used in the GLC analysis. Open tubular GLC is a better tool for this purpose.

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Lipid Composition of the Membrane Released After an In Vitro Acrosome Reaction of Epididymal Boar Sperm

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Prior to fertilization, mammalian sperm must undergo the acrosome reaction, which involves modifications of the plasma and outer acrosomal membranes followed by vesiculation and release of the membranes. The membrane fraction that was released from caudal boar sperm undergoing an in vitro acrosome-like reaction was isolated and characterized with respect to density, marker enzymes and lipid composition. This membrane had a lower phospholipid/protein ratio (mg/mg) than the sperm plasma membrane, whereas both membranes had similar molar sterol/phospholipid ratios. The major phospholipid was sphingomyelin, followed by phosphatidylethanolamine and phosphatidylcholine, whereas in the plasma membrane the order was reversed; the two major phosphoglycerides contained alkylacyl and alkenylacyl species in addition to the diacyl species. The released membrane also contained lower amounts of cholesterol sulfate and unsaturated fatty acids than the plasma membranes. These results, in combination with our studies on the changes of the sperm membranes during maturation and acrosome reaction, will allow a better understanding of the mechanism of the sperm acrosome reaction.
Lipids 21, 566-570 (1986).

Mammalian spermatozoa develop in the testis and mature in the epididymis and then must go through the acrosome reaction before fertilization can occur. We have recently examined the changes in plasma membrane lipid composition for boar sperm during epididymal maturation (1). We are now studying the sperm lipid changes during the acrosome reaction.

The acrosome reaction involves a number of modifications of the outer acrosomal membrane and the overlying sperm plasma membrane, followed by vesiculation and eventual loss of the vesiculated membranes (2,3). Evidence for the modification of these sperm membranes has been obtained from biochemical and ultrastructural studies (2). Presumably the membrane lipids play an important role in the acrosome reaction, but the available data for changes in lipids are from either the analysis of whole sperm lipid extracts (4–7) or the effect of exogenously added lipids (8,9). Therefore, we have isolated the membranes released from the sperm during the in vitro acrosome-like reaction and have determined the changes in lipid composition during the reaction.

MATERIALS AND METHODS

Epididymides were obtained from freshly slaughtered boars (American Meat Packing Corp., Chicago, Illinois) and laboratory processing was initiated within 1 hr. The distal caudal segments of the epididymides were minced with a razor blade in Ca^{2+}-free Tyrode's solution. Sperm were decanted from the tissue, strained through a nylon mesh and suspended in Ca^{2+}-free Tyrode's solution to give a concentration of about 5×10^7 spermatozoa ml^{-1}. The Ca^{2+} concentration was adjusted to 1.8 mM, and fatty acid–free bovine serum albumin and chloramphenicol were added to final concentrations of 0.1% and 100 μg/ml, respectively. The suspension was incubated with shaking in a gyrorotatory shaker at 37 C for 5 hr. The suspension was then mixed on a Genie vortex for 1 min and centrifuged two times at 2,000 × g for 5 min. The combined supernatant fractions were centrifuged again at 6,000 × g for 10 min and then at 100,000 × g for 30 min. The membrane pellet was resuspended in 10 mM Hepes/1 mM EDTA (pH 7.5) and layered on a linear gradient of 0.77 M to 2.12 M sucrose in 10 mM Hepes (pH 7.5) before centrifugation to equilibrium at 304,000 × g for 16 hr. A single band was found at a density of 1.16 ± 0.01 g/cm^3 which was removed and washed twice with 10 mM Hepes/1 mM EDTA (pH 7.5) by centrifugation at 100,000 × g for 30 min.

Lipids were extracted as described by Cohen et al. (10). The recovery was estimated to be 90 ± 5% based on recovery of either [^{3}H]phosphatidylinositol or [^{3}H]cholesterol added at the beginning of the extraction. Lipids were fractionated and quantitated as described previously (1). All techniques were evaluated by the use of appropriate internal standards and solvent blanks were always run.

All enzymatic activities were measured as described (1). Protein concentration was measured after precipitation with cold 5% trichloroacetic acid by the method of Lowry et al. (11) using bovine serum albumin as the standard.

Materials. All sterol, fatty acid, phospholipid and neutral lipid standards were purchased from Supelco Inc. (Bellefonte, Pennsylvania). [7(n)-^{3}H)]Cholesterol (sp act 5 Ci/mmol) and L-3-phosphatidyl[2-^{3}H]inositol (sp act 10 Ci/mmol) were obtained from Amersham (Arlington Heights, Illinois). The gas liquid chromatography columns packed with SP-2330 on 100/120 mesh Chromosorb and SP-2250 on 100/120 Supelcoport were from Supelco Inc. Organic solvents were of ACS grade or better from Fischer Scientific (Chicago, Illinois). Substrates for enzyme assays, enzymes and other chemicals were from Sigma Chemical Co. (St. Louis, Missouri). Silica gel H was obtained from EM Reagents (EM Laboratories Inc., Cincinnati, Ohio).

RESULTS

Caudal boar sperm were incubated in Tyrode's solution containing 0.1% bovine serum albumin similar to conditions commonly used to induce in vitro acrosome reactions in several species of mammalian sperm (12). The oxygen uptake increased fivefold during the first 30 min of incubation and reached a plateau after 3 hr. The in-

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TABLE 1

Specific Activities of Enzymes in the Released Membrane[a]

Enzyme	Homogenate	Released membrane	Enrichment
Acid phosphatase	14 ± 1	143 ± 29	10
Alkaline phosphatase	70 ± 4	3446 ± 401	49
5′-Nucleotidase	6 ± 1	142 ± 12	24
N-Acetylglucosaminidase	12 ± 1	43 ± 10	4
Hyaluronidase	7 ± 1	4 ± 1	0.6
Na^+/K^+-ATPase	1 ± 0.1	—[b]	0
Succinic acid dehydrogenase	30 ± 1	—	0

[a]Numbers represent nmol/min per mg protein. Each value is mean ± SE of four determinations. The assays were conducted as described (1). Homogenate is the sperm suspension following disruption by nitrogen cavitation.
[b]Not detectable.

creased oxygen uptake was accompanied by an increase in the sperm hyperactivated motility to >90% within 30 min of incubation (13) and remained activated over 5 hr. These data agree with the hyperactivation observed in other sperm species during in vitro capacitation and acrosome reactions (14,15). Also, two acrosomal enzyme activities, hyaluronidase and esterase, were released into the incubation medium. This release represented 70–90% of the total activities and agrees with previous observations by Yanagimachi (2,14) and Zao et al. (15). Finally, our preliminary observations by phase contrast microscopy indicated that about 70% of the sperm had gone through the acrosome-like reaction. Changes in the sperm morphology similar to those described for the boar sperm acrosome reaction (17,18) were observed by electron microscopy; these data will be presented as part of another study.

During the acrosome-like reaction, the amount of membrane released from the actively motile sperm reached a plateau near 5 hr. These released membranes were purified by previously described techniques (1) and found to have a density of 1.16 ± 0.01 g/cm^3. The membranes were analyzed for marker enzymes as shown in Table 1; they were enriched in phosphatase activities such as acid phosphatase (E.C. 3.1.3.2), alkaline phosphatase (E.C. 3.1.3.1) and 5′-nucleotidase (E.C. 3.1.3.5). The membranes were also enriched in N-acetylglucosaminidase activity (E.C. 3.2.1.30) and contained some hyaluronidase (E.C. 3.2.1.35) but no Na^+/K^+-ATPase (E.C. 6.3.1.3) or succinic acid dehydrogenase (E.C. 1.3.99.1) activities.

Table 2 shows the lipid composition of the released membranes. Phospholipids comprised 67% of the total membrane lipid; sterols were the second major class, comprising 17% of the lipid. The minor lipids in the fused membrane were diacylglycerols (DAG), a glycolipid characterized in previous studies (1) as monogalactosyl diglyceride sulfate and free fatty acids (FFA). The molar ratio of sterol to phospholipid and the weight ratio of phospholipid to protein of the membrane were 0.26 and 0.52, respectively. This lipid composition is similar to that which we reported for the plasma membranes (1). However, among the individual phospholipid classes, the major species were sphingomyelin (Sph; 32% of total), phosphatidylethanolamine (PE; 30%) and phosphatidylcholine (PC; 25%) (Table 3), whereas in the plasma mem-

TABLE 2

Lipid Composition of the Released Membrane[a]

Lipid class	nmol/mg Protein	Mol %
Phospholipid	699 ± 65	68
Sterols	180 ± 8	17
Diacylglycerols	85 ± 10	8
Glycolipid	46 ± 5	4
Free fatty acids	26 ± 4	3
Total	1037 ± 92	100.0
Phospholipid ÷ protein (mg/mg)[b]	0.52 ± 0.03	
Sterol ÷ phospholipid (mol/mol)	0.26 ± 0.03	

[a]Released membranes were isolated and lipids extracted as described in the text. Lipids were fractionated and quantitated as described previously (1). Each value is the mean ± SE of four determinations.
[b]A factor of 750 mg/mmol was used for conversion of μmol of lipid phosphate to mg of phospholipid. This factor is the weighted average of the mol wts of the individual phospholipids in the membrane as determined according to their relative abundance.

TABLE 3

Phospholipid Composition of Released Membrane[a]

Phospholipid	Mol %
Sphingomyelin	31.1 ± 0.2
Phosphatidylethanolamine	28.9 ± 0.2
Diacyl	16.2 ± 0.2
Alkylacyl	7.1 ± 0.2
Alkenylacyl	5.7 ± 0.1
Phosphatidylcholine	26.5 ± 0.3
Diacyl	8.0 ± 0.1
Alkylacyl	11.1 ± 0.3
Alkenylacyl	7.4 ± 0.2
Phosphatidylserine	9.0 ± 0.3
Phosphatidylinositol	3.0 ± 0.4
Lysophosphatidylcholine	1.3 ± 0.4
Phosphatidylinositol-4′-phosphate	trace
Phosphatidylinositol-4′,5′-biphosphate	trace

[a]Phospholipids were separated by two-dimensional TLC and the phospholipid species quantitated as described (1). Each value is mean ± SE of four to six determinations.

brane these species were 23%, 28% and 40%, respectively. For PE the diacyl species were the major form, followed by the alkylacyl and alkenylacyl species, while in PC, the major species were the alkylacyl, followed by the alkenylacyl and the diacyl forms. The total amount of ether phospholipids in the released membrane was 35% of the total phospholipid. Finally, the released membrane contained phosphatidylserine (PS; 9%), phosphatidylinositol (PI; 3%) and lysophosphatidylcholine (10%); polyphosphoinositides were barely detectable (< 0.1%).

The released membranes contained three sterol species (Table 4). Cholesterol was the major sterol (85%) and desmosterol and cholesterol sulfate were present at 14% and 1%, respectively.

The fatty acid composition of the whole membrane, each of the major phospholipids, DAG and FFA is shown in Table 5. A common characteristic in the fatty acid composition of the above lipids was a high percentage of saturated fatty acid, ranging from 58% in PE to 99% in Sph. The major saturated fatty acids found in the total lipid extract of the released membrane were 16:0, 18:0 and 14:0, whereas the major unsaturated fatty acids were 22:5, 18:1 and 22:6. PE, PC and PS generally had the same fatty acids (with a few exceptions like 20:0) as the whole membrane described above, although the molar ratios were not identical. However, the fatty acid composition of Sph was significantly different from the whole membrane primarily in 20:0 (53%), 16:0 (19%) and 18:0 (10.0%). PI contained only six fatty acids, four saturated (16:0, 18:0, 14:0 and 20:0) and two unsaturated (18:1 and 20:4). The fatty acid composition of the membrane diacylglycerols did not resemble the total membrane or any phospholipid since there were unusually high amounts of 14:0 (56%) and low amounts of 18:0 (5%) and 18:1 (2%). Finally, the major FFA of the membrane were the saturated 16:0, 18:0 and 14:0; the unsaturated 18:1 and 22:4 were found in lower amounts.

DISCUSSION

During the sperm acrosome reaction, the plasma membrane and the outer membrane of the acrosome vesiculate, resulting in the formation and release of membrane vesicles and much of the acrosomal contents. In this report, we incubated boar caudal sperm under conditions for an in vitro acrosome-like reaction, and five hours after the incubation we isolated the released membranes by differential and sucrose density centrifugation. It should be noted that we also isolated and analyzed the released membranes after 2 and 4 hr of incubation and found the same results as reported above.

The released membranes had a density of 1.16 ± 0.01 g/cm^3, denser than the purified plasma membrane (1.13 g/cm^3) we reported previously (1). The released membranes were enriched for several enzymatic activities usually associated with the acrosomes (2,19) and the plasma membrane (1), such as acid and alkaline phosphatases, 5′-nucleotidase and N-acetylglucosaminidase; this would be expected if the membrane were a hybrid of the plasma and the acrosomal membrane. The membrane was not enriched in hyaluronidase activity, which agrees both with data from this laboratory showing re-

TABLE 4

Sterol Composition of the Released Membrane[a]

Sterol	nmol/mg Protein	Mol %
Cholesterol	154 ± 4.2	85.5
Desmosterol	24.7 ± 5.4	13.7
Cholesterol sulfate	1.4 ± 0.5	0.8
Total	180.7 ± 8.5	100.0

[a]Amount of each sterol was determined after TLC separation of the lipid extract and GLC of the isolated sterol as described previously (1). Results are expressed in mean ± SE, n = 4.

TABLE 5

Fatty Acid Composition of the Membrane and Membrane Lipids[a]

	Composition (mol %)[a]							
Fatty acid	Total membrane	Sph	PE	PC	PS	PI	DAG	FFA
14:0	11.0 ± 1.0	6.1 ± 1.0	8.7 ± 2.9	11.4 ± 2.5	6.1 ± 0.5	11.5 ± 3.4	55.6 ± 3.8	21.4 ± 2.0
16:0	33.7 ± 0.8	18.9 ± 4.1	36.5 ± 3.8	41.5 ± 2.2	35.5 ± 1.0	35.8 ± 9.3	36.8 ± 3.2	40.3 ± 4.2
18:0	14.3 ± 0.3	10.0 ± 1.5	13.0 ± 8.5	14.6 ± 2.3	23.7 ± 1.4	28.8 ± 3.6	5.3 ± 1.6	26.3 ± 6.8
18:1	7.9 ± 0.5	—	9.9 ± 0.1	7.8 ± 1.5	15.7 ± 1.0	10.2 ± 1.1	2.4 ± 1.9	7.0 ± 0.7
18:2	2.3 ± 1.4	—	3.3 ± 1.4	2.6 ± 0.6	3.0 ± 0.3	—	—	—
18:3	—	—	—	0.7 ± 0.4	1.5 ± 0.5	—	—	—
20:0	7.9 ± 2.5	52.7 ± 6.1	—	—	—	7.8 ± 3.1	—	—
20:3	0.5 ± 0.3	—	1.3 ± 0.9	—	—	—	—	—
20:4	3.3 ± 0.5	—	6.2 ± 1.8	0.9 ± 0.6	—	5.9 ± 3.3	—	—
22:0	1.3 ± 0.5	7.6 ± 1.0	—	—	—	—	—	—
22:4	1.4 ± 1.8	—	1.3 ± 0.6	1.3 ± 0.5	3.0 ± 0.7	—	—	5.0 ± 2.5
22:5	10.0 ± 2.8	0.9 ± 0.5	13.7 ± 2.0	16.7 ± 1.7	11.4 ± 2.8	—	—	—
22:6	5.8 ± 2.3	—	6.0 ± 3.8	2.5 ± 1.2	—	—	—	—
24:0	0.6 ± 0.3	3.7 ± 2.5	—	—	—	—	—	—
% Saturated	68.8 ± 14.3	99.0 ± 16.9	58.2 ± 9.2	67.5 ± 7.0	65.3 ± 2.9	76.1 ± 19.4	97.7 ± 11.5	88.0 ± 13.0

[a]The lipids were transesterified and the resulting fatty acid methyl esters were analyzed as described previously (1). Each value is the mean ± SE of 4 determinations. Sph, sphingomyelin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; DAG, diacylglycerol; FFA, free fatty acid.

lease of the hyaluronidase into the incubation medium during this in vitro acrosome-like reaction and with other reports that localized hyaluronidase in the acrosomal matrix (20–22) and the inner acrosomal membrane (21). The absence of succinic acid dehydrogenase activity from the membrane suggests no mitochondrial contamination, whereas the absence of Na^+/K^+-ATPase activity may indicate that this enzyme, which was present in the plasma membrane (1), was degraded during the acrosome reaction. Alternatively, the ATPase might be present in the membrane in an inactive form. It is known that membrane-bound enzymes are sensitive to changes in the membrane lipid composition (23,24). Specifically, Bruni et al. (25) reported that Sph inhibited calf heart Na^+/K^+- ATPase. Also, Emmelot and Bos (20) showed that the ATPase activity of rat liver plasma membrane required unsaturated fatty acids for maximal activity. As discussed below, the released membrane contained lower amounts of unsaturated fatty acids and higher amounts of Sph than the plasma membrane, which could have inhibited the ATPase.

The lipid composition of the fused membrane (Tables 2–4) was qualitatively similar to the lipid composition of the plasma membranes of boar (1) and ram (27). However, the fused membrane had some unique features in its lipid composition, namely a low phospholipid/protein ratio, lower amounts of ether phospholipids, polyphosphoinositides, cholesterol sulfate and unsaturated fatty acids, and higher amounts of Sph relative to the plasma membranes. Ether phospholipids, polyphosphoinositides, cholesterol sulfate and 22:5 and 22:6 unsaturated fatty acids are common components of the mammalian sperm (2,4,28,29) and sperm plasma membranes (1,27). We and others have shown that the relative amounts of these lipids increased during sperm maturation in the epididymis (1,2,7,27), suggesting a role for these lipids in the sperm membrane function, possibly during the capacitation and acrosome reaction process. The low amounts of the above lipids in the released membrane may be related to the fact that it is the product of the capacitation and acrosome-like reactions, and these lipids may have a role during these events.

Sph was the major phospholipid in the released membrane, followed by PE and PC, whereas in sperm plasma membrane (1) and whole sperm (28) PC was the major phospholipid, followed by PE and Sph. Sph and PC are both choline phospholipids, which seem to replace each other in biological membranes (30) and apparently in sperm membranes during the acrosome-like reaction, as shown above. Although both are classified as nonsoluble swelling amphipaths (31) and serve as the matrix of a biological membrane, certain differences in their physical properties exist (for review, see 32). Sph has phase transition temperatures in the physiological temperature range, whereas all naturally occurring PC have transition temperatures below 37 C. Furthermore, increasing Sph content results in increases in the apparent microviscosity of model membrane bilayers. Finally, Sph has increased capabilities for hydrogen bonding, which influences its interaction with membrane sterols and protein and possibly passive transport processes.

Our study shows that the released membrane, the product of the in vitro acrosome-like reaction, has a different lipid composition than the plasma membrane. This might indicate that the lipid composition of the released membrane reflects the additive composition of the outer acrosomal and plasma membrane. The possibility can be tested by measuring the lipid composition of the outer acrosomal membrane; this work is in progress. Alternatively, the lipid composition of the released membrane might be different from the additive composition of the plasma and outer acrosomal membrane, which would suggest that the mechanism of the acrosome reaction is not a simple fusion of two membranes.

In summary, our previous studies on the modulation of the lipid composition of the sperm plasma membrane during the acrosome reaction have been extended by this report on the lipid composition of the membrane released after an in vitro acrosome-like reaction. Presumably, these results in combination with the lipid analysis of the acrosomal membrane, will result in a better understanding of the mechanism of the acrosome reaction of mammalian sperm.

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Rat Hepatocyte Plasma Membrane Acyl:CoA Synthetase Activity

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The presence of long chain acyl:CoA synthetases in mammalian microsomes and mitochondria has been established previously by Aas (*Biochim. Biophys. Acta 231*, 32-47 [1971]). The presence of a plasma membrane-associated enzyme was investigated in rat hepatocyte plasma membranes, where an enzyme exhibiting high activity and with a preferred substrate of 18-carbon chain length was discovered. The results are consistent with the presence of a single enzyme. The effect of the degree of unsaturation of the fatty acid substrates was not as pronounced as that arising from the length of the carbon chain. The pattern of substrate preference of the enzyme was ω3 polyenoic fatty acids > ω6 polyenoic acids > ω9 monoenoic acids > saturated acids. This may relate to the similar substrate preference pattern exhibited by the fatty acyl desaturase enzymes. The role played by long chain acyl:CoA synthetase in hepatocyte metabolism is uncertain, but it may relate to the incorporation of polyenoic fatty acids from the circulation into cell membranes and the trapping of other fatty acids within the cell for further metabolism. *Lipids 21,* 571-574 (1986).

The exact role of the plasma membrane long chain acyl:CoA synthetase is not clear. It may be associated with a mechanism whereby fatty acids are transported across the membranes (1). It may also provide a means of retention of fatty acids within the cell, as the plasma membrane is permeable to free fatty acids but not to acyl:CoA.

The requirement of the coenzyme A (CoA) thiol esters of fatty acids has long been established (2), and the metabolism of fatty acids is primarily that of these "activated" forms. The enzymes responsible for this activation step are the acyl:CoA synthetases (fatty acid:CoA ligases, E.C. 6.2.1.1-3), which can be subdivided into two classes depending on their requirement for specific nucleotide triphosphates (3). The most metabolically active classes are the acyl:CoA synthetase (AMP-forming) enzymes, which require ATP (4). There are three enzyme species within this class relating to fatty acid substrate specificity. Short chain acyl:CoA synthetases (E.C. 6.2.1.1) exhibit the highest activity with acetate as substrate (5), while medium chain acyl:CoA synthetases (E.C. 6.2.1.2) have a preferred substrate of 7:0 chain length (6). The long chain acyl:CoA synthetase enzymes (E.C. 6.2.1.3) exhibit a much broader range of activity, from 6:0 to 24:0 (7); however, the preferred chain lengths are reported as 12:0 to 16:0 (8). In all tissues examined to date, the highest activity of the mitochondrial enzyme has been with 16:0 as substrate (9), while both 12:0 and 16:0 have been reported as the preferred substrates of the microsomal fractions (10). Until recently, all work has concentrated on these subcellular organelles (10-14).

A putative role for a plasma membrane-bound or plasma membrane-associated long chain acyl:CoA synthetase has been postulated previously (15,16). This possibility was investigated in rat hepatocyte plasma membranes, and high levels of activity were obtained. The pattern of activation rate (ω3 > ω6 > ω9 > saturated) was the same as that exhibited by human erythrocytes (17), but the rates of activation by hepatocyte plasma membranes were ca. three times higher. Marker enzyme assays (performed to check membrane purity) proved negative for microsomal and mitochondrial contamination. The enzyme from this source activated saturated fatty acids, fatty acids with increasing degrees of unsaturation and fatty acids from different polyenoic families. The chain lengths of the saturated fatty acids ranged from 12:0 to 24:0, of the monoenoics from 16:1ω9 to 24:1ω9 and of the polyenoics from 18:2ω6 to 22:6ω3. The dependence of the plasma membrane long chain acyl:CoA synthetase on the presence of ATP has been demonstrated previously (18).

MATERIALS AND METHODS

The hepatocyte plasma membranes were prepared according to the method of Neville (19) as modified by Emmelot et al. (20). Protein was assayed by the Bio-Rad procedure. Acyl:CoA synthetase activity was assayed by the method of Polokoff and Bell (12) as modified by Murphy and Spence (13), in which fatty acid substrates at 50 μM concentration were incubated with 10 mM ATP, 8 mM Mg^{2+}, 50 μM ^{3}H-CoASH and 50 μg plasma membrane protein. Incubations were terminated after 5 min by the rapid addition of ice-cold 0.5 M TCA and were filtered through Millipore HAWP filters.

It has been shown that neither CoASH nor free fatty acid will adhere to the filters used in the assay; thus any radioactivity associated with the filters is attributable to acyl:CoA (12,13). All fatty acids were obtained from Sigma Chemical Co (St. Louis, Missouri). The ^{3}H-CoASH (sp. act. 0.547 Ci/mol) was obtained from New England Nuclear (Boston, Massachusetts). The HAWP filters required were supplied by Millipore Corp. (Sandton, South Africa) and the Aquagel scintillant cocktail by Chemlab (Sandton, South Africa).

RESULTS

Marker enzyme assays (5′-mononucleotidase for plasma membranes, NADPH-cytochrome c reductase and glucose-6-phosphatase for microsomes, and cytochrome c oxidase and monoamine oxidase for mitochondria) demonstrated the presence of plasma membranes and the lack of contamination of this fraction by microsomes or mitochondria. Varying the concentration of fatty acid substrate did not affect the rate of the reaction. The optimum incubation time and protein concentration determined were identical to those reported elsewhere (12-14).

Table 1 shows the levels of activation with the 12 to 24 saturated fatty acids as substrates. The substrate exhibiting the maximal rate of activation was stearic acid (18:0) (32.38 nmol/min/mg protein), and the activation profile of all the saturated substrates approximated a flattened bell-shaped curve. This

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TABLE 1

Comparison of the Maximal Rates of Activation of 22 Fatty Acids with the Long Chain Acyl:CoA Synthetase of Rat Hepatocytes Relative to 16:0

	Degree of unsaturation							
Chain length	0	1ω9	2ω6	3ω6	3ω3	4ω6	5ω3	6ω3
12	24.75 ± 0.52[a]							
14	29.47 ± 0.46[a]							
16	30.88 ± 0.33	31.74 ± 0.36[a]						
17	31.12 ± 0.62[b]							
18	32.38 ± 0.46[a]	33.94 ± 0.41[a]	34.38 ± 0.42[a]	34.92 ± 0.33[a]	35.41 ± 0.38[a]			
20	31.66 ± 0.42[a]	32.24 ± 0.40[a]	32.64 ± 0.28[a]	33.02 ± 0.41[a]	33.51 ± 0.27[a]	28.75 ± 0.41[a]	29.86 ± 0.31[a]	
22	29.28 ± 0.41[a]	31.03 ± 0.27[b]						27.52 ± 0.53[a]
24	27.28 ± 0.38[a]	28.05 ± 0.59[a]						

All the t-tests calculated relative to 16:0 (palmitic acid). Results obtained are expressed as nmol/min/mg protein of fatty acyl:CoA produced.
[a] $p < 0.005$.
[b] $p < 0.1$.

probably indicates the presence of only one enzyme. The maximal activation of the monoenoic substrates was again with the 18-carbon moiety, oleic acid (*cis*-18:1ω9) (33.94 nmol/min/mg protein). All the monoenoic substrates exhibited enhanced activation relative to their saturated counterparts. Table 1 also depicts the activation of the ω6 polyenoic fatty acids, i.e., linoleic, γ-linolenic, dihomolinoleic, dihomo-γ-linolenic and arachidonic acids (*cis*-18:2ω6, *cis*-18:3ω6, *cis*-20:2ω6, *cis*-20:3ω6 and *cis*-20:4ω6, respectively). There were slight differences between the two 18-carbon moieties (34.38 and 34.92 nmol/min/mg protein, with 18:2ω6 and 18:3ω6, respectively), and between the three 20-carbon compounds (32.64, 33.02 and 28.75 nmol/min/mg protein, with 20:2ω6, 20:3ω6 and 20:4ω6, respectively); however, the differences were not statistically significant, except for arachidonic acid. Within the ω6 polyenoic fatty acids, the only statistically significant differences related to chain length (34.38 and 34.92, 18 carbons, compared to 32.64, 33.02 and 28.75, 20 carbons) ($p < 0.005$). The rates of activation of the ω3 polyenoic fatty acids are shown (α-linolenic, dihomo-α-linolenic, eicosapentaenoic and docosahexaenoic acids; *cis*-18:3ω3, *cis*-20:3ω3, *cis*-20:5ω3 and *cis*-6ω3, respectively), and these exhibited a very similar pattern to that of the ω6 polyenoic fatty acids. The difference between the rates of activation of the two 20-carbon fatty acids was significant (33.51 and 29.86 nmol/min/mg protein for 20:3ω3, respectively) ($p < 0.005$); however, they were not consecutive members of the ω3 family of compounds, and thus the difference may not reflect a "real" effect. The differences in activation rate related to chain length among the ω3 polyenoics were again highly significant ($p < 0.005$) (35.41 nmol/min/mg protein for 18:3ω3 vs 33.51 nmol/min/mg protein for 20:3ω3; 29.86 nmol/min/mg protein for 20:5ω3 vs 27.52 nmol/min/mg protein for 22:6ω3).

Table 2 illustrates the influence of both degree of unsaturation and position of unsaturation on the activity of the enzyme with 18-carbon fatty acids. The order was α-linolenic > γ-linolenic > linoleic > oleic > stearic; all differences were significant ($p < 0.005$) except between γ-linolenic and linoleic (35.41, 34.92, 34.38, 33.94 and 32.38 nmol/min/mg protein, respectively). The activation rates of the 20-carbon fatty acids are also shown in Table 2. The trend is similar to the rates of activation seen with the 18-carbon substrates. When one considers the chain-elongated homologues of the 18-carbon compounds, the order of activation was dihomo-α-linolenic > dihomo-γ-linolenic > dihomolinoleic > dihomoleic > arachidic (33.51, 33.02, 32.64, 32.24 and 31.66 nmol/min/mg protein); all differences were significant ($p < 0.005$) except between dihomo-γ-linolenic and dihomolinoleic. The two most unsaturated fatty acids, arachidonic and eicosapentaenoic, showed lower rates of activation (28.75 and 29.86 nmol/min/mg protein, respectively) relative to their precursors, dihomo-α-linolenic and dihomo-γ-linolenic acids (33.51 and 33.02 nmol/min/mg protein, respectively). The highest rate of activation (33.51 nmol/min/mg protein) was, once more, exhibited by the trienoic ω3 compound.

DISCUSSION

The possibility of plasma membrane-linked long chain acyl:CoA synthetases has been proposed previously (15,16). The results obtained clearly indicate the presence of such a long chain acyl:CoA synthetase in rat heptocyte plasma membranes. The activities are such as

TABLE 2

Comparison of the Maximal Rates of Activation of All the 18- and 20-Carbon Fatty Acids with the Long Chain Acyl:CoA Synthetase of Rat Hepatocytes Relative to the Saturated Fatty Acid at Each Chain Length

	Degree of unsaturation						
Chain length	0	1ω9	1ω6	3ω6	3ω3	4ω6	5ω3
18	32.38 ± 0.46	33.94 ± 0.42[a]	34.38 ± 0.42[a]	34.92 ± 0.33[a]	35.41 ± 0.38[a]		
20	31.66 ± 0.42	32.24 ± 0.40[a]	32.64 ± 0.28[a]	33.02 ± 0.41[a]	33.51 ± 0.27[a]	28.75 ± 0.40[a]	29.86 ± 0.31[a]

All t-tests calculated relative to the saturated fatty acid for each chain length.
[a]$p < 0.005$.

to compare favorably with results on other tissue plasma membranes (Davidson, B.C., and Cantrill, R.C., in press) (14). Recent work has demonstrated the existence of an erythrocyte long chain acyl:CoA synthetase in humans (17), and it was shown that erythrocyte ghosts resealed with ATP and CoASH inside exhibited palmitoyl:CoA synthetase activity, that the enzyme was located on the inside of the membrane and that it exhibited a requirement for both ATP and CoASH (18).

The preferred substrates in microsomes were lauric (12:0) and palmitic acids (16:0) (21), while that of mitochondria was palmitic acid (16:0) (22). The plasma membrane enzyme, however, exhibited maximal activity with the 18-carbon substrates, and this increased with the degree of unsaturation of the fatty acid. The higher activity with other substrates may reflect the requirement for the incorporation of these moieties within the phosphoglyceride structures of the membrane. The activity cascade of the enzyme (ω3 > ω6 > ω9 > saturated) indicates a definite preference for the ω3-series polyenoic fatty acids relative to the ω6, and these to the ω9 and the saturated. This follows exactly the substrate preference pattern exhibited by Δ6-desaturase (23), and possibly Δ5 and Δ4-desaturase as well. It is possible, therefore, that the pattern of substrate preference of the plasma membrane long chain acyl:CoA synthetases and the desaturases are related in some way.

Morand et al. (24–26) have shown, using fluorescent derivatives of saturated fatty acids, that the process of incorporation appears to occur in four stages: initial incorporation into a freely exchangeable outer membrane pool, transfer to a nonexchangeable inner pool, activation and addition onto lysophosphatidylcholines, and transesterification to cytosolic triacylglycerols. They showed that the position of substitution of the fluorescent complex onto the fatty acid chain influenced how far the substituted fatty acid could be metabolized by the above process. If the group was substituted at or close to the carboxyl group, the fatty acid did not appear even to associate with the fatty acid pool of the membrane outer layer. If the group was substituted in approximately the middle of the molecule, the fatty acid could be partially processed as far as the inner membrane pool. Finally, however, if the substitution occurred at or close to the methyl terminal carbon atom, then the fatty acid could be processed as far as inclusion into both phosphoglycerides and triacylglycerols. The rate of incorporation of [^{14}C]-labeled palmitic acid into intact parenchymal cells has been measured in the rat (27). This occurred very rapidly (within 5 min). After ca. 10 min, most of the radiolabeled fatty acid had been transesterified to the cytosolic triacylglycerols. These results illustrate the primary importance of the plasma membrane long chain acyl:CoA synthetase for the incorporation of circulating fatty acids into the intracellular environment of liver cells and the very rapid nature of this process. The results also demonstrate the intermediate role played by lysophosphoglycerides in this mechanism, and thus the membrane structure can be influenced by activation of fatty acids in situ.

The results obtained in this study demonstrate the ability of rat hepatocyte plasma membranes to trap fatty acids from the circulation by formation of CoA derivatives, which may be one mechanism whereby elevated levels of specific fatty acids are reduced. The trapping of dietarily derived and circulation fatty acids allows for the modification of these fatty acids, e.g., by the processes of desaturation and chain elongation. This may provide substrates suitable for further metabolism by other tissues as well as the liver.

The enzyme may have other functions within the hepatocyte membrane. It may fulfill the role of supplying activated fatty acids for the membrane phosphoglycerides and thus help maintain the fluidity and integrity of those membranes. Alternatively, the enzyme may provide suitably activated fatty acids as substrates for the mitochondrial carnitine:acyl transferase enzymes, and thus β-oxidation. The importance of 18-carbon fatty acids as major components of membranes, significant energy sources and progenitors of the eicosanoid precursors (dihomo-γ-linolenic, arachidonic and eicosapentaenoic acids) may explain the higher activation rates of these fatty acids by the hepatocyte enzyme. Thus, the enzyme may act as a selective mechanism whereby polyenoic fatty acids of specific chain lengths and degrees of unsaturation are preferentially removed from circulation and retained within the liver cell. Whether they are for incorporation into cellular structures, further metabolism and/or subsequent export to the body may depend on the general lipid status of the body at any particular time.

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A Hydrophilic Bile Acid Effects Partial Dissolution of Cholesterol Gallstones in the Prairie Dog

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Gallstone formation and dissolution were studied in a prairie dog model of cholesterol (CH) cholelithiasis. Gallstones were induced in 49 prairie dogs by feeding 1.2% CH in a nutritionally adequate semisynthetic diet for 6 wk (period 1). At 6 wk, gallstones had developed in all animals examined. The diets were modified by reducing the amounts of CH to 0.4, 0.2, 0.1 and 0.0% (diets 1–4); hyodeoxycholic acid (HDA; 30 mg/kg/day) was added to these diets (diets 5–8). All animals were fed the modified experimental diets for an additional 8 wk (period 2). At week 14, spontaneous gallstone dissolution had not occurred, even in the groups given no added dietary CH during period 2 (group 4). Addition of HDA to the diet tended to reduce the incidence of biliary CH crystals and the size and number of CH gallstones. Biliary CH remained elevated and the lithogenic indices in all groups were found to be greater than 1.0 at the end of the experiment. Liver and plasma CH levels tended to be lower in the groups fed HDA. In these groups, HDA and 6βHDA became the major biliary bile acids. This study demonstrates that HDA achieved partial dissolution of gallstones in bile supersaturated with CH.
Lipids 21, 575–579 (1986).

The prevention of cholesterol (CH) gallstones has been reported in several animal models (hamsters, 1–3; prairie dogs, 4,5) as well as in man (6). Dissolution of established CH gallstones has been studied extensively in man using bile acid therapy (chenodeoxycholic acid and ursodeoxycholic acid) (7–10). In the National Cooperative Gallstone Study (NCGS) and a Canadian study, the success rates of dissolution with chenodeoxycholic acid (maximum dose 750 mg/day) have been only about 15%, making the development of newer gallstone-dissolving agents (possibly another bile acid or bile acid derivative) highly desirable (7).

Our laboratory has studied the biochemical and physiological effects of a hydrophilic bile acid, hyodeoxycholic acid (HDA), on CH cholelithiasis in animal models (2,5). HDA was shown to prevent gallstones in the hamster in the presence of supersaturated bile. The bile, abnormal in color and appearance to transmitted light, was found to contain CH in a liquid crystalline state (2). The hydrophilic bile acid was able to maintain CH in "solution" and prevent the phase transition from liquid crystal to solid CH monohydrate. Since the hamsters were fed a fatty acid–deficient diet which was "not physiologic," we examined the prairie dog model of CH cholelithiasis and found that HDA could also prevent stones when fed with CH in a nutritionally adequate diet (5). In the prairie dog, gallstone formation is dependent upon the dose of CH in the diet (i.e., stones form in 2–4 wk with 1.2% CH, 8 wk with 0.4% CH and 6 mo with 0.2% CH) (4). In theory, the bile acids currently used to dissolve gallstones do so by decreasing hepatic CH secretion and/or altering the CH/bile acid ratio in bile. In addition, as suggested by Carey, a hydrophilic bile acid should be able to dissolve CH gallstones via the formation of liquid crystalline vesicles (11,12).

Recently, the mechanism of action of gallstone dissolution was described for biles rich in chenodeoxycholic acid and ursodeoxycholic acid (13). These results suggested that dissolution with ursodeoxycholic acid was associated with development of a CH-phospholipid layer on the stone surface followed by dispersion of the layer into liquid crystalline vesicles (13). With chenodeoxycholic acid, micelle formation was the only possible mode of action for gallstone dissolution. In our prairie dog model, HDA was more effective than ursodeoxycholic acid for gallstone prevention, presumably due to its ability to form a liquid crystalline phase. Consequently, a dissolution study using this bile acid seemed warranted.

MATERIALS AND METHODS

Male prairie dogs (*Cynomys ludovicianus*, trapped in the wild) were purchased from Otto Martin Locke (Braunfels, Texas). The animals were quarantined for 2 wk in individual rabbit cages, during which time they received Purina rat chow and water ad libitum. The 49 animals were then weighed and the diet was changed to a semisynthetic diet (SSD) + 1.2% CH (Teklad, Madison, Wisconsin). The diet consisted of sucrose, 56.5%; cornstarch, 13.9%; soy protein, 20.2%; corn oil, 1.6%; cellulose 2.6%; and mineral mix (Teklad 40060), 1%. CH was incorporated into the diet as egg yolk (0.6%) and crystalline CH (0.6%). This diet was fed to all animals for 6 wk (period 1). At wk 6, five animals (group 0) were killed to determine the incidence of CH gallstones and CH crystals. As expected, incidence was 100% (see Results). The remaining 44 animals were divided into eight groups (minimum five animals/group) and given the following diets: group 1, SSD + 0.4% CH; group 2, SSD + 0.2% CH; group 3, SSD + 0.1% CH; group 4, SSD + 0.0% CH; group 5, SSD + 0.4% CH + HDA (30 mg/kg/day); group 6, SSD + 0.2% CH + HDA; group 7, SSD + 0.1% CH + HDA; group 8, SSD +0.0% CH + HDA. These diets were administered for an additional 8 wk (period 2) (total feeding period 14 wk). During the 24 hr prior to termination of the experiment, the animals were starved to assure adequate quantities of bile in the gallbladder for biliary lipid analyses. The animals were anesthetized with 100 mg of ketamine hydrochloride (Bristol Labs, Syracuse, New York) and 20 mg of xylazine (Haver-Lockhart, Shawnee, Kansas); they then were exsanguinated, and blood was collected for determination of serum cholesterol. The gallbladder was removed and bile aspirated with a 50 μl Hamilton syringe. The fresh bile and the gallbladder contents were

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examined by polarized light microscopy (Olympus MCHAP microscope, Olympus Corp., Lake Success, New York) to determine the presence of CH crystals, liquid crystals and CH gallstones. The liver was excised and weighed, and aliquots were removed for CH determination and preparation of microsomes.

Thin layer chromatography. To determine the pattern of bile acid conjugation, 0.5 μl of bile was applied directly to 0.25 mm Silica Gel G Plates (Analtech, Newark, Delaware). The developing solvent was n-butanol/acetic acid/water (17:2:1, v/v/v).

Gas liquid chromatography (GLC) and GLC-mass spectrometry. GLC of CH in liver, plasma and bile was carried out as described earlier (5). Biliary bile acids were analyzed after preparation of their methyl ester acetates on 2% OV-210 and 0.5% SP-525 (5). Positive identification of the bile acids was made in selective cases using a Hewlett-Packard 5992B mass spectrometer with the following conditions: 4 ft glass column, 2 mm ID, 4 mm OD, packed with 3% SP 2250 on 100/120 mesh Supelcoport; column temperature 260 C; injector temperature 265 C; source pressure 2×10^{-6} torr; source temperature 140 C.

Reference compounds. HDA (Sigma Chemical Co., St. Louis, Missouri) was analyzed as its methyl ester trimethylsilylether derivative on an SE-30 column and found to be greater than 97% pure. 3α,7α-Dihydroxy-12-keto-5β-cholanoic acid (Steraloids, Wilton, New Hampshire) was used as a recovery standard for determination of biliary bile acids.

Biliary lipid composition. Gallbladder bile obtained at sacrifice was centrifuged at 2000 g for 10 min, and aliquots were used for determination of the three biliary lipids. The lithogenic indices were calculated from Carey's tables (14).

Enzyme assays. Liver microsomes were prepared from fresh liver aliquots. HMG-CoA reductase was determined as previously described (5).

Statistical calculations. The data are reported as mean ± SEM. Analysis of variance was used to determine the F statistic; Student's t-test was applied to those values where the F statistic was significant (15,16). Chi square was used to determine statistical significance between groups (17).

RESULTS

The effects of the different dietary regimens in prairie dogs were observed after 6 and 14 wk. The average initial and final weights of the animals in groups 1–8 were similar, and all animals gained weight during the experimental period. Food intake and fecal outputs for the different groups of animals were also similar.

After 6 wk, five prairie dogs fed 1.2% CH were examined to determine the incidence of CH gallstones. Examination of the bile under polarized light microscopy revealed the presence of both CH gallstones and CH crystals (Table 1). The average lithogenic index exceeded 1.0 (Table 2), indicative that the bile was supersaturated with CH. The animals were randomly divided into eight groups and the modified diets (1–8) were fed for 8 wk, at which time all animals were autopsied. CH gallstone incidence ranged from 100% (groups 1 and 2) to 80% (groups 3 and 4). Although the diet of group 4 had no added CH during the 8-wk regression period, CH gallstones did not dissolve spontaneously. Incidence of gallstones in this group was also 80%. In the animals fed HDA, gallstone incidence ranged from 40% (group 7) to 83% (group 5). In most cases, incidence of CH crystals in bile paralleled the incidence of gallstones. Only in groups 5 and 7 were there variations between the incidence of gallstones and crystals.

Biliary lipid compositions for the various groups are listed in Table 2. In general, decreasing the percentage of CH in the diet from 0.4 to 0.0% led to a decrease in the mol % of CH and in lithogenic indices (group 1 through group 4 vs group 5 through group 8). The mol % of phospholipids and bile acids did not vary significantly between the groups and ranged from 6.3 to 12.8 mol % and 72.5 to 89.5 mol %, respectively (groups 1–4

TABLE 1

Incidence of Cholesterol Gallstones and Cholesterol Crystals in Prairie Dogs

Group	Diet[a]	No. of animals	Incidence of	
			Cholesterol gallstones	Cholesterol crystals
0	SSD + 1.2% CH	5	5/5[b]	5/5
1	SSD + 0.4% CH	6	6/6	6/6
2	SSD + 0.2% CH	5	5/5	5/5
3	SSD + 0.1% CH	5	4/5	4/5
4	SSD + 0.0% CH	5	4/5	4/5
5	SSD + 0.4% CH + HDA	8	7/8	1/8[c]
6	SSD + 0.2% CH + HDA	5	3/5	3/5
7	SSD + 0.1% CH + HDA	5	2/5	3/5
8	SSD + 0.0% CH + HDA	5	3/5	3/5

[a]SSD, semisynthetic diet; CH, cholesterol; HDA, hyodeoxycholic acid.
[b]Incidence determined after 6-wk gallstone induction period. In all other groups, gallstone incidence was determined after an 8-wk regression period. For details, see Materials and Methods.
[c]Differs from group 1–4, $p < 0.02$ by chi square.

and 5–8). In groups 5 and 8, where the amount of added CH in the diet was 0%, the lithogenic indices failed to fall below 1.0.

CH concentrations in the various tissues (liver, plasma and bile) decreased with less CH in the diet (groups 1–4) (Table 3). Liver CH ranged from 5.12 mg/g in group 1 to 2.63 mg/g in group 4. Plasma CH ranged from 1080 mg/dl to 220 mg/dl (group 1 to group 4, respectively). Biliary CH levels followed a similar pattern and ranged from 0.49 to 0.17 mg/ml. HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis, increased in activity as the amount of CH in the diet decreased (from 0 pmol/mg protein/min in group 1 to 121 pmol/mg protein/min in group 4). Addition of HDA to the various diets (groups 5 through 8) lowered the CH levels in liver, plasma and bile compared to the groups without the additional HDA (groups 1–4). Plasma CH in groups 7 and 8 approached that of chow-fed prairie dogs, 129 mg/dl (10). Interestingly, HMG-CoA reductase activity was significantly elevated in all groups fed HDA.

The biliary bile acid compositions are summarized in Table 4. As observed previously, the major bile acids in the CH-fed groups (0 through 4) were cholic acid and chenodeoxycholic acid. These bile acids were present exclusively as the taurine conjugates as determined by thin layer chromatography. The amount of cholic acid in the bile tended to increase slightly as the amount of CH in the diet decreased from 0.4 to 0.0%. Secondary bile acids were rehydroxylated to primary bile acids (17) and comprised 6% or less of the total biliary bile acids. In the animals fed HDA, this bile acid and its 6β-epimer comprised 60–70% of the total biliary bile acids.

TABLE 2

Effect of Diet and Hyodeoxycholic Acid on Lipid Concentration in Prairie Dogs[a]

Group	Diet[b]	Mol % biliary lipid			
		Cholesterol	Phospholipids	Bile acids	Lithogenic index
0	SSD + 1.2% CH	6.3 ± 0.7	10.5 ± 2.8	83.2 ± 2.8	1.60 ± 0.29
1	SSD + 0.4% CH	15.8 ± 2.1[c]	11.7 ± 1.8	72.5 ± 1.5	4.62 ± 1.60[d]
2	SSD + 0.2% CH	6.0 ± 0.5	10.6 ± 1.0	83.4 ± 1.5	1.37 ± 0.09
3	SSD + 0.1% CH	5.7 ± 0.5	9.9 ± 1.8	84.4 ± 1.4	1.40 ± 0.23
4	SSD + 0.0% CH	4.2 ± 0.6	6.3 ± 2.8	89.5 ± 2.9	1.23 ± 0.43
5	SSD + 0.4% CH + HDA	9.4 ± 2.6	8.8 ± 2.2	81.8 ± 4.5	2.46 ± 0.49[e]
6	SSD + 0.2% CH + HDA	6.2 ± 1.0	11.6 ± 1.1	82.2 ± 1.8	1.70 ± 0.43
7	SSD + 0.1% CH + HDA	6.8 ± 0.8	12.8 ± 3.0	80.4 ± 3.3	1.46 ± 0.45
8	SSD + 0.0% CH + HDA	5.5 ± 0.8	11.6 ± 3.2	82.9 ± 3.8	1.30 ± 0.14

[a]Mean ± SEM.
[b]SSD, semisynthetic diet; CH, cholesterol; HDA, hyodeoxycholic acid.
[c]Differs significantly from groups 2–4 and 6–8, $p < 0.01$.
[d]Differs significantly from groups 2–4 and 6–8, $p < 0.025$.
[e]Differs significantly from group 8, $p < 0.05$.

TABLE 3

Cholesterol Concentrations and Enzyme Activity in Tissues*

Group	Diet[†]	Average liver wt (g)	Cholesterol			Hepatic HMG-CoA reductase pmol/mg protein/min
			Liver (mg/g)	Plasma (mg/dl)	Bile (mg/ml)	
0	SSD + 1.2% CH	20.7	4.59 ± 0.64	610 ± 29	0.41 ± 0.07	—
1	SSD + 0.4% CH	33.3	5.12 ± 0.63	1080 ± 103[a]	0.49 ± 0.17[‡]	0[b]
2	SSD + 0.2% CH	30.0	6.79 ± 0.63[c]	739 ± 95[d]	0.46 ± 0.13[e]	75[f] ± 12
3	SSD + 0.1% CH	33.5	3.73 ± 0.33	290 ± 92	0.34 ± 0.06	50[g] ± 7
4	SSD + 0.0% CH	28.9	2.63 ± 0.17	220 ± 66	0.17 ± 0.05	121[h] ± 17
5	SSD + 0.4% CH + HDA	26.4	4.05 ± 0.63	440 ± 13[a]	0.29 ± 0.08[‡]	219[b] ± 35
6	SSD + 0.2% CH + HDA	26.4	3.20 ± 0.19[c]	260 ± 19[d]	0.23 ± 0.08[e]	389[f] ± 87
7	SSD + 0.1% CH + HDA	31.3	2.58 ± 0.31	180 ± 27	0.51 ± 0.06	317[g] ± 70
8	SSD + 0.0% CH + HDA	28.0	2.80 ± 0.28	140 ± 24	0.25 ± 0.03	313[h] ± 38

Numbers with identical superscripts differ significantly, $p < 0.01$.
*Mean ± SEM.
[†]SSD, semisynthetic diet; CH, cholesterol; HDA, hyodeoxycholic acid.
[‡]Differ significantly, $p < 0.025$.

TABLE 4

Bile Acid Composition (%)[a]

Group	Diet	CA	CDA	HDA	6β-HDA	DA	LA
0	SSD + 1.2% CH	54.8 ± 5.4	39.0 ± 8.2	—	—	5.1 ± 3.0	1.1 ± 0.04
1	SSD + 0.4% CH	56.5 ± 3.6	41.4 ± 4.4	—	—	1.4 ± 0.4	0.7 ± 0.3
2	SSD + 0.2% CH	46.7 ± 4.1	49.9 ± 5.2	—	—	2.7 ± 1.2	0.7 ± 0.3
3	SSD + 0.1% CH	59.3 ± 3.2	36.0 ± 3.0	—	—	3.6 ± 1.6	1.1 ± 0.3
4	SSD + 0.0% CH	68.5 ± 5.5	29.6 ± 4.8	—	—	1.6 ± 0.2	0.3 ± 0.04
5	SSD + 0.4% CH + HDA	19.6 ± 0.9	3.0 ± 0.2	46.9 ± 3.4	27.6 ± 2.3	2.8 ± 1.0	0.1 ± 0.1
6	SSD + 0.2% CH + HDA	23.9 ± 2.1	7.8 ± 4.9	45.1 ± 2.4	22.1 ± 3.2	1.1 ± 0.5	—
7	SSD + 0.1% CH + HDA	20.8 ± 2.0	3.9 ± 1.1	46.7 ± 2.2	27.2 ± 1.7	1.4 ± 0.6	—
8	SSD + 0.0% CH + HDA	33.3 ± 3.1	3.1 ± 0.8	38.8 ± 3.2	23.6 ± 4.3	1.2 ± 0.2	—

Bile was obtained from the same number of animals shown in Table 1.
[a]Mean ± SEM. CA, cholic acid; CDA, chenodeoxycholic acid; HDA, hyodeoxycholic acid; 6β-HDA, 3α, 6β-dihydroxy-5β-cholanoic acid; DA, deoxycholic acid; LA, lithocholic acid; SSD, semisynthetic diet; CH, cholesterol.

DISCUSSION

The dissolution studies of CH gallstones in the prairie dog model of cholelithiasis posed several new problems not associated with the previous gallstone prevention experiment (4,5). First, animals must be fed a lithogenic diet high in CH to produce a high incidence of gallstones (90–100%) in a short time. Second, the composition of the diet given to the animals in the dissolution phase of the study must not allow spontaneous gallstone disappearance to occur. In other words, a small amount of CH in the regression diet might be necessary so that one can assess the gallstone-dissolving potential of the compound of interest. If gallstone dissolution is incomplete, the duration of the study may have to be increased.

The present study represents the first attempt to dissolve CH gallstones in the prairie dog using bile acid therapy. This study consisted of two phases: (a) gallstone formation by feeding prairie dogs 1.2% CH in a semisynthetic diet for 6 wk; and (b) gallstone regression by reducing the amount of dietary CH to 0.4%, 0.2%, 0.1% and 0.0% and feeding the CH with and without the hydrophilic bile acid HDA. The use of the gallstone-forming diet was adopted from previous studies which showed that gallstone incidence is 100% in 3–4 wk (18). However, no previous studies for gallstone regression or dissolution were carried out using semisynthetic diets. We felt it preferable to feed a defined semisynthetic diet rather than a standard chow diet, since chow is of variable composition; thus, the variability of chow might make gallstone regression studies more difficult to reproduce.

The animals in our study were fed 1.2% CH in a semisynthetic diet for 6 wk and had a gallstone incidence of 100%. The animals were divided into the eight experimental groups and the modified diets (period 2) were fed for 8 wk. The results of the dissolution study showed that spontaneous gallstone dissolution did not occur using any of the experimental diets. We had postulated that animals fed a semisynthetic diet + 0.0% added CH should have no gallstones after 8 wk (group 4); similarly, we postulated that the addition of HDA to this diet should accelerate the rate of dissolution (group 8). Surprisingly, the incidence of CH gallstones and CH crystals in the group receiving no additional CH (group 4) did not differ significantly from the groups with added CH (groups 1–3). This result was different from earlier studies where spontaneous dissolution of preformed CH gallstones occurred in 3 wk (19). The one important difference, however, was that chow was used in the previous study whereas a semisynthetic diet was used in this one.

The animals given the bile acid HDA showed several interesting effects. Although complete gallstone dissolution was not achieved, the animals given the bile acid tended to have a lower incidence of both gallstones and biliary CH crystals. If we combine all the animals in groups 5–8 given HDA, we find a significant reduction in the incidence of CH crystals compared to animals in groups 1–4 (10/23 vs 19/21, respectively; $p < 0.025$ by chi square). A similar comparison for incidence of CH gallstones shows no significant differences. It has previously been demonstrated that HDA prevents gallstones by inhibiting the phase transition of liquid crystalline vesicles to solid CH monohydrate and thus is able to solubilize increased amounts of CH in supersaturated bile (1,4,11). It has been suggested that a hydrophilic bile acid might dissolve CH gallstones and CH crystals by a similar mechanism (11). From the present study, it appears that this hypothesis is valid to a certain extent; namely, HDA reduces CH crystals in bile possibly by forming vesicles. However, the CH gallstones, being large aggregates of CH crystals, may not be able to dissolve in the short time course of this experiment. A longer experimental trial seems warranted to test this hypothesis.

Other measurements made during the study suggest that addition of HDA to the lithogenic diet produces several desirable physiological effects. First, HDA and its 6β-isomer, 3α,6β-dihydroxy-5β-cholanoic acid, became the major biliary bile acids, comprising 60–70% of the total bile acids in groups 5–8. 3α,6β-Dihydroxy-5β-cholanoic acid was shown to be at least as effective as HDA in preventing gallstones in the prairie dog (10). Second, the HDA-fed animals had reduced levels of CH in liver, plasma and bile while at the same time they had elevated levels of HMG-CoA reductase, the rate-limiting enzyme of CH synthesis. This suggests that HDA might reduce CH absorption, which could lead to increased HMG-CoA reductase activity. Further absorption measurements are needed to verify this point.

HDA was used for the dissolution study of gallstones in the hope that it would be more effective than chenodeoxycholic acid or ursodeoxycholic acid in dissolving CH gallstones. Both chenodeoxycholic acid and ursodeoxycholic acid prevented gallstones in the prairie dog to a limited extent (4,20). A hydrophobic bile acid such as chenodeoxycholic acid probably acts via a micellar mechanism; however, the hydrophilic bile acid ursodeoxycholic acid seems to act via a dual mechanism, namely, formation of liquid crystalline vesicles and micelles (11). From the present study, it appears that HDA is capable of dissolving CH crystals but that dissolution of larger CH stones was not complete. The property of hydrophilicity alone may not be sufficient for dissolution. As suggested by Salvioli et al., alternate enrichment of the bile with a hydrophilic bile acid, such as HDA or ursodeoxycholic acid, and a hydrophobic bile acid, such as chenodeoxycholic acid, may be more effective than either therapy alone because dissolution by a dual mechanism (liquid crystal and micelle formation) will be enhanced (11).

Gallstone dissolution in man using bile acid therapy remains an active area of investigation (21–26). The use of a hydrophilic bile acid to dissolve gallstones offers a potentially new mechanism of action, namely, formation of liquid crystals, to accomplish the dissolution. However, HDA itself may not be effective in man because after oral administration it is largely excreted into the urine and does not accumulate in the bile (27). Further studies, including time and dose-response studies as well as studies with conjugated derivatives, must be carried out before the efficiacy of this mechanism can be determined.

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Fast Atom Bombardment and Tandem Mass Spectrometry of Phosphatidylserine and Phosphatidylcholine

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Fast atom bombardment (FAB) desorption of phosphatidylserine and various phosphatidylcholines produces a limited number of very informative negative ions. Especially significant is the formation of $(M-H)^-$ ions for phosphatidylserine, a compound which does not yield informative high mass ions by other ionization methods. Phosphatidylcholines do not yield $(M-H)^-$ ions but instead produce three characteristic high mass ions, $(M-CH_3^+)^-$, $[M-HN(CH_3)_3^+]^-$ and $[M-HN(CH_3)_3^+-C_2H_2]^-$. Both classes of lipids also yield anions attributed to the carboxylate components of these complex lipids. FAB desorption in combination with collisional activation allows for characterization of fragmentation and determination of structural features. Collisional activation of the carboxylate anion fragments from the complex lipids is especially informative. Structural characterization of the fatty acid chain can be achieved as the released saturated carboxylate anions undergo a highly specific 1,4-elimination of H_2, which results in the losses of the elements of CH_4, C_2H_6, C_3H_8 . . . in a fashion entirely consistent with the chemistry of carboxylate anions desorbed from free fatty acids. These C_nH_{2n+2} losses begin at the alkyl terminus and progress along the entire alkyl chain. Modified fatty acids undergo a similar fragmentation; however, the modification affects the series of C_nH_{2n+2} losses in a manner which permits determining the type of modification and its location on the fatty acid chain.
Lipids 21, 580–588 (1986).

Complex lipids derived from glycerophosphoric acid are major components of cell membranes of living organisms. Analysis of these important compounds, which include phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, phosphatidylinositols and cardiolipin, has proven to be challenging. Living organisms contain a diverse collection of complex lipids; hence, if a specific lipid is to be studied, it must be isolated from the organism and then separated from a mixture of similar compounds. Classical methods of analysis (1–3) are time-consuming, multistep processes that usually involve degradation of the complex lipids into simpler constituents and analysis of the component parts by using a variety of chromatographic and spectroscopic methods. The arrangement of components on the glycerol of the original molecular is typically established using a lipolytic enzyme specific for one position of the glycerol (1).

Mass spectrometry (MS) has been applied in a variety of ways for characterizing complex lipids. Studies by Djerassi and coworkers of these compounds from marine sponges (4–13) provide an example of the scope of the problem and the value of MS methods. The thermally labile, low volatility phospholipids usually decompose under electron ionization (EI) conditions to give characteristic fragment ions but no molecular ions (14–17). Rapid heating of a nonderivatized lecithin on a gold support (18) and, recently, in-beam electron ionization (19) are the only methods reported to give molecular ions under EI conditions. Stenhagen and coworkers (20–24) demonstrated that the methyl esters of fatty acids are amenable to EI analysis. EI methods are also useful for studying derivatives of the polar head groups.

Since phospholipids have low volatility and are not thermally stable, soft ionization methods are a better approach for obtaining molecular weight information. Field desorption (FD) and chemical ionization (CI) have been applied, and their use was reviewed by Games (25) and Wood (26).

Because FD yields abundant M^+ and $(M+H)^+$ ions and limited but informative fragment ions (26–34), it is useful both for obtaining molecular weight information and characterizing mixtures. However, problems of ion beam instability, mass spectral irreproducibility and high sensitivity to impurities, especially sodium, which may completely mask the mass spectrum, plague the FD method. Intermolecular methyl transfer can also create ambiguities for assigning molecular weight (31).

Isobutane CIMS of phospholipids was first introduced by Foltz (35). However, ammonia appears to be the reagent gas of choice for most applications of CIMS because it favors the formation of very abundant ions containing molecular weight information (36–39). Desorption chemical ionization of lecithins, which can be introduced to the ionization chamber on tungsten wire (40), gold wire (41) or gold-plated tungsten coils (42), is also useful when ammonia is used as the reagent gas. Ayanoglu et al. (42) reported that, under conditions of carefully controlled source temperature, protonated molecular ions may be observed for all classes of phospholipids, except phosphatidylserine when the gold-plated tungsten coil sample introduction system is used.

Other soft ionization methods for phospholipids include laser desorption (LD) (43), secondary ion mass spectrometry (SIMS) (44–46) and fast atom bombardment (FAB) (42,45–52). FAB desorption of positive ions has been the most widely used of these methods. This desorption method yields abundant $(M+H)^+$ ions in most cases; phosphatidylserine is a notable exception. A limited amount of fragmentation is also observed comparable to that produced by FD. However, FAB is more advantageous than FD because a stable spectrum over a considerably longer time scale is more readily produced. The spectra are more reproducible and less sensitive to instrumental parameters.

Liquid chromatography-MS (LC-MS) techniques are also attractive for complex mixtures of phospholipids from natural sources because the method provides both separation (LC) and structural information (MS). Interfacing the two systems is a problem; however, phospholipids were successfully analyzed using both the mov-

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ing belt (53–55) and the split LC flow (56,57) interfaces.

Although characterization of the entire phospholipid is desirable, often that is not possible, and methods for elucidating specific portions such as the polar head groups or the fatty acids are often used. Gas chromatography-MS (GC-MS) utilizing both EI and CI can be used for identifying derivatized constituents of phospholipids (58–68). MS is especially useful for characterizing fatty acids. We have reviewed such methods in detail elsewhere (69). While considerable fragmentation is obtained by using this method, molecular ions and other informative high mass ions are of low abundance, and location of double bonds and other structural features is obscured by rearrangements. Pyrrolidide (70–73) and more recently picolinyl derivatives (74–76) were shown to be useful derivatives for locating the double bond position in unsaturated acids and for providing some enhancement of the abundance of high mass ions.

Tandem MS (MS-MS) is also useful for fatty acid analysis. We showed recently that collisional activation of FAB-desorbed carboxylate anions of free fatty acids produces fragment ions that can be used to identify the acid and to determine the presence and location of structural modifications on the fatty acid chain (77–80). Consequently, it was expected that MS-MS combined with FAB desorption of negative ions would be even more useful than FAB alone for investigating phospholipids. This paper is a report of our examination of FAB and MS-MS for analysis and structural determination of phospholipids. Preliminary versions of this work have been presented at recent conferences (81,82) and published (83).

EXPERIMENTAL

MS. All mass spectra and collisionally activated decomposition (CAD) spectra were obtained with a Kratos MS-50 triple analyzer tandem mass spectrometer described previously (84). This instrument consists of a high resolution MS-I of EB configuration followed by an electrostatic analyzer used as MS-II. The instrument was equipped with a standard Kratos FAB source (Manchester, United Kingdom) and an Ion Tech saddle field ion gun (Teddington, United Kingdom). The instrument was operated in the negative ion mode for this study. The FAB gun was operated at 6–8 KV, and xenon atoms were used to ionize desorptively the sample in a triethanolamine matrix loaded on a copper FAB probe tip. Mass spectra of the FAB-produced negative ions were acquired at a resolution of ca. 2000 by scanning MS-I and leaving the voltage of MS-II fixed to pass all ions.

CAD spectra were obtained by focusing the desired ion with MS-I into the collision cell located in the third field free region and colliding the selected ions with sufficient helium to cause a 50% reduction in the abundance of the selected ions. Daughter ions formed in this process were detected by scanning MS-II. Fifteen to 30 scans were signal-averaged for each spectrum. Signal averaging and processing of the spectra were accomplished with a standard DS-55 data system using software written at this laboratory (85). It is noted that the mass resolving power of MS-II of the tandem mass spectrometer is ca. 100 due to unavoidable energy release in the decompositions. Hence, mass assignments are made from the centroids of these broadened peaks. The validity of these assignments was established previously (77).

Materials. All phosphatidylcholines used for this study were obtained from Sigma Chemical Co. (St. Louis, Missouri). Samples of phosphatidylserine were obtained from Sigma and Supelco Inc. (Bellefonte, Pennsylvania).

RESULTS AND DISCUSSION

Negative ions were chosen for this study for a number of reasons. First, collisional activation of the fatty acid carboxylate fragments from complex lipids should yield the same highly distinctive structural information as do the free fatty acid carboxylates desorbed by using FAB. Second, MS-MS offers the opportunity to study mixtures of both fatty acids and more complex lipids. Third, fatty acid composition of isomeric complex lipids can be determined by analyzing the daughter ion spectra even if the constituent fatty acids are similar in mass (e.g., two constituents could be either 18:1 and 18:1 or 18:0 and 18:2). The determination can be made if MS-II has approximately unit resolution. This was recently demonstrated by Sherman et al. (86), who used a BEB triple sector MS-MS instrument. However, in the positive ion mode, relatively abundant $(M\text{-}H)^+$ ions are generated along with $(M+H)^+$ ions of fatty acids, and the former ions will overlap with other fatty acid fragments containing one or more sites of unsaturation (49). This problem is much less severe for negative ions. Fourth, FAB of phosphatidylserines has the potential for generating negatively charged, high mass ions which contain molecular weight information. To date, however, no useful positively charged, high mass ions have been reported to be produced by using any soft ionization method, including FAB, for phosphatidylserines.

Phosphatidylserine. Two samples of phosphatidylserine were obtained from different commercial sources, and they contained a substantial number of impurities as evidenced in the FAB mass spectra. However, MS-MS methods allowed for the study of the ions of interest in the presence of contaminants. One sample (spectrum given in Fig. 1) contained a prominent contaminant that was seen as an ion of m/z 885 in the full mass spectrum. Collisional activation of this ion and comparison of its spectrum with spectra of other known phospholipids indicates that the contaminant is phosphatidylinositol (82).

Satisfactory determination of any phosphatidylserines using positive ion MS methods has not been accomplished to date. However, as shown in Figure 1a, $(M\text{-}H)^-$ ions (m/z 788 for oleoyl stearoyl phosphatidylserine) may be FAB-desorbed from a triethanolamine matrix containing the phosphatidylserine. Collisional activation of the $(M\text{-}H)^-$ ion causes a fragmentation involving the loss of serine, which leads to an abundant ion of m/z 701 (Fig. 1b). The ion of m/z 701 is also formed in the desorption process, as seen in FAB mass spectrum (Fig. 1a). Ions attributable to the carboxylates of stearic acid (m/z 283) and oleic acid (m/z 281) are also seen in the mass spectrum of desorbed ions. Collisional activation of these ions confirms their identities (see Figs. 1c and 1d for comparison of the CAD spectra of m/z 281 and authentic oleate).

Phosphatidylcholine. Investigations of FAB-desorbed

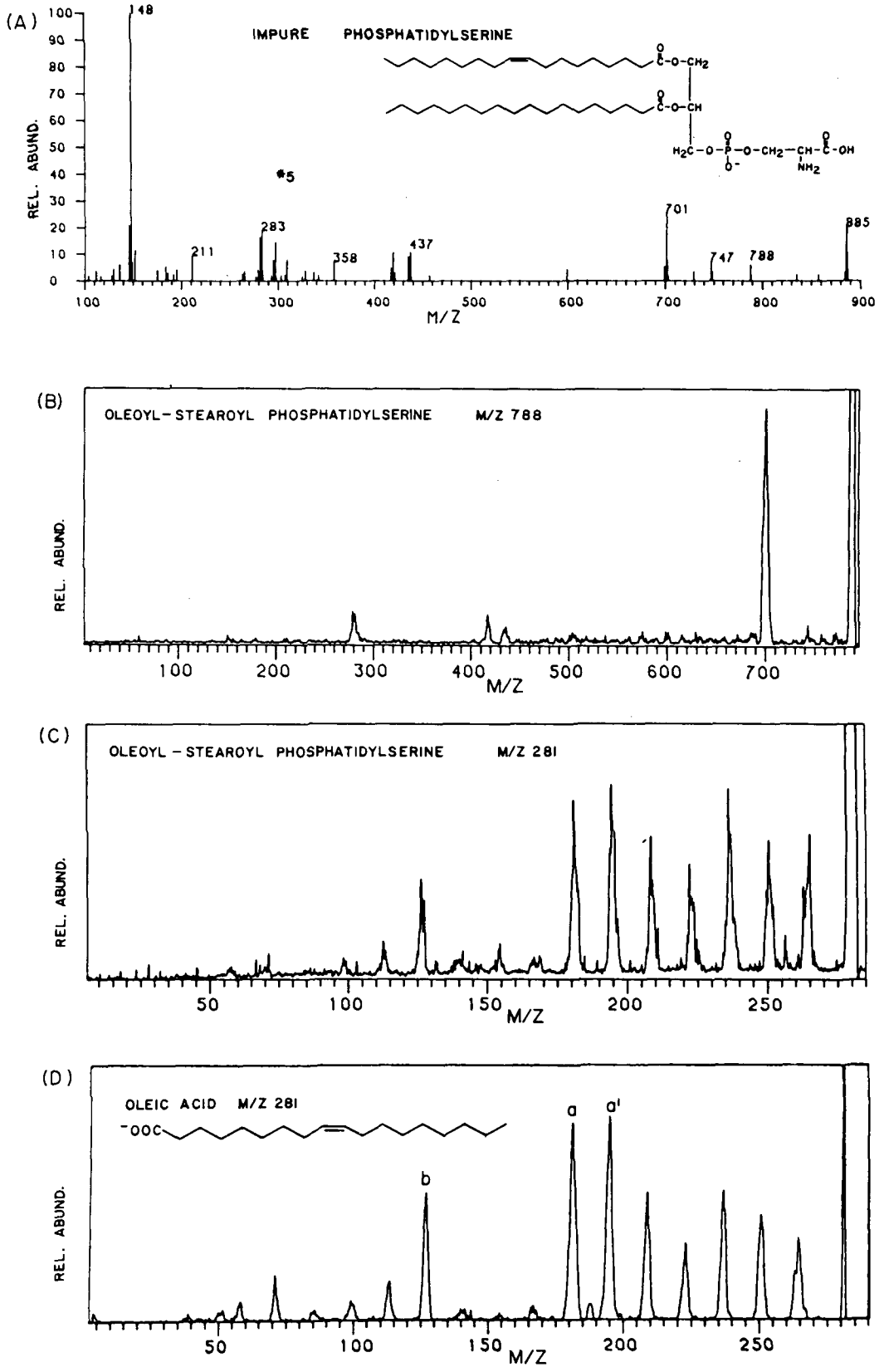


FIG. 1. (A) Mass spectrum of negative ions produced by FAB desorption of phosphatidylserine. The ion of m/z 788 is the $(M-H)^-$ ion. Collisional activation showed the ion of m/z 887 to be an impurity of phosphatidylinositol. (B) Spectrum of the daughter ions produced by collisionally activating $(M-H)^-$, m/z 788. (C) Spectrum of the daughter ions produced by collisionally activating the fragment ion of m/z 281. (D) Spectrum of the daughter ions produced by collisionally activating the $(M-H)^-$ ion, m/z 281, of authentic oleic acid.

negative ions of phosphatidylcholines and their CAD spectra are expected to be complementary to those of positive ions. The CAD spectra should be especially informative for identifying and verifying the structure of the acid chains. The series of phosphatidylcholines studied (see structure **A,** Scheme 1) have fatty acid compositions commonly found in nature.

FAB mass spectra of negative ions contain five major ions. The two low mass ions are apparently the carboxylate anions derived from the fatty acid portions of the complex lipid, and the three high mass ions arise from losses of various portions of the choline moiety to give ions **B, C** and **D** (Scheme 2).

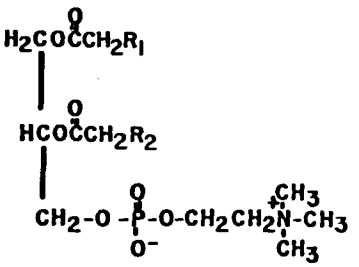
The FAB mass spectrum and the CAD spectrum of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine are typical examples of the information that may be obtained from negative ions (see Figs. 2 and 3). For the 1-palmitoyl-2-oleoyl lipid, these ions are of m/z 281, 255, 673, 699 and 744. Similar fragmentations occur for all the phosphocholines studied. Masses and relative abundances of the characteristic ions for the various lipids studied are listed in Table 1.

For all compounds except 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine, the carboxylate fragment from the 2-position is more abundant than that from the 1-position in the full mass spectra. Thus, it appears that a distinction can be made between the acids located in the 1- and 2-positions by using FAB, provided a pure sample of the lipid is available. Hayashai and Morita (87) recently noted that distinction is possible for FAB-desorbed positive phosphatidylcholine ions.

Collisional activation of carboxylate anions causes fragmentation that can be used to characterize the fatty acid substituents. In previous papers, we described remote charge site fragmentation of closed shell species (77–81). For carboxylate anions, collisional activation of the $(M-H)^-$ ion yields a series of fragments arising by losses of the elements of C_nH_{2n+2} from the alkyl terminus remote from the charge site. The identification of a carboxylic acid and the determination of the length of its carbon chain may be verified because a series of fragment ions is formed starting with the loss of CH_4 and ending with the loss which yields an ion of m/z 58. The spectrum of the ion of m/z 255 which is the palmitoyl portion of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (Fig. 2B) is representative. Note that the only other ion of major consequence results from the loss of H_2O from the fatty acid carboxylate. The pattern of relative abundances seen for the palmitoyl anion is typical for saturated acids.

The presence of unsaturation or substituents on the acid chain causes a perturbation of this fragmentation pattern. For example, the CAD spectrum of the ion of m/z 281 from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine is identical to that shown in Figure 1D. The double bond present in this m/z 281 fragment anion and in the oleic acid anion (Fig. 1D) causes an alteration of the series of fragment ions in a fashion which allows the presence of the double bond and its location on the chain to be identified. Two abundant ions (a and a′) are followed by three low abundance ions at lower mass and then a third abundant ion (b). Two abundant ions a and b arise from a hydrogen transfer and cleavage of the allylic bonds, one on the carboxyl side to lose C_nH_{2n} to give b and the other on the hydrocarbon side of the double bond to lose C_nH_{2n+2} to yield a. Ion a′ also forms by loss of the elements of C_nH_{2n+2}.

Both the decomposition modes of each of the three high mass ions (structures **B, C** and **D**, Scheme 2) and the identities of the 1- and 2-substituents can be determined by collisional activation. The principal fragmentations of the two highest mass ions are the losses of the two fatty acid carboxylates. For 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocoline, the high mass ions



A

I R_1CH_2CO = PALMITOYL, R_2CH_2CO = OLEOYL

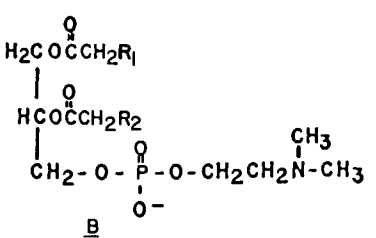
II R_1CH_2CO = OLEOYL, R_2CH_2CO = PALMITOYL

III R_1CH_2CO = OLEOYL, R_2CH_2CO = STEAROYL

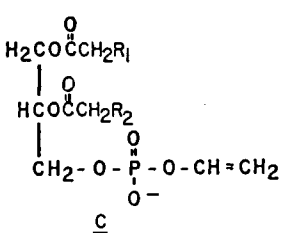
IV R_1CH_2CO = STEAROYL, R_2CH_2CO = OLEOYL

V R_1CH_2CO = PALMITOYL, R_2CH_2CO = LINOLEOYL

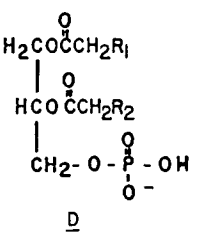
SCHEME 1



B



C



D

SCHEME 2

of m/z 744 and 699 fragment to give the two carboxylate fragments of m/z 255 and 281 (Figs. 3B and 3C). The carboxylate anion expelled from the 2-position has a relative abundance about twice that of the acid arising from the 1-position. This pattern is characteristic for all the phosphatidylcholines studied, including 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine. This latter phosphatidylcholine did not show enhanced production of the carboxylate anion located at the 2-position ion in the mass spectrum of the desorbed ions.

The lowest mass ion (m/z 673, Fig. 3A) of the three high mass ions arises from the loss of the choline moiety to give $[M\text{-}HN(CH_3)_3^+\text{-}C_2H_2]^-$ (see structure D) and fragments in a slightly different but very informative manner compared to the two other high mass ions. The carboxylate anions are again major fragments; however, the 1-carboxylate is substantially more abundant (ca. twofold) than the 2-carboxylate–a reversal of the abundances of the two carboxylates compared to the relative abundances of those from the two higher mass ions. For 1-stearoyl-2-oleoyl and 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine, the mass resolution is not sufficient in the full CAD spectra of the high mass ions to distinguish oleate (m/z 281) and stearate (m/z 283). Nevertheless, narrow mass scans of the CAD spectrum under conditions of narrowed energy slits and substantial signal averaging show partial resolution of the doublet and confirm this fragmentation behavior.

Additional fragmentations of the high mass ion formed by loss of $HN(CH_3)_3^+$ and C_2H_2 are losses of R_1CHCO, R_2CHCO, R_1CH_2COOH and R_2CH_2COOH. The fragmentation of the ion of m/z 673 from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine is representative of this fragmentation behavior (Fig. 3A). Ions of m/z 435 and 409 arise from the losses of R_1CHCO and R_2CHCO, respectively, while ions of m/z 417 and 391 arise from losses of R_1CH_2COOH and R_2CH_2COOH, respectively. The losses of R_2CHCO and R_2CH_2COOH occur more readily than losses of their 1-counterparts, and the RCH_2COOH loss occurs approximately twice as readily as the RCHCO loss.

Lysophosphatidylcholine. 1-Oleoyl-lysophosphatidylcholine, formed as the result of enzyme hydrolysis and toxic to cells, has an unsubstituted OH group at the 2-carbon of the glycerol. Ions desorb and fragment in a manner similar to the phosphatidylcholine with two acid

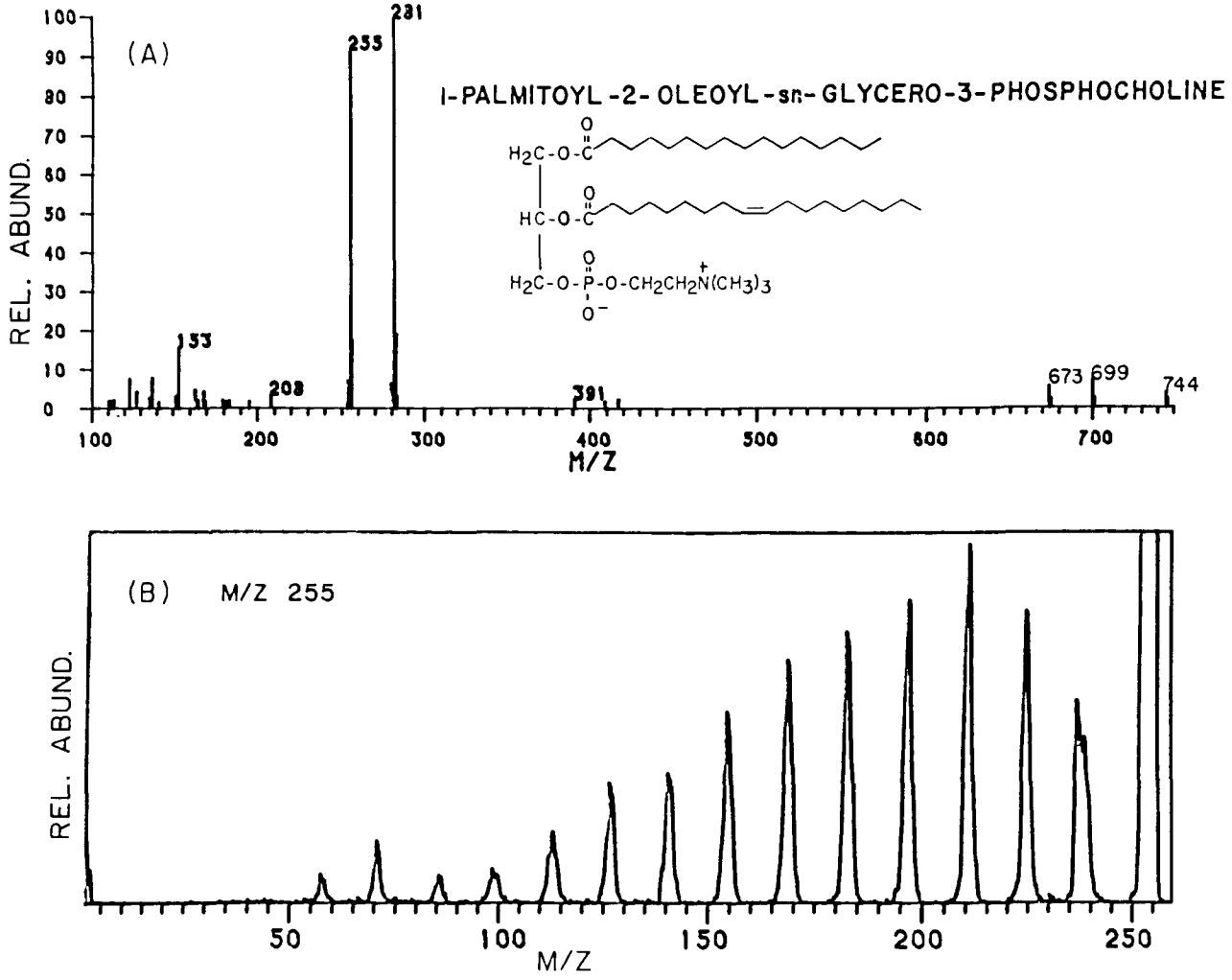


FIG. 2 (A) Mass spectrum of negative ions produced by FAB desorption of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. (B) CAD spectrum of the daughter ions produced by collisionally activating the ion of m/z 255 (palmitate). The CAD spectrum of the ion of m/z 281 is identical to that in Fig. 1D.

substituents. Three high mass ions of m/z 506, 461 and 435 are formed as a result of the same losses of various portions of the choline moiety that phosphatidylcholines undergo. The product ions are analogous in structure to those for the aforementioned diacyl phosphatidylcholines (see structures **B-D**). A lower mass carboxylate ion of m/z 281 is also seen. Collisional activation of each of these ions causes fragmentations similar to those of their disubstituted counterparts. The ion of m/z 281 fragments identically to the ion of m/z 281 from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (Fig. 2c) and is readily identified as the oleate anion. Ions of m/z 506 and 461 show little fragmentation other than the formation of the carboxylate anion. The principal fragmentation of m/z 435 ions is the loss of R_1CH_2COOH; however, loss of R_1CHCO and formation of the carboxylate ion also occur.

Pyrenyl phosphatidylcholine. The replacement of a

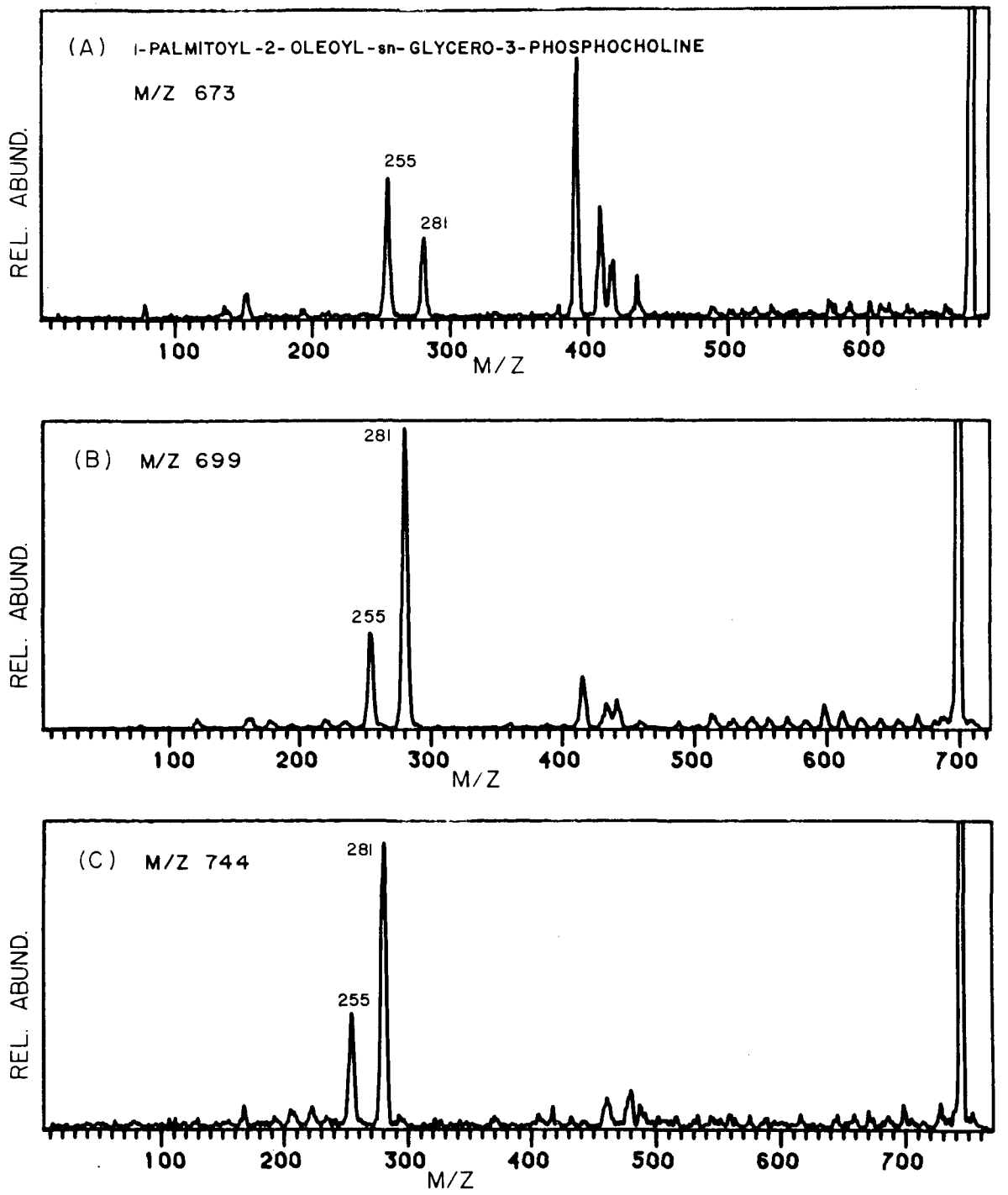
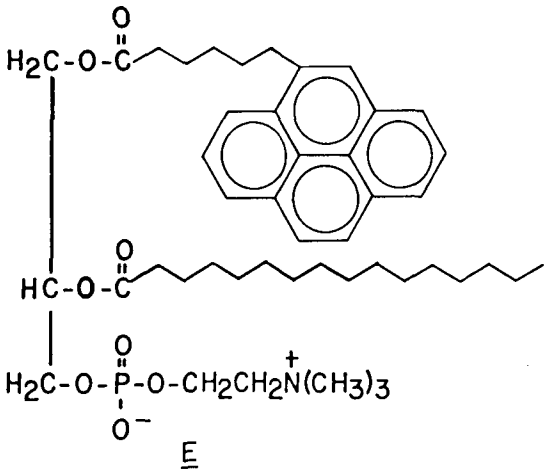


FIG. 3. Spectrum of the daughter ions produced by collisionally activating the fragment ions of (A) m/z 673, (B) m/z 699 and (C) m/z 744 of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

fatty acid substituent with a pyrene functionality creates a fluorescent molecule useful as a biological probe. The principal ion in the mass spectrum of ions desorbed from 1-(pyren-1-yl)-hexanoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine, **E** (Scheme 3), is that of m/z 778, which is $(M\text{-}CH_3^+)^-$ and may be attributed to the loss of one of the methyl groups of the choline.

An ion of the mass of a palmitate anion (m/z 255) is also present. Collisional activation of the m/z 255 ion confirms it to be a straight chain, 16–carbon atom carboxylate. The ion of m/z 778 fragments upon collisional activation to form ions of m/z 255 and 315, which may be attributed to the palmitoyl and (pyren-l-yl)-hexanoyl portions of the molecule, and to a lesser extent to lose R_2CHCO and R_2CH_2COOH.

More fragmentation of 1-palmitoyl-2-(pyren-1-yl)-hexanoyl-*sn*-glycero-3-phosphocholine occurs upon desorption than of its isomer mentioned above. Ions of m/z 778 and 707, arising from loss of a positively charged methyl from the choline group and of the entire choline group, respectively, are seen. Collisional activation of the ion of m/z 255 yields the expected fragmentation for a palmitate anion. The principal CA fragmentations of the ion of m/z 315 are losses of 18 and 44, presumably water and CO_2. The CA fragmentations of m/z 707 ions are similar to those observed for other phosphocholine fragment ions of structure type **D** (Scheme 2). The most abundant ions result from losses of R_2CH_2COOH and R_1CH_2COOH, and the ion resulting from loss of R_2CH_2COOH is formed to a greater extent than the ion resulting from the loss of R_1CH_2COOH. R_2CHCO and R_1CHCO losses also occur, and ions of m/z 255 and 315 (palmitoyl and pyrenyl-hexanoyl) are also present. Collisional activation of the ion of m/z 778 principally causes formation of an ion of m/z 315 (the ion derived from the 2-acyl group) and to a lesser extent an ion of m/z 255 from the 1-group.



E

SCHEME 3

In summary the combination of FAB for producing negative ions and MS-MS for collisionally activating them is particularly useful for determining phospholipids and their fatty acid components. Structure identity and position of attachment of the acid may be obtained from a study of the intact lipid without degrading or derivatizing it. FAB and MS-MS are also appropriate for obtaining $(M\text{-}H)^-$ ions for phosphatidylserine and for determining its structure.

As is usual, purity of sample is not critical because MS-MS methods are suited to mixture analysis. Collisional activation of $(M\text{-}H)^-$ ions for phosphatidylserines or of the characteristic high mass negative ions of a mixture of phosphatidylcholines may be used to identify acid substituents of a given component. Complete characterization of the carboxylate fragments (that is, both verification of compound type and characterization of structural features) of a pure complex lipid is readily accomplished by selecting the appropriate ion followed by collisional activation. For mixtures of complex lipids, acid identification (from mass only) and extent of unsaturation can always be done, but the feasibility of detailed characterization of acid components is dependent on the composition of other lipids in the mixture. If each lipid gives unique fatty acid carboxylates, then complete structural analysis is possible just as in the case of pure complex lipids. However, if two or more lipids of a mix-

TABLE 1

Phosphatidylcholine Full Scans

Structure	Phospholipid	Acyl, 1-acid	Fragments, 2-acid	High mass ions		
				A	B	C
I	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine	225(92)	281(100)	673(5.6)	699(7.5)	744(4.0)
II	1-Oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine	281(81)	255(100)	673(0.9)	699(1.9)	744(.75)
III	1-Oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine	281(86)	283(100)	701(9)	727(17)	772(16)
IV	1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine	283(81)	281(100)	701(8)	727(10)	772(8)
V	1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine	255(100)	279(95)	671(10)	697(17)	742(17)
VI	1-Oleoyl-*sn*-glycero-3-phosphocholine	281(100)		435(30)	461(34)	506(49)

ture give fatty acid carboxylates of the same mass, MS-MS-MS (88) would be needed, first, to produce the carboxylates of a selected lipid ion and, second, to activate by collision the various carboxylates produced in the first step.

ACKNOWLEDGMENT

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Absorption and Distribution of Deuterium-Labeled *trans*- and *cis*-11-Octadecenoic Acid in Human Plasma and Lipoprotein Lipids

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Triglycerides of deuterium-labeled *trans*-11-, *trans*-11-*cis*-11- and *cis*-9-octadecenoic acid (11t-18:1-^{2}H, 11c-18:1-^{2}H) were simultaneously fed to two young adult male subjects. Plasma lipids from blood samples collected periodically for 48 hr were analyzed by gas chromatography-mass spectroscopy. The results indicate (i) the Δ11-18:1-^{2}H acids and 9c-18:1-^{2}H were equally well absorbed; (ii) relative turnover rates were higher for the Δ11-18-1-^{2}H acids in plasma triglycerides; (iii) incorporation of the Δ11-18:1-^{2}H acids into plasma phosphatidylcholine was similar to 9c-18:1-^{2}H, but distribution at the 1- and 2-acyl positions was substantially different; (iv) esterification of cholesterol with 11t-18:1 was extremely low; (v) chain shortening of the Δ11-18:1-^{2}H acids was 2–3 times greater than for 9c-18:1-^{2}H; (vi) no evidence for desaturation or elongation of the 18:1-^{2}H acids was detected; and (vii) a 40% isotopic dilution of the 18:1-^{2}H acids in the chylomicron triglyceride fraction indicated the presence of a substantial intestinal triglyceride pool. Based on our present knowledge, these metabolic results for Δ11-18:1 acids present in hydrogenated oils and animal fats indicate that the Δ11 isomers are no more likely than 9c-18:1 to contribute to dietary fat–related health problems.
***Lipids* 21, 589–595 (1986).**

The *trans*-11- and *cis*-11-octadecenoic acids (11t-18:1 and 11c-18:1) are widely distributed in food products that contain partially hydrogenated vegetable oil and ruminant fats (1,2). In addition, dietary fats from nonruminants contain 11c-18:1, which is produced endogenously by chain elongation of *cis*-9-hexadecenoic acid (3,4).

The metabolism of 11t- and 11c-18:1 has been reasonably well studied in animals (5–10), but the only information available for humans is composition data for tissue fatty acids (11,12). These data indicate that the *trans* monoene fractions from human liver, heart and adipose tissue contain 22–32% 11t-18:1 and that the *cis* monoene fractions contain 4–9% 11c-18:1. Compared to these human data, the monoene fractions from liver and heart phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of rats fed 15% hydrogenated safflower oil contained up to 31% 11t-18:1 and 37% 11c-18:1 (9).

The purpose of this study with adult male subjects was to compare directly absorption, distribution in plasma lipids, turnover rates and interconversion of 11t- and 11c-18:1 with 9c-18:1. These data were obtained by simultaneously feeding mixtures of triglycerides (TG) containing deuterium-labeled 11t-, 11c- and 9c-18:1. The results are compared to data from previous human studies, in which other *trans*- and *cis*-18:1 positional isomers were fed, and to animal and in vitro studies with 11t- and 11c-18:1.

EXPERIMENTAL

Deuterium-labeled fat mixture and sampling schedule. The amounts and identities of the deuterium-labeled fatty acids in the TG mixtures fed are summarized in Table 1. Synthesis of the labeled fatty acids (11t-18:1-15,15,16,16-$^2H_4$, 11c-18:1-14,15-$^2H_2$, 11c-18:1-14,14,15,15,-17,18-$^2H_6$, 9c-18:1-9,10-$^2H_2$ and 9c-18:1-14,14,15,15,17,18-$^2H_6$) has been described previously (13–15).

The mixture of deuterated TG (ca. 22–24 g) was heated to ca. 65 C and with a high speed blender was emulsified with 30 g calcium cassinate, 30 g dextrose and 15 g sucrose in 200 ml water, which also had been warmed to ca. 65 C. This mixture containing the labeled fats was fed at 8 a.m. in place of the subjects' normal breakfast. A light lunch at ca. 12:45 p.m. and a normal evening meal at ca. 6:30 p.m. were allowed.

TABLE 1

Deuterium-Labeled Fatty Acid Content of Triglyceride (TG) Mixtures Fed

Fatty acids in mixture fed	Melting point of TG (C)	Wt (g)	Percentage	Ratio fed (isomer/9c-18:1)
Subject 1				
11c-18:1-$^2H_2$	3	8.56	38.13	1.26
11t-18:1-$^2H_4$	43	7.09	31.58	1.04
9c-18:1-$^2H_6$	–3	6.80	30.29	
Total		22.45		
Subject 2				
11c-18:1-$^2H_6$	2	8.04	33.06	1.02
11t-18:1-$^2H_4$	43	8.37	34.42	1.06
9c-18:1-$^2H_2$	4	7.91	32.52	
Total		24.32		

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Blood samples (ca. 38 ml each) were obtained by venipuncture at 0, 2, 4, 6, 8, 12, 15, 24 and 48 hr for plasma lipid class fatty acid analysis. Samples (ca. 38 ml) were also collected at 2, 4, 6, 8, 12, 15 and 24 hr for lipoprotein lipid class fatty acid analysis. Standard preparative ultracentrifuge methods were used to separate chylomicron (CHYLO), very low density (VLDL), low density (LDL) and high density lipoprotein (HDL) fractions (16). Samples were analyzed by electrophoresis to confirm the purity of the lipoprotein fractions.

Subjects. The subjects were two Caucasian males, ages 24 and 29. Medical histories, physical examinations and clinical blood profile data indicated that the subjects were in excellent health, had no history of congenital ailments and had not taken any medication for at least 3 wk before the study. Dietary histories confirmed that food selection was typical of American diets reported in the Hanes and USDA surveys (17,18). The subjects' height/weight ratios (190 cm/79.9 kg and 178 cm/70.9 kg), blood pressure (110/70 and 100/60), and serum cholesterol (175 mg/dl and 176 mg/dl), fasting TG (46 mg/dl and 59 mg/dl) and HDL levels (42 mg/dl and 50 mg/dl) were within normal ranges.

The subjects were requested to follow, for 1 wk prior to feeding, the standard diet for diabetics recommended by the American Diabetic Association. The purpose was to help the subjects select diets of ca. 40% fat, 40% carbohydrate and 20% protein. No significant changes in the subjects' weights were observed during this period, which indicated a stable energy balance. The subjects were fasted for 10–12 hr before the experimental meals were fed.

Blood lipid analysis and analytical methodology. Standard methods were used to extract and isolate plasma and lipoprotein TG, free fatty acids (FFA), cholesteryl ester (CE), PE, phosphatidylserine-phosphatidylinositol, PC, lysophosphatidylcholine, sphingomyelin (SM), 1-acylphosphatidylcholine (PC-1) and 2-acylphosphatidylcholine (PC-2) fractions.

Total lipids were extracted with $CHCl_3$/MeOH (2:1, v/v) (19), neutral and phospholipid classes were separated by preparative thin layer chromatography (TLC) (20,21), and methyl esters were prepared with HCl-MeOH (22). Phospholipase A_2 hydrolysis of PC with *Ophiophagus hannah* venom (23) was followed by TLC separation and methylation of the reaction products. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis of the products were used to determine the distribution of deuterium-labeled fatty acids in the 1- and 2-acyl positions of PC.

Quantitation of deuterium-labeled fatty acids incorporated into plasma lipids was achieved by GC-MS analysis of their methyl esters. A Finnigan 4500 quadrupole mass spectrometer was operated in a chemical ionization mode with isobutane as the ionization reagent gas. GC separations of the plasma methyl esters were achieved with a 30 M × 0.32 mm DB-1 (methylsilicone) bonded fused silica capillary column purchased from J&W Scientific.

The MS methodology included selected ion monitoring of each GC peak, followed by integration of the peak areas at appropriate mass numbers. The specific operating conditions and computer-assisted storage and processing of the MS data has been described previously (24).

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

Fatty acid composition of plasma lipid classes was also determined by GC with a Packard model 428 chromatograph equipped with SP2330 (30 M × 0.2 mm) and SP2560 (100 M × 0.25 mm) fused silica capillary colmns supplied by Supelco (Bellefonte, Pennsylvania). Methyl ester GC peaks were identified and quantitation was confirmed by analysis of authentic standards and mixtures of known composition and by MS data.

Calculation and application of ratio data. Experimental data from early dual-labeled radioisotopic tracer studies document the use of ratios for comparison of metabolically similar compounds (25). These ratios reflect the sum of the overall rate constants for the metabolic reactions involved and consistently agree with results obtained by nonisotope methods. One definite advantage of the use of ratios is automatic compensation for much of the biological variation and experimental error. There is no experimental evidence to suggest that unequal isotopic dilution of labeled compounds by unlabeled endogenous compounds influences these ratios. Thus, the difference in removal of 11t-18:1-^{2}H compared to 9c-18:1-^{2}H from plasma TG is not affected by the large difference in pool size for 9c-18:1-^{1}H and 11t-18:1-^{1}H. Also, for example, the higher ratios of the chain-shortened products (9t-16:1-^{2}H and 9c-16:1-^{2}H compared to 7c-16:1-^{2}H) are not a function of the difference in specific activity of their precursors.

In this paper, "selectivity values" indicate the preferential incorporation or discrimination of the 11t- and 11c-18:1-^{2}H acids into plasma lipids relative to the "control" fatty acid (9c-18:1-^{2}H). Selectivity values are calculated according to the following equation: selectivity value = $\log_{10}$ (experimental ratio/fed ratio), where experimental ratio equals the ratio of 11t- or 11c-18:1-^{2}H to 9c-18:1-^{2}H in the plasma lipid samples, and fed ratio equals the ratio of the corresponding fatty acids in the fed mixture.

The use of logarithms results in nonlinear values; they provide a positive value greater than 0.0 when 11t- or 11c-18:1-^{2}H is preferentially incorporated and, conversely, a negative value less than 0.0 when discrimination against incorporation of 11t- or 11c-18:1 occurs.

"Percent difference values" are used in Table 2 as an alternative to selectivity values for comparing the isotopic data; they are more easily visualized since they avoid the nonlinearity of logarithm functions. These values are calculated by the following equation: percent difference value = (experimental ratio/fed ratio – 1.0) × 100. If the value for the experimental ratio/fed ratio is less than 1.0, then the reciprocal is calculated before subtraction of 1.0 and the percent difference value is given a negative sign to indicate discrimination. Selectivity

and percent difference values are generally calculated from the ratio of the areas under the curves defined by eight data points. This approach provides a weighted average.

The purpose of these calculations is to provide data that can be readily compared. Actual percentage enrichment data are plotted in the figures to show the magnitude of the actual percentages of each labeled fatty acid incorporated and the variation in the time at which maximum enrichment occurred.

RESULTS

Incorporation of 11t- and 11c-18:1 fatty acids into plasma lipids. The uptake and turnover of the Δ11-18:1-^{2}H acids relative to 9c-18:1-^{2}H are plotted in Figures 1, 2 and 3 for plasma TG, FFA, PE, PC and CE. The data are plotted as the percentage of deuterated 18:1 acids in the total fatty acids from various lipid classes. Data for total percent 18:1-^{2}H enrichment, time of maximum 18:1-^{2}H incorporation, overall curve shapes and the selectivity values were similar for both subjects. Large differences between utilization of the 11t- and 11c-18:1-^{2}H acids and 9c-18:1-^{2}H were obvious for the CE and PE samples. The rate of removal of 11t-18:1-^{2}H and 11c-18:1-^{2}H from plasma TG was higher than for 9c-18:1-^{2}H. Incorporations of the labeled fatty acids into plasma FFA and total PC were similar.

The validity of plotting these data as percent 18:1-^{2}H enrichment was confirmed by comparison with absolute concentration (μg/ml) data. An example of this comparison is shown in Figure 3 for plasma CE. The similarity of the curves for percent enrichment and absolute concentration illustrate the reliability of the data, even at 0.1% 18:1-^{2}H enrichment.

Selectivity and percent difference values are listed in Table 2 and attach a numerical value to the qualitative differences illustrated by the curves in Figures 1–3. (The calculations of these values are described in detail in the Experimental section.) A difference in selectivity values of 0.1 is substantial because they are log values. For example, selectivity values between 0.0 and 0.1 represent a difference of about 25% between the experimental ratio and the fed mixture ratio. An advantage of using ratio data is that they effectively compensate for the influence of uncontrollable variables (i.e., biological variation, dietary habits). In addition, the percent enrichment and absolute concentration data are similar, which adds to the confidence level of these results.

Absorption of 11t- and 11c-18:1-^{2}H. The CHYLO-TG data in Figure 4 were plotted as both percent 18:1-^{2}H enrichment and wt/ml of plasma. This figure provides evidence that the structure and physical properties of the

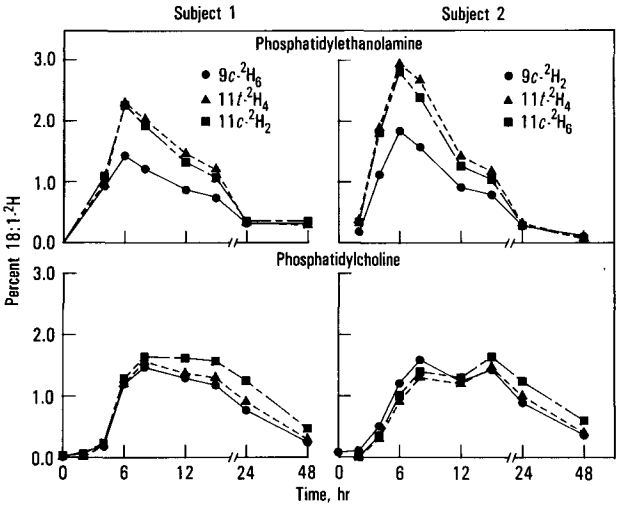

FIG. 2. Uptake and disappearance of 11t-18:1-^{2}H, 11c-18:1-^{2}H and 9c-18:1-^{2}H in human plasma phosphatidylethanolamine (PE) and phosphatidylcholine (PC) fractions. Data for each 18:1-^{2}H acid are plotted as percent isotopic enrichment in the total methyl esters from PE and PC.

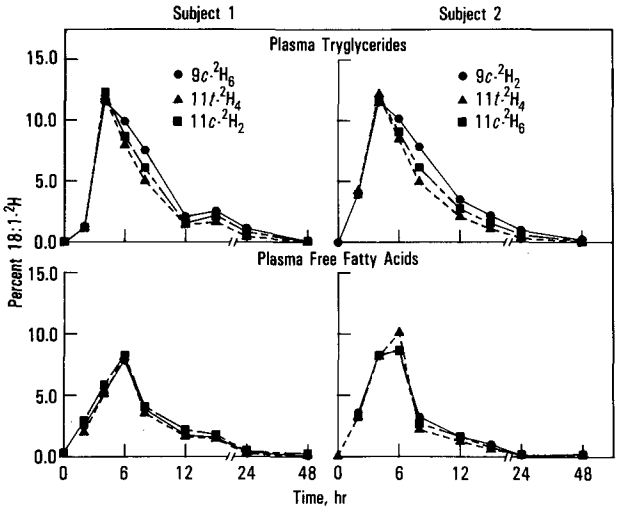

FIG. 1. Uptake and disappearance of 11t-18:1-^{2}H, 11c-18:1-^{2}H and 9c-18:1-^{2}H in human plasma triglyceride (TG) and free fatty acid (FFA) fractions. Data for each 18:1-^{2}H acid are plotted as percent isotopic enrichment in the total methyl esters from TG and FFA.

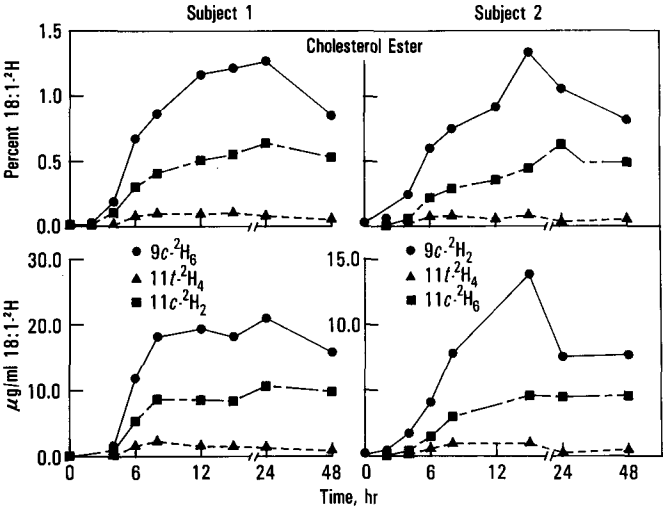

FIG. 3. Uptake and disappearance of 11t-18:1-^{2}H, 11c-18:1-^{2}H and 9c-18:1-^{2}H in human plasma cholesteryl ester (CE). Data for each 18:1-^{2}H acid are plotted both as percent isotopic enrichment and a μg/ml of 18:1-^{2}H in the total methyl esters from CE.

Δ11-18:1-^{2}H acids do not influence absorption. Both the percent enrichment and concentration (wt/ml) data for these subjects were nearly identical and indicate that the Δ11-18:1-^{2}H and 9c-18:1-^{2}H acids were equally well absorbed. Also, note that the 4-hr CHYLO-TG samples contain a maximum of about 60% deuterium-labeled and 40% nonlabeled fatty acids and that a definite second peak occurs 12 hr after feeding. These data indicate the presence of a substantial intestinal TG pool, which retains a portion of the deuterium-labeled TG from the fed mixture.

Incorporation of 11t- and 11c-18:1-^{2}H into lipoprotein lipid classes. Discrimination against incorporation of the Δ11-18:1-^{2}H acids into lipoprotein TG samples increased as the density of the lipoprotein particle increased (Fig. 5). This apparent discrimination is probably the result of the higher rate of removal of the Δ11-18:1-^{2}H acids from the circulation system, consequently lowering the concentration of Δ11-18:1-^{2}H acids available for subsequent incorporation into LDL- and HDL-TG.

Maximum percent enrichment, time of maximum 18:1-^{2}H incorporation and area selectivity values for lipoprotein lipid classes are summarized in Table 3. Maximum isotope enrichment and times are included to demonstrate that maximum enrichment of CHYLO-TG and FFA fractions occurred during absorption. The data also show that maximum enrichment of the lipoprotein neutral lipids is time-dependent, which suggests that exchange of lipoprotein TG is slow compared to sampling frequency. These lipoprotein data supplement the plasma lipid class data and are useful for confirming and understanding the plasma lipid class results. The lipoprotein PC data show a constant nonselective incorporation of the Δ11-18:1 acids, similar incorporation times and similar total 18:1-^{2}H enrichment. This similarity between the CHYLO-, HDL- and LDL-PC data indicates that significant amounts of 18:1-^{2}H acids were not incorporated into CHYLO membrane during absorption because the maximum level of deuterated fatty acids in the CHYLO fraction was found in the 12-hr sample rather than the 4- or 6-hr samples. The maximum percent enrichment data also indicate that either exchange of intact PC between the different lipoprotein fractions or deacylation-reacylation of PC is rapid compared to sampling frequency.

Chain shortening, desaturation and elongation of 11t- and 11c-18:1-^{2}H acids. The methyl esters of plasma and lipoprotein lipids were analyzed by capillary GC-MS for deuterium-containing C_{16}, C_{18} and C_{20} fatty acids. Elongation and desaturation products from 11t-, 11c- or 9c-

TABLE 2

Incorporation of 11t- and 11c-18:1-^{2}H Compared to 9c-18:1-^{2}H into Human Plasma Lipid Classes

Lipid class[a]	Selectivity values[b]		Percent difference[b]	
	11t/9c	11c/9c	11t/9c	11c/9c
TG	-0.18	-0.07	-51	-17
CE	-1.20	-0.35	-1485	-124
FFA	-0.07	-0.03	-17	-7
PE	0.15	0.10	41	26
PC	0.0	0.06	0	15
PC-1	0.59	0.21	289	62
PC-2	-0.87	-0.07	-641	-17
SM	-0.10	0.07	-25	17

[a]TG, triglyceride; CE, cholesterol ester; FFA, free fatty acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PC-1, 1-acylphosphatidylcholine; PC-2, 2-acylphosphatidylcholine; SM, sphingomyelin.
[b]Positive values indicate selective incorporation; negative values indicate discrimination against incorporation.

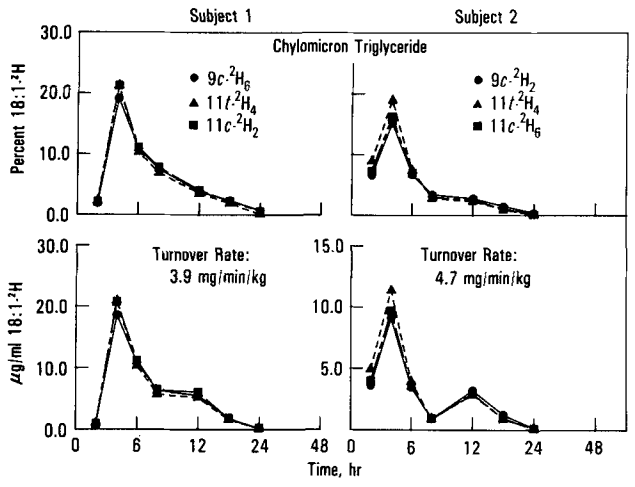

FIG. 4. Incorporation and turnover of 11t-18:1-^{2}H, 11c-18:1-^{2}H and 9c-18:1-^{2}H in human chylomicron triglycerides. Data for each 18:1-^{2}H acid are plotted both as percent isotopic enrichment and as μg/ml of 18:1-^{2}H in the total triglyceride methyl esters.

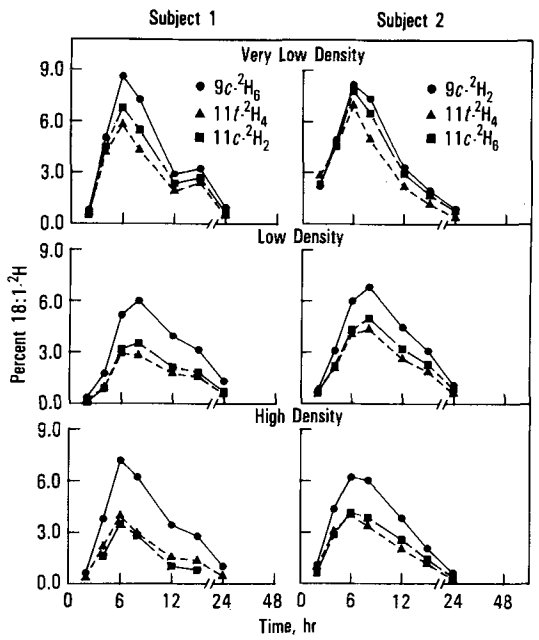

FIG. 5. Uptake and distribution of 11t-18:1-^{2}H, 11c-18:1-^{2}H, 9c-18:1-^{2}H in human lipoprotein triglyceride fractions. Data for each 18:1-^{2}H isomer are plotted as percent isotopic enrichment in the total methyl esters from lipoprotein triglycerides.

18:1-^{2}H fats were not detected by MS and, based on instrument sensitivity, are estimated to be below 0.001%. The 9t-, 9c- and 7c-16:1-^{2}H acids present in plasma TG samples (Fig. 6) were formed by chain shortening 11t-, 11c- and 9c-18:1-^{2}H, respectively. These products were readily detected in both plasma and lipoprotein TG samples, but were generally not detectable in other lipid classes. Maximum levels of 9t-16:1-^{2}H in the lipoprotein TG samples ranged from 0.19 to 0.5% of the total TG and were consistently 3–4 times higher than 7c-16:1-^{2}H. The levels of 9c-16:1-^{2}H (0.1 to 0.28%) were about twice as high as 7c-16:1-^{2}H percentages. These data indicate that formation of the 16:1-^{2}H chain-shortened products from 11t- and 11c-18:1-^{2}H were 2–3 times greater than for 9c-18:1-^{2}H products.

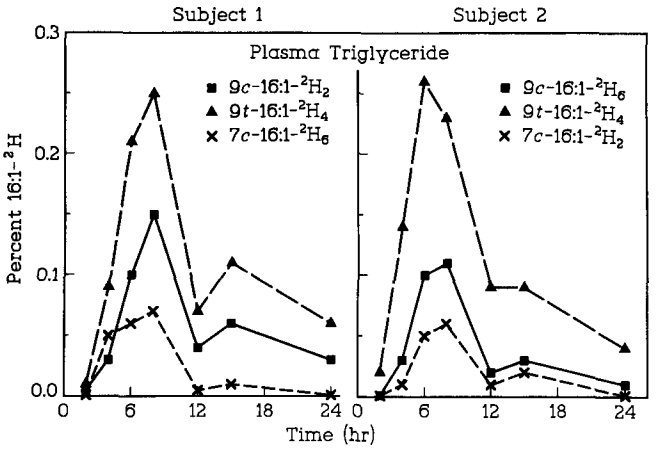

FIG. 6. Percent of 9t-16:1-^{2}H, 9c-16:1-^{2}H and 7c-16:1-^{2}H formed by chain shortening of 18:1-^{2}H subtrates and incorporated into plasma triglyceride samples. Data are plotted as percent isotopic enrichment in the total methyl esters from plasma triglyceride.

DISCUSSION

Although the results reported for the Δ11-18:1 acids are from only two subjects, the data are believed to be representative of the metabolism of normal young adult males. This conclusion is based in part on the fact that the 9c-18:1-^{2}H data for various plasma and lipoprotein

TABLE 3

Lipoprotein Lipids: Distribution of 11t-, 11c- and 9c-18:1-^{2}H at Maximum Enrichment and Area Selectivity Values[a]

Lipid class[b]	Time (hr)	Percent of total fatty acids			Selectivity value	
		11t-18:1-^{2}H	11c-18:1-^{2}H	9c-18:1-^{2}H	11t/9c	11c/9c
Triglyceride						
CHYLO	4	12.15	12.02	11.65	-0.19	-0.07
VLDL	6	6.40	7.30	8.40	-0.17	-0.09
LDL	8	3.60	4.25	6.45	-0.28	-0.22
HDL	6	4.00	3.80	6.70	-0.30	-0.27
Cholesteryl ester						
CHYLO	8	0.24	0.38	1.09	-0.79	-0.39
VLDL	15	0.23	0.46	1.13	-0.76	-0.44
LDL	24	0.16	0.52	1.18	-0.88	-0.44
HDL	15	0.18	0.61	1.34	-0.88	-0.36
Free fatty acid						
CHYLO	6	9.11	8.50	9.32	-0.07	-0.02
VLDL	8	1.46	1.41	1.64	-0.14	-0.09
LDL	8	1.10	1.22	1.45	-0.19	-0.13
HDL	6	1.66	1.13	1.97	-0.16	-0.25
Phosphatidylethanolamine						
CHYLO	4	1.20	1.10	0.86	0.08	0.10
VLDL	6	0.35	0.50	0.40	-0.12	0.05
LDL	6	1.09	1.09	0.80	0.11	0.05
HDL	6	1.84	1.30	0.99	0.26	0.02
Phosphatidylcholine						
CHYLO	12	1.50	1.63	1.45	0.02	0.01
VLDL	12	1.12	1.18	1.09	0.03	0.05
LDL	12	1.54	1.71	1.52	0.00	0.04
HDL	12	1.69	1.40	1.47	0.05	-0.01

[a]Average of two subjects.
[b]CHYLO, chylomicron; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

fractions are consistent with similar 9c-18:1-^{2}H data from nine subjects used in previous studies (26–30). In addition, medical information and clinical data revealed no abnormalities related to fat metabolism. Also, fatty acid composition data for plasma, red blood cell and platelet lipid classes are consistent with data reported for large numbers of subjects.

The curves in Figure 4 plus the selectivity data in Table 3 for the CHYLO-TG samples clearly show that the 11t-, 11c- and 9c-18:1 fatty acids are equally well absorbed. Comparable CHYLO-TG data for labeled 11t- and 11c-18:1 from animals are not available, but plasma TG data from rats fed hydrogenated oils and unlabeled 11c-18:1 indicate that the 11t- and 11c-18:1 acids are well absorbed (5–10,31).

CHYLO-TG turnover rates were calculated as previously described (30). Total plasma volume was estimated by using the standard value of 39 ml/kg body weight. The total weight of labeled plus unlabeled TG that was absorbed and cleared was estimated at 37.4 g (subject 1) and 40.5 g (subject 2). These values are based on the total amount of deuterated TG fed and the approximate 60% deuterated fat enrichment in the 4-hr CHYLO-TG samples. Total absorption was assumed to be complete in a 2-hr period. The turnover rates of 3.9 and 4.7 mg/min/kg calculated in this manner are minimum values, since the minimum isotopic dilution value was used to determine the total weight of CHYLO-TG cleared. These minimum turnover rates are lower than the 5.18 mg/min/kg value reported for a subject fed 27.4 g of a mixture containing 10t-, 10c- and 9c-18:1-^{2}H. Calculation of these turnover rates on a per-gram-of-deuterated-fat-fed basis gave turnover rates of 0.174, 0.193 and 1.89 mg/min/kg/g of deuterated TG fed. These values are very consistent, considering the limited control we had over the experimental variables.

The Δ11-18:1-^{2}H content of plasma and lipoprotein lipid classes plus the selectivity values in Table 2 and 3 show a substantial discrimination against incorporation of 11t-18:1 into CE and the 2-acyl PC position, but only the CE fraction strongly excluded 11c-18:1. The exclusion of 11t-18:1 from plasma CE was more extreme than observed in similar studies with deuterium-labeled 9t-, 10t-, 12t- and 13t-18:1 acids (26–30). Since discrimination was very strong for all isomers, the differences between the negative selectivity values for the various *trans* positional 18:1 isomers may not be physiologically significant. The negative plasma CE selectivity value of -0.35 for 11c-18:1was intermediate between the extremes of -0.16 for 12c-18:1 and -0.72 for 10c-18:1. These differences for the *cis* 18:1 isomers values are large, and they reflect metabolic differences that may be physiologically important.

The exclusion of the Δ11-18:1-^{2}H acids from CE is the result of both low PC acyl transferase and lecithin:cholesterol acyl transferase (LCAT) activities because the fatty acid in the 2-acyl position of PC supplies the fatty acid used to esterify cholesterol. Thus, the 11t-18:1 negative selectivity value of -0.87 for PC-2 is responsible for about 43% of the apparent discrimination against incorporation of 11t-18:1 into CE. In contrast, the 11c-18:1 selectivity value of -0.35 for CE is primarily due to LCAT, since the 11c-18:1 selectivity value for PC-2 is -0.07. The negative PC-2 selectivity value for 11t-18:1 contrasts with the preferential incorporation of 11t-18:1 into the PC-1 position. Thus, PC-1 acyl transferase utilizes 11t-18:1 more like 18:0 than 9c-18:1. In contrast, PC-2 acyl transferase activity for 11c-18:1 is similar to 9c-18:1.

Plasma PE selectivity values are positive and not greatly different for 11t- and 11c-18:1. These data contrast with selectivity values found in similar human studies with other deuterated 18:1 positional isomers (26–30). For example, incorporation of 10t-, 12t- and 13t-18:1 was similar to 9c-18:1, but 9t- and 11t-18:1 values were positive. Selectivity values for 11c-18:1 were similar to 12c-18:1, which was in contrast to the strong exclusion observed for 13c-18:1.

A comparison of data for human plasma CE, PC and PE with results reported for the rat and laying hen indicate substantial differences in enzyme specificities. These differences suggest that the Δ11-18:1 acids may have a different biochemical function or role depending on the species. In vivo studies with rats report 11c-18:1 levels about twice as high as 9c-18:1 in tissue phospholipids. Even when the diets contain no 11c-18:1, the 18:1 fraction of heart PC and PE contains 60–70% 11c-18:1 (9,10). For the laying hen, a similar twofold preferential incorporation of radioisotope-labeled 11c-18:1 relative to 9c-18:1 occurred in egg PC (32). Data for 11t- and 11c-18:1 incorporation into tissue lipids are available for rats fed hydrogenated soybean oil (5), hydrogenated peanut oil (6) and hydrogenated safflower oil (7). The rat phospholipid data show strong preferential incorporation of 11c-18:1, in contrast to the human PC, PE and SM data (Table 2). The levels of 11t-18:1 incorporated into rat heart PC and PE were 10–25% lower than dietary levels, and for liver lipids (6) the 11t-18:1 content was about 40% lower than dietary levels. These data differ substantially from the 0–40% preferential incorporation of 11t-18:1-^{2}H in human plasma PE and PC. Rat serum CE data indicated that 11c-18:1 incorporation was similar to dietary levels (5,31), but 11t-18:1 incorporation was 75% lower (5). Compared to CE data from in vivo rat studies (33), the more negative selectivity values for human CE fatty acids indicate a substantial difference between species. This difference is probably the result of the fact that CE synthesis in rats follows the acyl CoA:cholesterol acyl transferase pathway, which prefers oleic acid as a substrate, whereas CE synthesis in human plasma follows mainly the LCAT pathway, which prefers linoleic acid as its substrate (34).

Interconversion of 11t- and 11c-18:1-^{2}H. Negative plasma and lipoprotein TG selectivity values for 11t- and 11c-18:1 were consistent with the negative values previously observed in human studies with the Δ10-, Δ12- and Δ13-18:1 isomers (26–30). These data indicate that those positional 18:1 isomers without a Δ9 double bond are preferentially removed from TG. In addition, the higher ratio of 9t- and 9c-16:1-^{2}H to 7c-16:1-^{2}H suggests a higher initial β-oxidation rate for 11t- and 11c-18:1-^{2}H compared to 9c18:1-^{2}H. These results for the Δ11-18:1 acids were consistent with 16:1 data from rat (7) and human (30) studies, which indicated higher oxidation rates for 10t- and 10c-18:1.

No evidence for chain elongation or desaturation of the Δ11-18:1-^{2}H acids was noted in any lipid fraction. These products can easily be detected at the 0.01% level by the GC-MS methodology used. Since 7–8 g of the 11t- and 11c-18:1-^{2}H isomers were fed, the failure to detect

these products indicates that these reactions have little impact on the metabolism of the Δ11-18:1 isomers in normal diets.

Isotopic dilution during absorption. The percentage isotopic enrichment in CHYLO-TG samples (Fig. 4 and Table 3) indicate that substantial isotopic dilution of the 18:1-^{2}H acids occurred during absorption. The percent isotopic enrichment in the CHYLO-TG samples was a very consistent feature of the data from these two subjects and the nine subjects studied previously (26–30). A plot of the weight of 18:1-^{2}H per ml plasma vs time confirms the percent enrichment data and shows a distinct second peak at 12 hr. These combined data strongly suggest that the intestinal mucosa cells retain an estimated 12–15 g of TG from previous meals, which is displaced by TG from subsequent meals. These data are consistent with recent rat data, which indicated that as much as 50% of the fat incorporated into CHYLO-TG was from an endogenous source (35).

The fact that fasting for 10–12 hr does not deplete the intestinal TG pool may have a significant impact on $^{13}CO_2$ breath tests used to assess fat absorption and oxidation rates. The reason is that if a portion of the isotopic carbon-labeled fats fed is trapped temporarily in this TG pool, it would distort results based on expired $^{13}CO_2$ or $^{14}CO_2$ data.

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Inhibition of the Antidiuretic Hormone Hydroosmotic Response by Phospholipids and Phospholipid Metabolites

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Phospholipid metabolities and phospholipids containing arachidonic acid (AA) inhibited the antidiuretic hormone (ADH)-induced increase in transepithelial water flow in the toad urinary bladder, but had no effect on basal water flow when added to the serosal bathing solution. Other fatty acid–substituted phospholipid metabolites had no effect on osmotic water movement in the presence or absence of ADH. Indomethacin attenuated the inhibitory effects of the AA containing phospholipid metabolities (PMAA), suggesting that the PMAA response required AA release and prostaglandin (PG) formation. PMAA increased PGE formation as measured by radioimmunoassay. PG have been reported to inhibit ADH-stimulated water flow by inhibiting adenylcyclase. PGE_2 (10^{-8} M) had no effect on cyclic AMP-stimulated water flow, whereas exogenous AA and PMAA attenuated the hydroosmotic response to added cyclic AMP. Indomethacin only partially reversed the inhibition by AA of the cyclic AMP–associated water movement, suggesting that the inhibition by AA and PMAA may involve other metabolites of AA than PG.

PG and the AA cascade have been implicated as cellular modulators of the ADH hydroosmotic response. The present results offer additional support to the theory that this system may regulate the intracellular events that are transduced following receptor activation by ADH.
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Antidiuretic hormone (vasopressin, or ADH) is an essential hormone in maintaining water homeostasis. Little is known about the mechanism controlling the cell's responsiveness to hormone or to the cellular effects initiated by hormone receptor interaction. Several studies have shown that phospholipids may play a significant role in the transport of ions and fluid across membrane lipid barriers (1) and contribute to the cell's permeability properties (2). Recently, differences in the lipid content of a transporting epithelium, frog skin, have been related to changes in permeability to ions and water (3,4), and exogenous phospholipid metabolites have been shown to stimulate transepithelial sodium transport (5). Phospholipase C, a phospholipid phosphodiesterase, increases sodium transport across frog skin (6) and inhibits the response to ADH in increasing osmotic water flow in toad urinary bladder (7). The relationship between phospholipid turnover and the inhibition of the ADH-induced hydroosmotic response has been suggested to be the result of prostaglandin (PG) production. Many laboratories, using the in vitro toad urinary bladder preparation, have shown that exogenous PG inhibit the increase in water flow induced by ADH (8). Arachidonic acid (AA), a precursor to the formation of PG and a metabolite of phospholipase action, also inhibits vasopressin-stimulated water flow (9), while indomethacin and meclophenamate, inhibitors of PG biosynthesis, enhance the ADH-induced increase in water flow (10–12). It was first suggested by Grantham and Orloff (13) that PG synthesized in ADH-sensitive epithelia modulate the actions of the hormone, and it subsequently has been shown that this attenuation is due to inhibition of the ADH-sensitive adenylcyclase (13–16). ADH stimulates PG production in isolated epithelia (11–17), including an increase in thromboxane formation (17,18). Yorio et al. (12) observed that indomethacin blocked the effect of calcium ionophore A23187 in decreasing ADH and methylxanthine-stimulated water flow. These investigators suggested that PG may act as regulators of the hydroosmotic response through a feedback mechanism on adenylcyclase activity. Most recently, Burch and Halushka (19) have shown that PG increase the concentration of intracellular calcium in toad urinary bladder epithelium, and Berl and Erichson (20) have suggested that PG attenuate the ADH response by primarily interfering with membrane calcium transport. PG thus appear to play a significant role in the regulation of the water flow response to ADH.

The present study examines the hypothesis that the turnover of membrane phospholipids and the production of arachidonate metabolites are involved in regulation of the water permeability response of ADH and are responsible, in part, for returning the cell to its prehormonal state. The effects of several fatty acid–substituted phospholipid and phospholipid metabolites on ADH and cyclic AMP (cAMP)-stimulated water flow were investigated using the toad urinary bladder preparation, a common model for the mammalian collecting tubule.

METHODS

Toads (*Bufo marinus*) were obtained from Nasco (Ft. Atkinson, Wisconsin) and kept in terraria at 25 C.

Toad urinary bladders were prepared as sacs as described elsewhere (21). Toads were double-pithed, and the bladder was excised, cut in half and tied with silk thread onto the end of the glass tube with the mucosal (urinary) side facing inward. Since each toad provided two lobes, one was used as a control and the other for the experimental treatment. The size of the bladder sacs was kept constant to minimize variation of surface area, and each sac was filled with 4 ml of Ringer's solution and placed in a test tube containing 30 ml of Ringer's solution. The bladders were equilibrated for at least 30 min prior to the start of the experiment.

Water movement was measured gravimetrically according to Bentley (21). Water transfer is measured in the presence of an osmotic gradient. The osmotic gradient was established by diluting the mucosal Ringer's solution 1:10. The bladder sacs were weighed to the nearest milligram on the analytical balance. The bladder was then immersed in 30 ml of Ringer's solution, with the experimental group having the phospholipid or

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PG present for 15 min prior to the measurement of osmotic water movement. After 30 min of incubation, the bladder was removed from the bathing solution and reweighed. Following this period, the ADH was added to the serosal side at a concentration of 10 mU/ml to both control and experimental sacs. The bladder was incubated for an additional 30 min and reweighed.

PGE_2 was measured in the serosal fluid bathing hemibladders, which were mounted as sacs, using radioimmunoassay. Immunoreactive PGE was quantitated after rapid extraction according to Powell (22) using octadecylsilyl silica (ODS) column (Sep-pak C_{18} cartridges, Waters Associates, Milford, Massachusetts). This procedure allows for separation of PG from lipids and AA. This was necessary because of the relatively high concentration of exogenous lipid (arachidonate-containing) added to the serosal media. Briefly, the media was acidified to pH 3.5 with formic acid, the acidified sample was applied to the column and the column was washed with 20 ml of water. To remove the polar lipids, 20 ml of 15% aqueous ethanol was passed through the column followed by 20 ml of water to remove the ethanol. The water was removed with 20 ml of petroleum ether, and the fatty acids, including arachidonate, were eluted with 20 ml of petroleum ether/chloroform (65:35, v/v). PGE was removed using 10 ml of methylformate. The PGE eluate was dried under N_2 and reconstituted in RIA assay buffer.

Radioimmunoassay of iPGE was performed using ^{125}I-PGE_2 and antibodies purchased from Seragen Inc. (Boston, Massachusetts). The antibody crossreactivity at 50% B/Bo was 21% for PGE_1, but less than 1% for all other PG. Recovery of ^{3}H-PGE_2 added to the serosal media was greater than 90%.

The Ringer's solution had the following composition (in mmol): NaCl, 111; KCl, 3.35; $CaCl_2$, 2.7; $MgCl_2$, 0.5; $MaHCO_3$, 4.0; and glucose, 5. This solution was aerated, and the pH was 8.0 at the beginning of the incubation period and remained at this value for the duration of the experiment.

The statistical analysis was performed using a Radio Shack TRS-80 microcomputer with statistical programs from Tallarido and Murray (23). Student's t-test for paired observations between two sample means was applied to the data.

Arginine vasopressin (ADH), PGE_2, indomethacin, phosphatidic acid, cardiolipin, dioleoylphosphatidylcholine, stearoyl lysophosphatidylcholine, lysophosphatidylchloline oleoyl and cyclic AMP were purchased from Sigma Chemical Co. (St. Louis, Missouri). 1-Stearoyl-2-arachidonylglycerol (SAG) and diarachidonylglycerol (DAG) were synthesized by Avanti Polar Lipids Inc. (Birmingham, Alabama). Diarachidonylphosphatidylcholine and lysophosphatidylcholine arachidonyl were bought from Serdary Research Laboratory (London, Ontario, Canada), and arachidonic acid came from Calbiochem-Behring Corp. (La Jolla, California).

All of the lipids were suspended in chloroform, dried with nitrogen gas and sonicated (2-sec bursts) in normal Ringer's solution prior to incubation with the toad urinary bladders.

RESULTS

Effect of DAG on osmotic water flow in the presence and absence of ADH. Preincubation of toad bladders with DAG decreased the hydroosmotic response of ADH (Table 1). DAG had no effect on basal water flow in the absence of hormone. Since DAG contains two molecules of AA, the inhibitory effect seen with DAG could be due to the release of AA and subsequent PG synthesis. To test this possibility, indomethacin (10^{-5} M), a PG synthesis inhibitor, was added to the serosal side of the toad bladder prior to adding DAG. Indomethacin, either alone or in combination with DAG, had no effect on basal water flow (Table 1), but indomethacin partially reversed the inhibition normally observed with DAG on ADH-stimulated water flow (Table 1).

To determine at which position AA was being re-

TABLE 1

Effect of Diarachidonylglycerol (DAG) on the ADH Hydroosmotic Response[a]

	Water movement (mg/30 min)		
	Period I (no hormone)	Period II (with ADH)	P value[b] A vs B, period II
DAG (50 μg/ml) [6]	55 ± 10	704 ± 103	<.05
Control [6]	67 ± 45	1207 ± 109	
DAG (100 μg/ml)[c] [12]	51 ± 7	482 ± 57	<.01
Control [12]	66 ± 21	1106 ± 95	
Indomethacin (10^{-5} M) [6]	32 ± 6	1240 ± 132	NS
Control [6]	31 ± 4	1223 ± 107	
DAG + Indomethacin (10^{-5} M) [12]	54 ± 7	698 ± 50	<.01
DAG (100 μg/ml) [12]	44 ± 7	329 ± 54	

[a]Paired hemibladders were incubated in Ringer's solution. Values are means ± S.E. Number of experiments is indicated in brackets.
[b]Statistical analysis of a paired sample t-test. NS, not significant.
[c]A 15-min preincubation with DAG preceded period II.
[d]Indomethacin was added 15 min prior to the DAG.

leased by endogenous phospholipase for PG biosynthesis, SAG was applied to the bathing medium on the serosal side. SAG, like DAG, had no effect on basal osmotic water movement, even at a concentration of 200 μg/ml. SAG at 100 μg/ml produced a significant inhibition of ADH-stimulated water flow (Table 2). Similar to DAG, the response to SAG was attenuated with indomethacin. In the SAG-treated bladder (100 μg/ml) the water flow response to ADH was 985 ± 92 mg/30 min, whereas if indomethacin was added 15 min prior to the addition of SAG, it was 1322 ± 40 mg/30 min ($p. < .05$ for a paired t-test, N = 6 pairs).

Effects of fatty acid–substituted phospholipids on transepithelial water transport. In the following study, the effects of several different fatty acid–substituted phospholipids were tested as to their ability to alter transepithelial water transport. Diarachidonylphosphatidylcholine (PC-dA) at 50 μg/ml had no effect on basal osmotic water flow or that induced by ADH (Table 3), whereas at 100 μg/ml this lipid inhibited the hydroosmotic response of ADH (Table 3). A time course of the inhibition of vasopressin-stimulated water flow by PC-dA was determined. Figure 1 shows that the onset of inhibition is at 10 min with an appreciable attenuation of the ADH hydroosmotic response and is maximal at 15 min. This attenuation remains throughout the 30-min period.

Other non-arachidonate-containing lipids, like dioleoylphosphatidylcholine (PC-dO) and dipalmitoylphosphatidylcholine (PC-dP) had no effect on osmotic water flow in either the absence or presence of ADH (Table 3). Cardiolipin and phosphatidic acid, similarly, had no effect on basal water flow or on the hydroosmotic response to ADH (Table 2). The effect of lysophospholipids, however, was quite different. Lysophosphatidylcholine arachidonyl (LPC-A), which has AA attached at the number one position of the phospholipid backbone, and lysophosphatidylcholine oleoyl (LPC-O), oleic acid in the number one position, also inhibited the water permeability response of the toad urinary bladder to ADH (Table 2). If the fatty acid was stearoyl in lysophosphatidylcholine, there was no effect on basal or ADH-stimulated water flow.

To determine if the inhibitory response on these lysolipids was a consequence of PG formation, the effects of indomethacin on the actions of LPC-A and LPC-O were investigated. Preincubation of toad bladders with indomethacin attenuated the inhibitory effect of LPC-A on ADH-induced osmotic water movement, but had no effect on the LPC-O response (Table 4).

Effects of fatty acid–substituted phospholipids on iPGE formation. The above findings suggest that the inhibitory effects of the AA-substituted phospholipids may be mediated in part by an increase in PG formation. This could occur either through an increase in the release of

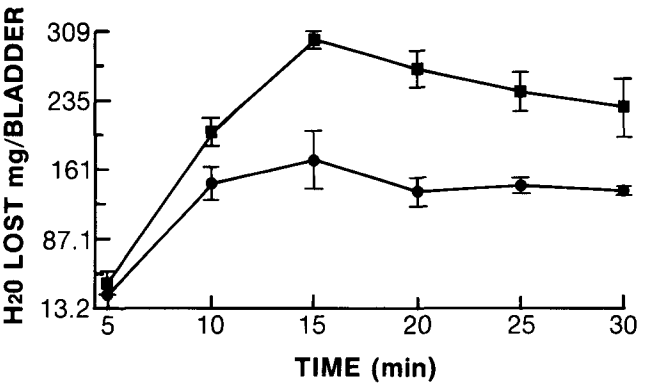

FIG. 1. Time course for the response of diarachidonylphosphatidylcholine (PC-dA) on transepithelial water flow in ADH-stimulated toad urinary bladder. ■, ADH-treated bladders; ●, water flow in the presence of PC-dA plus ADH (each curve's points are means ± S.E. of six paired experiments).

TABLE 2

Effect of Phospholipid Metabolites on Transepithelial Water Transport across Toad Urinary Bladder[a]

	Water movement (mg/30 min)		
	Period I (no hormone)	Period II (with ADH)	P value[b] A vs B, period II
SAG (50 μg/ml) [11]	47 ± 6	1016 ± 95	>.05
Control [11]	45 ± 9	1006 ± 120	NS
SAG (100 μg/ml) [12]	33 ± 7	1121 ± 48	<.05
Control [12]	31 ± 9	1543 ± 61	
Cardiolipin (100 μg/ml) [6]	35 ± 7	1087 ± 61	>.05
Control [6]	35 ± 9	1091 ± 30	NS
Phosphatidic acid (100 μg/ml) [12]	30 ± 5	1102 ± 49	>.05
Control [12]	33 ± 4	1223 ± 37	NS

[a]All lipids were added 15 min prior to the hormone. Values are means ± S.E. Number of experiments is indicated in brackets.
[b]Statistical analysis of a paired sample t-test. NS, not significant.
[c]SAG (stearoylarachidonylglycerol).

TABLE 3

Effect of Fatty Acid-Substituted Phospholipids on Transepithelial Water Transport[a]

	Water movement (mg/30 min)		
	Period I (no hormone)	Period II (with ADH)	P value[b] A vs B, period II
PC-dA (50 μg/ml) [6]	58 ± 15	1170 ± 61	>.05
Control [6]	42 ± 6	1290 ± 61	NS
PC-dA (100 μg/ml) [12]	47 ± 8	1109 ± 54	<.05
Control [6]	36 ± 6	1366 ± 73	
PC-dO (100 μg/ml) [6]	45 ± 10	1228 ± 130	>.05
Control [6]	44 ± 12	1300 ± 85	NS
PC-dP (100 μg/ml) [6]	67 ± 26	1268 ± 201	>.05
Control [6]	56 ± 17	1200 ± 115	NS
LPC-A (100 μg/ml) [6]	34 ± 7	1065 ± 118	<.05
Control [6]	73 ± 13	1563 ± 103	
LPC-O (100 μg/ml) [8]	52 ± 8	345 ± 119	<.05
Control [8]	81 ± 22	955 ± 135	
LPC-S (100 μg/ml) [8]	58 ± 7	709 ± 98	>.05
Control [8]	49 ± 6	832 ± 102	NS

[a]All lipids were added 15 min prior to the hormone. Values are means ± S.E. Number of experiments is indicated in brackets.
[b]Statistical analysis of a paired sample t-test. NS, not significant.
[c]PC-dA, diarachidonylphosphatidylcholine; PC-dO, dioleoylphosphatidylcholine; PC-dP, dipalmitoylphosphatidylcholine; LPC-A, lysophosphatidylcholine arachidonyl; LPC-O, lysophosphatidylcholine oleoyl; LPC-S, lysophosphatidylcholine stearoyl.

TABLE 4

Effect of LPC-A and LPC-O on the Hydroosmotic Response to ADH in the Presence of Indomethacin[a]

	Water movement (mg/30 min)		
	Period I (no hormone)	Period II (with ADH)	P value[b] A vs B, period II
Indomethacin (10^{-4} M) [8]	42 ± 8	1633 ± 61	<.05
Control [8]	40 ± 7	1401 ± 68	
Indomethacin + LPC-A [8]	31 ± 5	1050 ± 85	>.05
Indomethacin [8]	22 ± 3	1181 ± 89	NS
Indomethacin + LPC-O [6]	56 ± 9	359 ± 161	<.05
Indomethacin [6]	100 ± 25	983 ± 184	

[a]LPC-A, lysophosphatidylcholine arachidonyl; LPC-O, lysophosphatidylcholine oleoyl. Paired hemibladder sacs were incubated in Ringer's solution. Indomethacin was added 15 min before the additions of ADH or the lipid. The results are expressed as means ± S.E. Number of experiments is indicated in brackets.
[b]Statistical analyses of a paired sample t-test. NS, not significant.

AA from membrane lipids or, most likely, as a consequence of an increase in arachidonate release from the exogenous lipid by endogenous lipases. Immunoreactive PGE was measured in the serosal fluid bathing the hemibladders in the presence and absence of exogenous lipids following a 30-min incubation. In the control hemibladders, the basal iPGE synthesis was 0.11 ± .03 pmol min^{-1} $hemibladder^{-1}$, whereas in the presence of DAG it was 1.22 ± .15 pmol min^{-1} $hemibladder^{-1}$. Bladders treated with SAG and PC-dA had iPGE formation rates of 0.57 ± .06 and 1.88 ± .20 pmol min^{-1} $hemibladder^{-1}$, respectively, as compared to their paired controls of 0.08 ±.02 and .10 ± .02 pmol min^{-1} $hemibladder^{-1}$. The concentration of lipid was 100 μg/ml for all lipids tested. There was a significant difference in the rate of iPGE formation in arachidonate-containing lipids compared to their matched controls ($P < .001$ for a paired t-test, n = 6 pairs).

Effect of AA and PG on the hydroosmotic response to cAMP. Previous studies have suggested that PG inhibit the ADH response by preventing cAMP formation (8,12). Exogenous cAMP mimics the actions of ADH by increasing water flow in toad urinary bladder. AA attenuated the cAMP-mediated water flow response, whereas PGE_2 had no effect on this nucleotide response (Table 5). Indomethacin reduced, but did not totally prevent, the inhibition of the cAMP response by AA (Table 5). The phospholipid metabolite DAG also inhibited cAMP-mediated water flow, and this effect was reversed when indomethacin was present (Table 5). The inhibition of the cAMP response LPC-O was not altered by indomethacin.

DISCUSSION

The present results demonstrate that phospholipids and their metabolites containing AA inhibit the hydroosmotic response to ADH in the toad urinary bladder, a common model of the cortical collecting duct. Indomethacin, a cyclooxygenase inhibitor, reduces the inhibition produced by these arachidonate-containing lipids. These results suggest that the inhibition seen with these lipids may be due, in part, to the release of AA with subsequent PG formation. It is well known that exogenous PG inhibit the ADH-induced increase in water flow, and some investigators have localized this effect to inhibition of cAMP formation (8,12,16). Burch and Halushka (18) have shown an increase in PG biosynthesis in isolated epithelial cells from toad urinary bladder in response to ADH, and Zusman et al. (11) observed a similar effect in the intact bladder epithelium. The latter authors have suggested that PG may act as a negative feedback control for the osmotic water flow response to ADH. The present study offers additional support of this contention, but also includes actions of AA metabolites beyond the cAMP generating step.

When DAG was applied to the bathing solution on the corium side (serosal side), an inhibition of ADH-induced water flow was observed. A similar response was seen with SAG, but it was less effective. This could be due to the presence of only one arachidonate molecule per phospholipid as compared to DAG, resulting in less free AA. The actions of these diacylglycerides mimic the response reported for phospholipase C on transepithelial water transport (24). Diacyglycerol is a product of phospholipase C action. Other phospholipid metabolites, such as phosphatidic acid and cardiolipin, had no effect on osmotic water movement in either the presence or absence of ADH. This was rather surprising, as these compounds have been reported to possess ionophoric activity (25). PC-dA, LPC-A and LPC-O inhibited the hydroosmotic effect of ADH, whereas PC-dO, PC-dP and lysophosphatidylcholine stearoyl (LPC-S) had no effect

TABLE 5

Effect of AA, PGE_2, DAG and LPC-O on Cyclic AMP-Induced Transepithelial Water Transport[a]

	Water movement (mg/30 min)		
	Control bladder lobe (no lipid)	Experimental bladder lobe (+ lipid)	P value
Cyclic AMP (2 mM)			
AA (50 μg/ml) [9]	730 ± 192	249 ± 40	<.05
DAG (100 μg/ml) [6]	653 ± 64	380 ± 49	<.05
PGE_2 (10^{-8}) [6]	801 ± 228	574 ± 155	>.05, NS
LPC-O (100 μg/ml) [6]	732 ± 89	160 ± 15	<.01
Indomethacin + cyclic AMP			
10^{-4} M Indomethacin + AA (50 μg/ml) [6]	601 ± 66	513 ± 97	>.05, NS
10^{-4} M Indomethacin + DAG (100 μg/ml) [6]	601 ± 66	513 ± 97	<.05, NS
10^{-4} M Indomethacin + LPC-O (100 μg/ml) [6]	778 ± 154	141 ± 13	>.01

[a]AA, arachidonic acid; PGE_2, prostaglandin E_2; DAG, diarachidonylglycerol; LPC-O, lysophosphatidylcholine oleoyl. Paired hemibladders were incubated in Ringer's solution. Values are means ± S.E. Number of experiments is indicated in brackets. Indomethacin was added 15 min prior to the lipids. Bladder sacs were preincubated with lipids for 15 min before addition of the cyclic AMP.

on ADH-stimulated water flow. In general, only the phospholipids containing AA were active in preventing the increase in water movement induced by ADH; the exception was the action of LPC-O derived from egg lecithin. The reason for the inhibition by LPC-O is not known. Lysophospholipids, however, have detergent-like effects and have been reported to inhibit adenylcyclase in bovine renal medulla (26) and mouse fibroblasts (27). This effect on adenylcyclase may contribute to the inhibitory response, although this is unlikely as LPC-O also inhibited exogenous cAMP-induced water movement, suggesting an action subsequent to the formation of this nucleotide. Additional experiments are needed to delineate the mechanism of action of this lipid.

Indomethacin reversed the effects of DAG, SAG and LPC-A on the ADH hydroosmotic response. This reversal could be a result of inhibition of PG formation, or, as has been previously shown (12), indomethacin could potentiate the actions of ADH. This latter effect also is presumably related to inhibition of PG formation. As predicted, indomethacin did not alter the response seen with LPC-O, suggesting that the PG cascade is not involved in its inhibiting adenylcyclase (12,14). Yorio et al. (12) measured cAMP concentrations in toad urinary bladder epithelial cells and found that PGE_2 (10^{-8} M) decreased cAMP formation, whereas at higher concentrations of PGE_2 (10^{-5} M), cAMP formation was enhanced. In the present study, an increase in iPGE formation was produced by the administration of the arachidonate-containing lipids DAG, SAG and PC-dA. These results suggest that these lipids were acted on by endogenous lipase to release arachidonate and increase PG formation. The increase in PG could mediate the inhibitory effects of the lipids.

PG synthesis is dependent upon phospholipase and cyclooxygenase activity and the concentration of the precursor fatty acid, arachidonate (28). AA has been shown to produce a dose-dependent inhibition of the hydroosmotic response to ADH (29). The present observations concerning the actions of PGE_2 and AA on the water flow response to administered cAMP are, however, confusing. PGE_2 (10^{-8} M) had no effect on the cAMP-stimulated water flow, similar to what others have reported (13,30,31), whereas AA (50 μg/ml) still attenuated the hydroosmotic response to cAMP. The reason for this difference is not clear. AA may be converted to metabolites other than PG and exert its action through this mechanism. This could explain why only a partial reversal of the AA inhibition was observed using indomethacin. Recently, Schlondorff et al. (32) suggested that exogenous PG at high concentrations interfere with vasopressin's response on water flow at a step distal from cyclic nucleotide formation. AA at 10^{-4} M may give rise to high concentrations of PG, which may influence mechanisms subsequent to cAMP formation.

PG and some phospholipid metabolites have been reported to play a role in regulating intracellular calcium concentrations (19,25,33), and calcium ion has been shown to be an important coupling factor in the ADH response. Verapamil, which is a calcium antagonist, prevents the increase in water movement normally accompanying the addition of ADH in toad urinary bladder (34). Schlondorff et al. (32) suggested that the hydroosmotic response of ADH involves the activation of several second messengers, including calcium. Calcium and calcium regulator protein have been implicated in the actions of ADH on transepithelial water flow (35) and in the control of the microtubule-microfilament assembly/disassembly process (36). The microtubule-microfilament system is essential for the expression of the ADH response (37). Burch and Halushka (38) observed that the colchicine-induced inhibition of the ADH-stimulated water flow is prevented by PG synthesis inhibitors. PG increase calcium uptake into toad bladder epithelial cells (19), and it has been reported that increases in intracellular calcium stimulate the disassembly of the microtubular system (39). The actions of the arachidonate-containing lipids may involve an action on the microtubule-microfilament system. The observation that these lipids also inhibited cAMP-mediated water flow is consistent with their action at a subsequent step, perhaps at the level of the cytoskeleton.

AA and PG have been implicated as cellular modulators of the hydroosmotic response of ADH. The present study offers additional support of this system may function to modulate the intracellular events transduced following receptor activation by ADH.

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A Relationship Between Essential Fatty Acid and Vitamin E Deficiency

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To test whether vitamin E deficiency might influence the course of essential fatty acid (EFA) deficiency, Long Evans rats were fed diets containing a marginal amount (1.5% of calories) of 18:2ω6 or 18:3ω3 fatty acid with complete absence of the other and with or without vitamin E. Vitamin E contents decreased continuously in serum and liver in all rats fed the E-free diets but in the brains of only the rats fed the marginal 18:3ω3, E-free diet. It is considered that the vitamin E is cooxidized in the liver with 22:6ω3, since this fatty acid is very low in livers of the rats fed the marginal 18:2ω6 diet but much higher in livers of the rats fed the marginal 18:3ω3 diet. Brain 22:6ω3 values are comparable for both groups. The source of 22:6ω3 is evidently in the mother's milk, since following weaning there is a precipitous drop in 22:6ω3 in serum, liver and carcass of rats on the 18:2ω6–containing diet. No significant signs of EFA deficiency were seen in the E-deficient rats.
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It has been reported from several laboratories (1,2) that oxygen-centered free radicals, whether generated within membrane systems or impinging on them from an external source, can bring about oxidation of membrane polyunsaturated fatty acids and consequent membrane damage. With this information, it is logical to assume that marginal levels of essential fatty acids (EFA) might be reduced to the point of EFA deficiency by a deficiency of vitamin E, a condition leading to signs of free radical damage (3,4). Indeed, under special conditions, signs of EFA deficiency have been produced by vitamin E deficiency in the presence of marginal dietary linoleic acid (5,6).

The present experiments were designed to check these results under more stringently controlled conditions. In addition, the study was planned to distinguish between the effects of dietary linoleic and linolenic acids at marginal levels, each in the complete absence of the other.

MATERIALS AND METHODS

Diets. Linoleic acid (18:2ω6) from Sigma Chemical Co. (St. Louis, Missouri), was methylated with 1% methanolic H_2SO_4 and purified by passage through a silica gel column. Analysis of the methyl ester by capillary gas liquid chromatography (GLC) showed it to consist of 99.0% 18:2 and 1.0% 18:1. Ultraviolet spectrophotometry revealed 0.55% conjugated diene and 0.39% conjugated triene. Since the methyl esters had been purified by silica gel chromatography, the conjugation was considered not to be due to hydroxy or hydroperoxy derivatives. Sigma Chemical Co. methyl linolenate (18:3ω3) was similarly found to be 99.26% 18:3 and to contain 1% conjugated diene. Tripalmitin was purchased from U.S. Biochemical Corp. (Cleveland, Ohio), and was found by GLC to contain traces of 12:0, 14:0, 18:0 and 18.1.

For the linoleate diet, methyl linoleate was mixed with tripalmitin in such proportion that the final fatty acid composition was, in percent, 12:0, 0.16; 14:0, 0.21; 16:0, 85.97; 18:0, 0.23; 18.1, 0.41; 18:2ω6, 13.02. Similarly, the composition of the linolenate fat mixture was, in percent; 12:0, 0.14; 14:0, 0.17; 16:0, 86.93; 18:3ω3, 12.76. The appropriate fat mixture was added to the fat-free diet as 5% (for the linoleate diet) or 5.16% (for the linolenate diet) by weight, providing 1.5% of total calories as linoleate or linolenate. The fat-free portion of the diets was purchased from ICN Nutritional Biochemicals (Cleveland, Ohio), and had the following composition, by weight percent: vitamin-stripped casein, 20; cornstarch, 15; sucrose, 50; fiber, 5; AIN minerals, 3.1; d,l-methionine, 0.3; AIN vitamins (without vitamin E [−E]), 1.0; and choline bitartrate, 0.2. This is the American Institute of Nutrition AIN-76A semipurified modified rat diet (7). Both diets were prepared weekly and the fat mixtures, which were stored at −70 C, were checked weekly for fatty acid composition.

Animals. Black-hooded Long Evans mothers with 6-day-old pups were purchased from Simonsen Laboratories (Gilroy, California). It has been reported (Mustafa, M., School of Public Health, UCLA, personal communication) and confirmed in this laboratory that this strain of rats is more susceptible to vitamin E deficiency than are other commonly used strains and consequently develops deficiency signs earlier.

The marginal linoleate study. For this study, eight mothers with a total of 45 pups were divided into three dietary groups: group 1 was fed the linoleate diet without vitamin E; group 2 was fed the same diet supplemented with 110 mg all racemic α-tocopheryl acetate per kg diet and was pair-fed to group 1; group 3 was fed the same diet as group 2 ad libitum. The male pups were fed the same diets after weaning and were killed at 25, 35 and 45 days of age (19, 29 and 39 days on the diet). They were weighed every 10 days.

The marginal linolenate study. Following analyses of the results of the linoleate study, it appeared that subsequent experiments could be somewhat simplified with no significant change. Sixteen male weanling rats were purchased from Simonsen Laboratories and divided into two groups: group 4 was fed the linolenate diet with no vitamin E; group 5 was fed the same diet supplemented with 110 mg all racemic α-tocopheryl acetate per kg diet. Since the pair-fed and ad libitum-fed groups, 2 and 3, showed no differences, no pair-fed group was used and both 4 and 5 were fed ad libitum. The groups were fed their respective diets for 36 days until killed.

Analyses. In all cases, serum, brain and liver were analyzed for fatty acids and vitamin E. Tissues were frozen on dry ice immediately after dissection and were kept at −70 C until extraction. Only tissues from male rats were analyzed and, following preliminary analyses

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indicating that the pair-fed and ad libitum-fed linoleate groups (2 and 3) were not different, these analyses were averaged.

Lipids were extracted with chloroform/methanol (2:1, v/v) containing 3 mg BHT/100 ml using a Tekmar high speed tissue disintegrator (Cincinnati, Ohio) according to the method of Folch et al. (8). Total lipids were methylated with 1% methanolic H_2SO_4, extracted with ether/petroleum ether (1:1, v/v), washed with water, dried over anhydrous sodium sulfate and purified by silica gel chromatography.

The resulting methyl esters were analyzed by GLC using a Hewlett-Packard model 5830A gas chromatograph (Palo Alto, California) in conjunction with a model 18850A GC terminal integrator and fitted with a 1.8 m × 3.1 mm SP2330 column (Supelco, Bellefonte, Pennsylvania). Temperature was programmed from 180 C to 210 C at 1 C/min with injector and detector at 250 C. A Hewlett-Packard model 5880 gas chromatograph with a model 5880A terminal integrator and equipped with a 60-m glass capillary SP 2340 column was also used. Temperature was programmed from an initial value of 180 C held for 15 min, to a final value of 210 C at 2 C/min. Injector temperature was 225 C and detector temperature 250 C. Values from both chromatographs were compared with the same samples and found to be the same.

Methyl ester peaks were identified partially by direct comparison of retention times with those of known standards. For further identification, in the case of the total liver lipids, methyl esters were separated into classes depending on degree of unsaturation by silver nitrate silica gel column chromatography (9), chromatographed by GLC, hydrogenated and rechromatographed. Both the original unsaturated methyl esters and their saturated derivatives were identified by comparison of their retention times with those of known standards.

Vitamin E was extracted from brain and liver according to the method of Vatassary and Hogan (10) and from serum by the method of Tangney et al. (11) and determined by high pressure liquid chromatography using a Varian 8500 instrument (Palo Alto, California) with a Hewlett-Packard model 3380A integrator, a 25 cm x 4.6 mm Lichrosorb RP 18 (10 μ particle size) column and a fluorescence detector (Fluorichrom, Varian Instruments, Palo Alto, California) with a 220 nm excitation filter and 325 and 360 nm emission filters. Methanol was the eluting solvent at a flow rate of 80 ml/hr.

The values are expressed as the means ± S.D. and the P value was determined using Student's t test.

RESULTS

In Table 1 are listed the total body, brain and liver weights; total brain and liver lipids; and vitamin E content of serum, brain and liver of vitamin E-deficient rats (group 1) after consuming the E-deficient, 18:2ω6 marginal diet for 39 days and of controls fed the same diet supplemented with vitamin E (groups 2 and 3). Serum vitamin E levels are also included for rats that were fed the E-deficient and control diets for 29 and 19 days, respectively. No significant differences were noted between controls and experimental animals in any of the measures except the vitamin E content of liver and serum. Liver

TABLE 1

Characteristics and Tissue Vitamin E Content of Long Evans Rats Fed 18:2 as 1.5% of Total Calories With and Without Vitamin E

	Without vitamin E (group 1)	With vitamin E (groups 2 and 3)	P
Serum vitamin E (μg/ml)			
25 days old	2.32 ± 0.17 (n = 8)	19.85 ± 0.97 (n = 4 + 4)	<0.001
35 days old	0.84 ± 0.05 (n = 8)	14.2 ± 2.7 (n = 4 + 4)	<0.001
45 days old	0.35 ± 0.09 (n = 8)	11.8 ± 2.7 (n = 3 + 4)	<0.001
45-day-old rats only (fed diet for 39 days)			
n	6	7 (3 + 4)	
Body wt	133 ± 14	140 ± 24	NS
Brain wt	1.68 ± 0.07	1.68 ± 0.07	NS
Liver wt	8.02 ± 1.65	7.96 ± 1.37	NS
Brain, total lipid (mg/g)	85.6 ± 0.9	84.4 ± 1.4	NS
Liver, total lipid (mg/g)	55.9 ± 16.1	60.6 ± 11.3	NS
Brain, vitamin E (μg/g)	17.8 ± 8.8	20.9 ± 7.9	NS
Liver, vitamin E (μg/g)	0.49 ± 0.17	28.4 ± 7.5	<0.001

Values are mean ± S.D. Significance is determined by Student's t-test. NS, not significant.

TABLE 2

Characteristics and Tissue Vitamin E Content of Long Evans Rats Fed 18:3 as 1.5% of Total Calories With and Without Vitamin E[a]

57-day-old rats, fed diet for 36 days	Without vitamin E (group 3)	With vitamin E (group 4)	P
Body wt	240 ± 16	233 ± 25	NS
Brain wt	1.86 ± 0.06	1.85 ± 0.08	NS
Liver wt	11.6 ± 1.4	9.5 ± 1.3	NS
Brain, total lipid (mg/g)	85.8 ± 8.3	90.4 ± 7.7	NS
Liver, total lipid (mg/g)	74.8 ± 17.6[a]	62.3 ± 10.0	NS
Serum vitamin E (μg/g)	0.18 ± 0.08	8.3 ± 1.1	<0.001
Brain, vitamin E (μg/g)	6.1 ± 1.1	24.9 ± 7.7	<0.001
Liver, vitamin E (μg/g)	0.22 ± 0.08	22.4 ± 5.4	<0.001

Values are mean ± S.D. for 8 rats.
[a]Fatty livers noted in all animals.

TABLE 3

Fatty Acid Composition (Percentage of Total FID Response) of Liver and Brain Total Lipids of Rats fed a Marginal 18:2 Diet, With or Without Vitamin E, for 39 Days

	Liver		Brain	
Fatty acid	with vitamin E (n = 4)	without vitamin E (n = 4)	with vitamin E (n = 7)	without vitamin E (n = 6)
14:0	0.9 ± 0.3	0.8 ± 0.2	1.1 ± 0.3	1.0 ± 0.5
14:1[a]	–	–	1.0 ± 0	0.1 ± 0
15:0[a]	–	–	2.2 ± 0.2	2.3 ± 0.08
16:0	26.4 ± 4.0	25.6 ± 2.7	17.5 ± 0.6	17.5 ± 0.2
16:1	11.0 ± 1.3	10.6 ± 3.4	0.5 ± 0.05	0.5 ± 0.05
17:0[a]	–	–	3.7 ± 0.2	3.7 ± 0.3
17:1[a]	–	–	2.5 ± 0.1	2.4 ± 0.05
18:0	11.4 ± 2.7	11.7 ± 2.3	19.5 ± 0.5	19.7 ± 0.2
18:1	27.9 ± 2.8	27.1 ± 3.1	19.4 ± 0.4	20.1 ± 0.2
18:2ω6	5.3 ± 0.7	5.8 ± 1.5	0.4 ± 0	0.5 ± 0.05
20.0	0.4 ± 0.2	0.3 ± 0.05	0.6 ± 0.08	0.6 ± 0.05
20.1, 18:3ω3	0.2 ± 0.2	0.1 ± 0.06	1.9 ± 0.1	2.1 ± 0.1
20:2	–	–	0.3 ± 0.01	0.4 ± 0.05
20:3ω9	1.3 ± 0.2	1.8 ± 0.6	0.2 ± 0.05	0.2 ± 0.05
22.0, 20:3ω6	0.9 ± 0.1	1.1 ± 0.2	0.9 ± 0.3	1.0 ± 0.05
20.4ω6	11.6 ± 3.3	11.9 ± 3.7	9.7 ± 0.09	9.7 ± 0.05
22:1	–	–	0.2 ± 0	0.2 ± 0.05
24.0	tr	tr	1.1 ± 0.6	1.1 ± 0.1
24:1, 22:4ω6	0.3 ± 0.06	0.3 ± 0.1	4.5 ± 0.2	4.7 ± 0.2
22.5ω6	1.6 ± 0.4	1.8 ± 0.6	3.1 ± 0.6	3.5 ± 0.3
22:6ω3	0.3 ± 0.1	0.4 ± 0.2	8.8 ± 0.6	8.6 ± 0.4
Total PUFA	21.5	23.2	29.8	30.7
ω6 PUFA	19.7	20.9	18.6	19.4
ω3 PUFA	0.5	0.5	10.7	10.7

[a]Tentative identification.

vitamin E was significantly lower at 39 days, and serum vitamin E was significantly lower at 29 and 19 days in the experimental group than in the controls. (It was felt unnecessary to include all the measurements for the shorter periods.) Liver and serum vitamin E levels confirm that the rats were indeed E-deficient. However, brain vitamin E levels were not significantly different in controls and E-deficient rats.

In Table 2 are listed similar data for vitamin E-deficient and control rats fed the marginal 18:3ω3 diet for 36 days (groups 4 and 5). Again, there were no significant differences in the major measurements of deficient and control rats, although total liver lipids of the E-deficient rats tended to be higher than in the controls. Actually, all rats fed the marginal EFA diets had increased total liver lipids when compared with the value of 45.9 ± 2.7 mg/g for normal Long Evans rats of this age. A gross examination of the livers revealed the appearance of fatty infiltration in all groups, and it may be significant that the marginal 18:3ω3 diet seemed to have had the greatest effect. Liver and serum reflected the vitamin E deficiency state and, in this case, the brain also contained significantly lower vitamin E levels.

It was first considered that the decreased vitamin E in the brains of the 18:3ω3-fed animals might be due to the occurrence of larger amounts of the highly unsaturated 22:6ω3 in these brains, since this fatty acid is a product of 18:3ω3 elongation and desaturation and normally accumulates in the brain lipids. It is also the most susceptible of the membrane fatty acids to oxidative attack.

Table 3 shows the fatty acid composition of brain and liver total lipids of the vitamin E-deficient and control rats fed the marginal 18:2ω6 diet. Table 4 gives the same data for rats fed the marginal 18:3ω3 diet. In both cases, only the animals fed the diets for 39 and 36 days, respectively, are considered, since there were no significant changes during the feeding period. Values thought to be of particular interest are underlined. In neither case did the vitamin E deficiency result in a significant increase in 20:3ω9, an indication of EFA deficiency.

As can be seen by a comparison of Tables 3 and 4, there is, as would be expected, a considerably smaller proportion of 22:6ω3 in the livers of rats fed the 18:2ω6 diet than in those fed the 18:3ω3 diet. However, the brain lipids of these animals contained substantial amounts of 22:6ω3 (9% compared with 13% for the 18:3ω3 diet). The source of this 22:6ω3 in the brains of rats not fed any 18:3ω3 past weaning was therefore sought in an experiment in which the 22:6ω3 content of the total body lipids of rats fed the 18:2ω6 diet to 46 days of age was analyzed. Two mothers with 6-day-old pups were fed the marginal 18:2ω6 diet and treated identically to group 3. Three pups were killed at birth and at 6, 21 and 46 days. Brains, livers, lungs and remaining carcasses were

TABLE 4

Fatty Acid Composition (Percentage of Total FID Response) of Liver and Brain Total Lipids of Rats Fed a Marginal 18:3 Diet, With or Without Vitamin E, for 36 Days

	Liver		Brain	
Fatty acid	with vitamin E (n = 4)	without vitamin E (n = 4)	with vitamin E (n = 7)	without vitamin E (n = 6)
14:0	0.8 ± 0.3	0.8 ± 0.4	0.8 ± 0.6	0.5 ± 0.3
14:1[a]	0.8 ± 0.2	0.8 ± 0.2	0.3 ± 0.2	0.1 ± 0
15:0[a]	–	–	2.3 ± 0.2	2.4 ± 0.2
16:0	30.4 ± 4.0	30.3 ± 2.9	16.7 ± 0.3	16.6 ± 2.0
16:1	6.2 ± 0.8	5.4 ± 0.9	0.4 ± 0.08	0.4 ± 0.07
17:0[a]	–	–	4.0 ± 0.2	4.2 ± 0.4
17:1[a]	–	–	3.0 ± 0.1	3.2 ± 0.2
18:0	12.1 ± 2.5	12.3 ± 2.4	18.8 ± 0.5	19.3 ± 1.4
18:1	28.4 ± 4.8	28.8 ± 5.1	22.9 ± 1.3	22.6 ± 0.7
18:2ω6	1.2 ± 0.4	1.3 ± 0.4	0.3 ± 0.06	0.2 ± 0.09
20.0	–	–	0.6 ± 0.06	0.7 ± 0.3
20.1, 18:3ω3	0.8 ± 0.2	0.9 ± 0.2	2.7 ± 0.2	2.5 ± 0.2
20:2	0.2 ± 0.1	0.3 ± 0.1	0.6 ± 0.06	0.6 ± 0.2
20:3ω9	0.9 ± 0.3	0.8 ± 0.2	0.2 ± 0.05	0.2 ± 0.04
22.0, 20:3ω6	–	–	0.4 ± 0.05	0.4 ± 0
20.4ω6	2.1 ± 0.8	2.2 ± 0.5	7.4 ± 0.6	7.8 ± 0.4
20:5ω3	5.6 ± 0.2	6.1 ± 1.1	0.2 ± 0.06	tr
24.0	0.2 ± 0.1	0.2 ± 0.09	1.0 ± 0.1	1.0 ± 0.1
24:1, 22:4ω6	0.1 ± 0.05	0.2 ± 0.1	3.4 ± 0.2	3.3 ± 0.3
22.5ω6	–	–	0.4 ± 0.08	0.4 ± 0.15
22.5ω3	1.1 ± 0.5	1.3 ± 0.5	0.3 ± 0.1	0.4 ± 0.1
22:6ω3	7.1 ± 2.9	7.4 ± 3.0	13.1 ± 0.8	12.8 ± 0.5
Total PUFA	19.1	20.5	29.0	28.6
ω6 PUFA	3.4	3.7	11.9	12.1
ω3 PUFA	14.6	15.7	16.3	15.7

[a]Tentative identification.

TABLE 5

Amounts of 22:6ω3 in Rats Fed an 18:3-Free Diet Containing 18:2ω6 as 1.5% of Calories (mg 22:6ω3/Total Tissue)

	Newborn	6 Days Old	21 Days Old	46 Days Old
Brain	0.33 ± 0.015	1.18 ± 0.06	5.56 ± 0.49	4.73 ± 0.36
Liver	0.36 ± 0.021	1.57 ± 0.03	3.92 ± 0.36	0.91 ± 0.05
Lung	0.06 ± 0.014	0.30 ± 0.06	0.18 ± 0.006	0.01 ± 0
Carcass	1.96 ± 0.027	12.4 ± 4.10	22.4 ± 4.77	2.62 ± 1.19
Total	2.71 ± 0.05	15.45 ± 4.21	32.06 ± 5.64	8.27 ± 1.57

weighed and the lipids were extracted and weighed as described above. The methyl esters of the fatty acids from the total lipids of each tissue were analyzed, and the amounts of 22:6ω3 in each fraction and in the total bodies of each rat were calculated in mg/total tissues. In Table 5 are listed the amounts of 22:6ω3 in each of the tissues and the totals of each age group. There is evidently a steady increase in total 22:6ω3 per rat during the 21-day period. In the nursing pups, the source is obviously the mother's milk, even though after 15 days some of the diet supplied is consumed. An analysis of milk from the stomachs of the 6-day-old pups showed 0.3% 18:3ω3 and 0.9% 22:6ω3. In addition, 20:5ω3 and 22:5ω3 were present at about 0.5% each. After weaning, of course, there is no dietary source of ω3 fatty acids, and a glance at Table 5 reveals that at 46 days, the only significant amounts are in the brains, with a precipitous drop of 22:6ω3 in all other tissues.

DISCUSSION

In the present experiments, no signs of EFA deficiency were seen either in the physical state of the rats or in a significant increase in the ratio of 20:3ω9 to 20:4ω6 which, in some circumstances, would signify a deficiency (12). In this study, moreover, this ratio would have had little meaning, since in the rats fed no 18:2ω6 the 20:4ω6 would decrease, and the ratio would thus increase even in the absence of any change in 20:3ω9. It is possible that in the previous studies (6) dietary deterioration could have accounted for a reduction of 18:2ω6 and 18:3ω3 below marginal levels. Other presently obscure factors could possibly also have been involved.

Of interest in the present study is the decrease in brain vitamin E in the rats fed marginal 18:3ω3 (Table 2) but not in those fed 18:2ω6 (Table 1). It would be difficult to ascribe this to a supposed higher concentration of the highly unsaturated fatty acid 22:6ω3 in the brain lipids, since the concentrations of this fatty acid in the brains of both dietary groups are quite similar (Tables 3 and 4). However, a comparison of the 22:6ω3 content of the liver lipids shows that this fatty acid is almost absent in the 18:2ω6-fed rats but quite high in the 18:3ω3-fed rats. It is reasonable, then, to assume that the destruction of vitamin E took place by cooxidation in the liver, followed by transport of the reduced amount to the brain. One problem with this interpretation is that there does not seem to be any decrease in 22:6ω3 in the 18:ω3-fed vitamin E-deficient rats. It is probable, however, that in all but the late stages of membrane lipid peroxidation this would not be seen. It has been shown in this laboratory (13) that the effect of tocopherol in decreasing lipid peroxidation is to introduce a lag period during which very little decrease in unsaturated fatty acid occurs. However, during this period, the tocopherol itself decreases rapidly until, when it has reached a level of about 10% of its initial value, protection ceases and fatty acid peroxidation begins (14).

Finally, of further interest are the increase in 22:6ω3 in growing rats, even nursing on mothers with no dietary 18:3ω3; the precipitous drop of 22:6ω3 following cessation of nursing but seen mainly in the non-neural tissue; and the stubborn retention of 22:6ω3 in the brains of the 18:3ω3-deficient rats. These observations have also been made by others (15–17).

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COMMUNICATIONS

Imprints of Virus Infection: Can Paramyxoviruses Permanently Modify Triacylglycerol Metabolism?

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We have examined whether the passage of a paramyxovirus in a cell (BGM, African green monkey kidney) or animal (Swiss mouse) can permanently modify its metabolism. In an in vitro model in which cells had been cured of a measles virus persistent infection, the cells retained the modifications affecting lipid metabolism and composition induced during the infection. In a canine distemper virus mouse model, the same virus-induced modifications were observed in mice after the virus had been eliminated.

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The lipid constituents of membranes, once considered merely a matrix for the implantation of proteins, have in recent years been shown to play a more active and functional role in the biochemistry of the cell. Although the diacyglycerol moieties of the phospholipids (PL) may still be considered structural elements of the membrane, certain of their attached fatty acids may be further metabolized into molecules with potent biological activities. Therefore, their modification may have either a direct or indirect effect on cellular metabolism.

Recently, we showed that lipid metabolism was modified in cells persistently infected with measles virus and that saturated and polyunsaturated radiolabeled fatty acids were accumulated in the triacylglycerols of these cells (1,2). This alteration of the neutral lipid metabolism was not accompanied by a modification in the turnover of membrane PL, which differed only in their fatty acid moieties (3).

In an attempt to reproduce our results in an animal model, we adapted canine distemper virus (CDV), which is immunologically related to measles (4), to grow in newborn Swiss mice (5). Inoculation of the virus into weanling mice gave a meningitis with up to 40% mortality. In the surviving animals, up to 30% of the mice became obese, but no virus antigens could be detected in these animals (5). In parallel, when the measles virus persistently infected cells were cloned, half of the cells cloned contained no detectable virus, although they retained other virus-induced modifications (6).

In the present communication, we compare the lipid composition and metabolism of cells containing no detactable virus from the in vitro and animal models. We show that the passage of a paramyxovirus in a cell or host can induce permanent modifications in the system. These results may be relevant to a number of human metabolic diseases of undefined etiology.

MATERIALS AND METHODS

Cells. BGM, an African green monkey kidney cell line, and its derivative line (BGM-MC) were cultivated as previously described (7). The latter cell line was obtained from a persistent infection of BGM cells with measles virus. No virus could be detected in this cell line (6).

Animals. Four-week-old Swiss mice (IFFA-CREDO) were inoculated intracerebrally with 20 μl of the mouse-adapted Onderstepoort strain of CDV (5). In the surviving group of animals, obesity was observed in up to 30% of animals surviving at 2 mo postinfection. Control animals were inoculated intracerebrally with cell growth medium. The animals used for the present experiments were taken 6 mo after inoculation. For metabolic studies, 150 μCi/mouse of [2- ^{3}H]glycerol in physiological solution (Amersham, Buckinghamshire, England) was injected intraperitoneally and the animals were killed 30 min later.

Lipid analysis. The lipids were extracted in chloroform/methanol (1:1, v/v) by a modification of the method of Folch et al. (8), as described by Anderton et al. (3). Neutral lipids were separated by thin layer chromatography (TLC) on precoated silica gel G-50 plates developed with hexane/diethyl ether/acetic acid (90:10:1, v/v/v). Triacylglycerols were quantified by enzymatic determination of glycerol after saponification of lipids by ethanolic-KOH (9). Total cholesterol was assayed as previously described (3) and free cholesterol was determined by Boehringer's enzymatic method (Boehringer, kit no. 139050, Mannheim, Federal Republic of Germany).

Total lipid phosphorus was determined by a modification of Bartlett's method. PL were separated by double dimensional chromatography on precoated silica gel G-60 plates (E. Merck, Darmstadt, Federal Republic of Germany) as described by Portoukalian et al. (10) and located with Dittmer and Lester's reagent (11). The separated PL were scraped into tubes and mineralized and the quantity of phosphate was determined (10).

The protein content was estimated on the delipidized residue, dissolved in 1 N NaOH, by the method of Lowry et al. (12) using bovine serum albumin as a standard.

Determination of [^{3}H]glycerol incorporation. Determination of the incorporation of [^{3}H]glycerol into the total lipid was performed as previously described (3). The PL, which were separated and detected on TLC as described above, were scraped into scintillation vials and suspended in 3 ml of ethanol/water (1:1, v/v) and 8 ml of picofluor-30 (Packard, Downers Grove, Illinois). Counting efficiency was determined by the external standard channels ratio method and the results were expressed in dpm. Neutral lipids and PL were separated on precoated silica gel G-60 plates in one dimension with isopropyl ether as solvent. The PL remain at the origin and the neutral lipids migrate with the solvent front. Less than 1% of the radioactivity migrates between the two fractions. Neutral lipids were separated along with standards on precoated silica gel plates with heptane/iso-

propyl ether/acetic acid (60:40:4, v/v/v) and the radioactivity was counted as described above.

RESULTS AND DISCUSSION

Lipid composition and metabolism in postinfected BGM cells. The two cell lines used in the experiment, BGM (parental) and BGM/MC (cured of virus infection), had similar growth rates and were examined during their exponential growth phase. The lipids were extracted from the parental BGM cell line and its derivative cured of measles virus infection, BGM/MC, and analyzed. Apart from a slight decrease in the PL of BGM/MC cells, the overall composition was similar in both cell lines. The BGM/MC cells showed a small but nonsignificant decrease in sphingomyelin; the other PL components remained unchanged (data not shown).

Incorporation of 1(3)[^{3}H]glycerol was greater into BGM/MC than BGM cells. This increase was determined to be exclusively at the level of triacylglycerols and corresponded to an increase of 2–3 times that found in the control BGM cells (Fig. 1). The distribution of the radioactivity among the different PL appears similar in both models (data not shown). These results are similar to those found in the measles virus persistently infected cell line (1,2). The cured clones have a modified karyotype similar to that observed in the infected cell line (Patet, unpublished observations). Thus, it is unlikely that there is a selection of a cell clone nonrepresentative of the original cell population.

Lipid composition and metabolism in postinfected mice. Swiss weanling mice were inoculated with either CDV or culture medium (controls). Up to 30% of the mice surviving the infection became obese (cured-obese). Six months after infection, the period in which the experiments were performed, these animals weighed ca. 70 g. Control and infected nonobese (cured-lean) animals weighed ca. 30 g.

Lipid composition of organs. Analysis of the lipid content of the tissues of the three groups of animals (control, cured-lean, cured-obese) showed a statistically significant increase in the triacylglyceride content in the liver of obese animals and a highly significant increase in the white adipose tissue (Table 1). The PL content in the liver of obese animals was similiar to the other two groups. There was a nonsignificant increase in the PL content of white adipose of these animals (Table 1). In obese mice the phospholipid content of wet adipose tissue decreases; this was accompanied by a parallel decrease in protein content, thus leaving the PL/protein ratio unchanged.

Compared with protein content, the amount of triacylglyceride per mg of protein was fourfold higher in the parametrial and mesenteric adipose tissue of obese

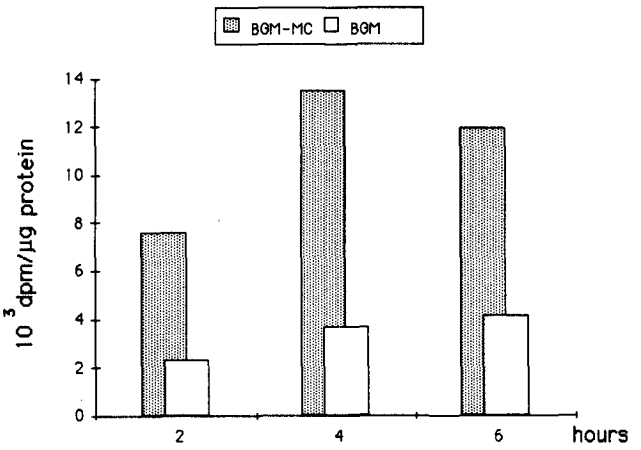

FIG. 1. Incorporation of 1(3)[^{3}H]glycerol in triacylglycerols of BGM and BGM-MC cells. The value of the standard error deviation is inferior to 10% of the mean, which represents the average of three experiments.

TABLE 1

Lipid Composition of the Liver and White Adipose Tissue in Control, Post-CDV-infected Lean and Obese Mice*

	Liver		
		Post-CDV-infected	
	Control	Lean	Obese
Phospholipids	19.4 ± 0.2	20.0 ± 0.3	20.3 ± 0.9
Triacylglycerols	16.4 ± 1.2	18.4 ± 2.3	35.8 ± 4.8[a,b]
Cholesterol			
Free	1.68 ± 0.02	1.68 ± 0.02	1.39 ± 0.05
Esterified	0.49 ± 0.04	0.49 ± 0.11	1.05 ± 0.25
Phospholipids/free Cholesterol	11.55 ± 0.01	11.91 ± 0.71	14.6 ± 0.32[a,b]
		Adipose tissue	
Phospholipids	13.2 ± 2.0	15.0 ± 4.7	18.9 ± 2.8
Triacylglycerols	2190 ± 190	3430 ± 1370	8010 ± 1550[a]

*Average ± S.M. is expressed as a percentage of tissue protein.
[a,b]Value with a significant difference ($p < 0.01$) to the control (*a*) and lean (*b*) group.

TABLE 2

Incorporation of 2[^{3}H]Glycerol into Lipids of Liver and White Adipose Tissue in Control and Post-CDV-infected Lean and Obese Mice*

	Liver		Adipose tissue: triacyclglycerols	
	Triacyglycerols	Phospholipids	Parametrial	Mesenteric
Control	65770 ± 6840	24240 ± 2390	11100 ± 750	17640 ± 1930
Lean	66290 ± 3530	24340 ± 1200	16880 ± 4430	16620 ± 3740
Obese	137000 ± 10000[a]	32100 ± 5050	48600 ± 4650[a]	69820 ± 5920[a]

*Results are expressed in dpm per 100 mg of tissue protein.
[a]See Table 1.

mice. There was no significant difference in the PL composition of the liver and adipose tissue of the three groups of animals (results not shown).

Incorporation of [2-^{3}H]glycerol. Although injected radioactive glycerol was rapidly incorporated into diacylglyerol and phosphatidic acid in the liver of rats (13–16), previously published results in rats (14) and our preliminary studies in mice determined 30 min after intraperitoneal injection as the time in which sufficient incorporation of radioactivity was achieved in triacylglycerols and the major PL of the liver and adipose tissues.

Mice were injected intraperitoneally with [2-^{3}H]-glycerol and killed 30 min later. There was an increased incorporation of the radioactivity into the total and neutral lipids in the liver and adipose tissue of the obese animals examined (Table 2). Although the increase in the adipose tissue was similar to the liver of the obese mice, the tissue has a greater mass in the obese animals (15–30 times greater than the control animals) and so the accumulated radioactivity in the total adipose tissue is much greater. Thus, there was an increased uptake of glycerol in the obese animals. The triacylglycerols represented 85–90% of the neutral lipid radioactivity. The rest was distributed between free cholesterol, diacylglycerol (7–10%) and monoacylglycerol (4–7%) fractions.

The incorporation of the [^{3}H]glycerol into the different PL was measured. No difference was observed between liver from the control and obese animals. Thus there is no alteration in the relative metabolism of the different PL. No virus or virus antigens could be found in any of the animal tissues. These observations parallel the in vitro measles-BGM model, in which even after the loss of the virus the cell clones retained several of the markers or "imprints" of the virus-infected cultures, i.e., changes in karyotype and topographical changes at the cell membranes (6).

In the present study, we have compared the lipid metabolism in two systems (in vitro and in vivo) in which, after an initial infection, the virus is eliminated. The aim of the experiments was to see if a virus can irreversibly modify the metabolism of a cell or host.

Comparison of the ratios of the liver PL/free cholesterol showed an increase in the obese mice. This suggests there is a modulation of the microviscosity of the lipid layers of the hepatic cell membranes (17). Alterations reported in the activity of endoplasmic reticulum enzymes in cells after certain treatment are normally accompanied by changes in the viscosity of the membranes (18). We have also observed variations in fluorescence polarization in measles virus persistently infected cells (19). There was no significant variation in the PL composition of the BGM/MC cell line or of the liver of the obese mice (data not shown). No differences were found in the incorporation of [^{3}H]glycerol into the PL for the BGM/BGM-MC or for the liver and adipose tissue of control/obese mice models. Differences in accumulation of radioactivity in the triacylglycerols in both models were observed, even though the virus is no longer present.

Our results on the BGM-MC cells and the CDV-induced obese mice confirm that the in vitro and the in vivo models have similar metabolic disorders. The in vitro (BGM-MC) and the in vivo (obese mice) models are examples of metabolic alterations developing after the virus has been eliminated. The mechanism of this metabolic change in both systems is still not clear.

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Hypolipidemic Activity of 3- and 4-Phenyl-piperidine-2,6-diones and Selected N-Substituted Derivatives

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Three- and 4-phenyl-piperidine-2,6-dione derivatives were investigated for hypolipidemic activity at 20 mg/kg/day intraperitoneally in rodents. The 3-phenyl compound afforded the best activity and effectiveness in both normal and hyperlipidemia-induced mice. The agent lowered lipids by blocking the de novo hepatic synthesis of cholesterol and triglycerides, specifically at the sites of ATP-dependent citrate lyase, acetyl CoA synthetase, *sn*-glycerol-3-phosphate acyl transferase and phosphatidylate phosphohydrolase. The agent caused a more rapid clearance of cholesterol by the fecal route. Cholesterol levels of the chylomicrons, very low density lipoprotein and low density lipoprotein (LDL) were reduced, whereas high density lipoprotein cholesterol was significantly elevated after drug administration. Triglyceride content was lowered in the chylomicron and LDL fractions. These modulations of lipid content of serum lipoproteins by the drug suggest a favorable situation for treatment of hyperlipidemic states.
Lipids 21, 617–622 (1986).

Previously, studies have demonstrated that glutarimide (piperidine-2,6-dione) as well as its N-substituted and 3-phenyl derivatives possess potent hypolipidemic activity in rodents (1). For example, 3-phenyl-piperidine-2,6-dione at 20 mg/kg/day lowered serum cholesterol levels 49% and serum triglyceride levels 36% after 16 days' dosing in mice (1). These agents were more potent than clofibrate administered at 150 mg/kg/day in the same system, which lowered serum cholesterol levels 13% and serum triglyceride levels 25% after 16 days' dosing in mice (1). Based on these observations, it was decided to investigate a series of 3- and 4-phenyl-piperidine-2,6-diones as well as their N-substituted derivatives for hypolipidemic activity. Furthermore, the effect of 3-phenyl-piperidine-2,6-dione on lipid metabolism was examined at its most effective dose.

MATERIALS AND METHODS

Chemical synthesis. Melting points were determined using a Thomas Hoover Capillary melting point apparatus (A.H. Thomas, Philadelphia, Pennsylvania) and are uncorrected. Nuclear magnetic resonance (NMR) data was obtained using a JEOL-FX-60 spectrophotometer (Peabody, Massachusetts). Elemental analyses were conducted by M-H-W Laboratories (Phoenix, Arizona) and were within ±0.4% of the theoretical values. The synthesis and physical characteristics of 3-phenyl-piperidine-2,6-dione (α-phenyl glutarimide) have been reported previously (2). 3-Phenylglutaric acid was purchased from Aldrich Chemical (Milwaukee, Wisconsin) and was sublimed under reduced pressure to afford 3-phenylglutaric anhydride for the preparations described herein.

4-Phenyl-piperidine-2,6-dione (β-phenyl glutarimide). The method of Bishop et al. (3) was used to prepare the compound from 3-phenylglutaric acid and ammonia. The product was recrystallized from ethyl acetate and afforded white crystals, mp 173.5–175 C, lit. (3), mp 176–177 C.

N-butyl-3-phenyl-piperidine-2,6-dione. To a suspension of 2-phenylglutaric anhydride (3.8 g, 0.02 mol) in toluene (75 ml) was added n-butyl amine (4.38 g, 0.06 mol). The resulting mixture was heated to reflux for three days, and water was removed azeotropically with a Dean-stark apparatus (Kontes; Vineland, New Jersey). Solvent was removed and the product was purified on column chromatography (silica gel; chloroform/ethyl acetate eluant, 8:3, v/v). The yield of the colorless oil was 1.3 g (27%). Anal: calc. C, 73.47; H, 7.75; n, 5.71. Found: C, 73.39; H, 7.87; N, 5.73.

N-(2'-carboxyethyl)-3-phenyl-piperidine-2,6-dione. A mixture of 2-phenylglutaric anhydride (6 g, 0.03 ml), β-alanine (5 g, 0.05 mol) in toluene (70 ml) was refluxed for 60 hr, and water was removed azeotropically with a Dean-Stark apparatus. The reaction mixture was diluted with water and extracted thoroughly with diethyl ether. The ether layer was washed with sodium bicarbonate solution, and the aqueous layer was acidified with 12 N HCl. The acidic solution was extracted with ether, and the extract layer was dried and evaporated to obtain a gummy material (yield = 200 mg [2%]). Anal: calc. C, 64.36; H, 5.74; N, 5.36. Found: C, 64.17; H, 5.68; N, 5.20.

N,3-diphenyl-piperidine-2,6-dione. A mixture of 2-phenylglutaric anhydride (1.9 g, 0.01 mol) and aniline (1.39 g, 0.015 mol) was heated at 160–180 C for 3 hr. The reaction mixture was cooled and the crude product was purified on column chromatography (silica gel, ethyl acetate eluant) to obtain a solid; mp = 82–84 C; yield = 625 mg (23%). Anal: calc. C, 76.98; H, 5.66; N, 5.28. Found: C, 76.75; H, 5.73; N, 5.26.

N-(1-butyl)-4-phenyl-piperidine-2,6-dione. To a suspension of 3-phenylglutaric anhydride (0.5 g, 26 mmol) in toluene (20 ml) was added n-butyl amine (0.58 g, 78 mmol). The resulting mixture was heated to reflux for three days, and water was removed azeotropically with a Dean-Stark apparatus. Solvent was removed and the product was purified via column chromatography (silica gel, ethyl acetate eluant), yielding 100 mg (16%) of a colorless oil. Anal: calc. C, 73.47; H, 7.75; N, 5.71. Found: C, 73.19; H, 7.83; N, 5.70.

N-(2'-carboxyethyl)-4-phenyl-piperidine-2,6-dione. A mixture of 3-phenylglutaric anhydride (1 g, 0.005 mol) and β-alanine (0.75 g, 0.008 mol) in toluene (80 ml) was heated to reflux for three days, and water was removed azeotropically with a Dean-Stark apparatus. The solvent was removed and the residue was heated at 160–180 C for 2 hr. The reaction mixture was cooled and treated with sodium bicarbonate solution. The aqueous layer was extracted with ether and subsequently acidified with 12 N HCl. The acidic aqueous portion was extracted with ether and the extract dried (Na_2SO_4). The solvent was evaporated to obtain a gum (yield = 90 mg [6%]). Anal: calc. C, 64.36; H, 5.74; N, 5.36. Found: C, 64.63; H, 5.77; N, 5.34.

N,4-diphenyl-piperidine-2,6-dione. A mixture of 3-phenylglutaric anhydride (0.5 g, 26 mmol) and aniline (0.28 g, 30 mmol) was heated at 160–180 C for 3 hr. The

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reaction mixture was cooled and the crude product was purified on column chromatography (silica gel, ethyl acetate eluant) to yield 100 mg (14%) of a colorless gum. Anal: calc. C, 76.98; H, 5.66; N, 5.28. Found: C, 76.93; H, 5.78; N, 5.26.

Hyperlipidemic screens in normal rodents. Test compounds were suspended in an aqueous 1% carboxymethylcellulose solution, homogenized, and administered either to CF_1 male mice (~25 g) intraperitoneally (IP) for 16 days at 20 mg/kg/day or to Sprague-Dawley male rats (~350 g) orally by an intubation needle for 14 days at 10 mg/kg/day. On days 9 and 14 or 16, blood was obtained by tail vein bleeding, and the serum was separated by centrifugation for 3 min. The serum cholesterol levels were determined by a modification of the Liebermann Burchard reaction (4). Serum was also collected on day 14 or 16, and the triglyceride content was determined by a commercial kit (Boehringer Mannheim Diagnostic Inc., 348201, Indianapolis, Indiana).

Testing in hyperlipidemic mice. CF_1 male mice (~25 g) were placed on a commercial diet (U.S. Biochemical Corp. basal atherogenic test diet) that produced a "hyperlipidemic" state (5). After the serum cholesterol and triglyceride levels were observed to be elevated, the mice were administered 3-phenyl-piperidine-2,6-dione at 10 mg/kg/day, IP for an additional 14-day period. Serum cholesterol and triglyceride levels were measured at that time.

Animal weights and food intake. Periodic animal weights were obtained during the experiments and expressed as a percentage of the animal weight on day 0. After dosing for 14 days with test drugs, selected organs (liver, kidney, spleen, lung, brain, heart, stomach, small intestine, large intestine and reproductive organs) were excised, trimmed of fat and weighed. Food consumption was determined daily as g/day/100 g body weight.

Toxicity studies. The acute toxicity (LD_{50} values) (6) was determined in CF_1 male mice (~25 g) by administering 3-phenyl-piperidine-2,6-dione IP from 100 mg to 1 g/kg as a single dose. The number of deaths was recorded over a seven-day period for each group.

Enzymatic studies. In vitro enzymatic studies were determined using 10% homogenates prepared in 0.25 M sucrose + 0.001 M (ethylenedinitrilo) tetraacetic acid, pH 7.2, of CF_1 male mouse liver incubated with 50–200 μM of test drugs. In vivo enzymatic studies were determined using 10% liver homogenates (prepared in 0.25 M sucrose + 0.001 M [ethylenedinitrilo]-tetraacetic acid, pH 7.2) obtained from CF_1 male mice after administering the agents for 16 days at a dose ranging from 10–60 mg/kg/day IP. The enzyme activities were determined by following the literature procedures (5): acetyl coenzyme A synthetase (7); adenosine triphosphate dependent citrate lyase (8); mitrochondrial citrate exchange (9,10); cholesterol-7α-hydroxylase (II); 3-hydroxy-3-methyl-glutaryl coenzyme A (12,13); acetyl coenzyme A carboxylase activity (14); *sn*-glycerol-3-phosphate acyl transferase activity (15), phosphatidate phosphohydrolase activity (16); acyl-CoA-cholesterol acyl transferase (17) and heparin-activated hepatic lipoprotein lipase (18). Protein was determined for all enzyme assays by the technique of Lowry et al. (19).

Liver, small intestine and fecal lipid extraction. CF_1 male mice that were administered 3-phenyl-piperidine-2,6-dione at 20 mg/kg for 16 days were placed in metabolic cages the last 24 hr to obtain a 24-hr fecal collection from each animal. The liver and small intestine were removed, extracted by the methods of Folch et al. (20) and Bligh and Dyer (21) and analyzed for cholesterol levels (4), triglyceride levels (Boehringer Mannheim 348201), neutral lipid content (22) and phospholipid content (23).

^{3}H-cholesterol distribution in rats. Sprague-Dawley

TABLE 1

Effects of 3- and 4-Phenyl-Piperidine-2,6-Diones and N-Substituted Derivatives on Serum Cholesterol and Triglyceride Levels of CF_1 Male Mice after 9 or 16 Days of Dosing at 20 mg/kg/day Intraperitoneally (n=6, X ± S.D.)

Compound	Serum cholesterol (mg%)		Serum triglyceride (mg %)
	Day 9	Day 16	Day 16
1% Carboxymethylcellulose	128 ± 6	130 ± 4	137 ± 6
Glutarimide[a]	102 ± 5[b]	100 ± 7[b]	105 ± 8[b]
N-(1-butyl) glutarimide[a]	121 ± 10	91 ± 5	87 ± 4[b]
β(N-glutaridimido) propionic acid[a]	124 ± 5[b]	113 ± 9	115 ± 7
3-Phenyl-piperidine-2,6-dione (α-phenylglutarimide)[a]	119 ± 5	66 ± 10[b]	88 ± 3[b]
N-(1-butyl)-3-phenyl-piperidine-2,6-dione	96 ± 7[b]	74 ± 6[b]	98 ± 7[b]
N-(2′-carboxyethyl)-3-phenyl-piperidine-2,6-dione	99 ± 8[b]	102 ± 7[b]	110 ± 8[b]
N,3-diphenyl-piperidine-2,6-dione	115 ± 9	107 ± 7[b]	126 ± 6
4-phenyl-piperidine-2,6-dione (β-phenylglutarimide)	86 ± 6[b]	79 ± 5[b]	97 ± 5[b]
N-(1-butyl)-4-phenyl-piperidine-2,6-dione	113 ± 6	105 ± 6[b]	109 ± 7[b]
N-(2′-carboxylethyl)-4-phenyl-piperidine-2,6-dione	121 ± 5	119 ± 6	126 ± 6
N,4-diphenyl-piperidine-2,6-dione	117 ± 7	113 ± 5[b]	115 ± 7[b]

[a]Activities for these compounds have been reported previously in ref. 1.
[b]$p \leq 0.001$ (Student's t-test).

TABLE 2

Effects of 3-Phenyl-Piperidine-2,6-Dione on Serum Cholesterol and Triglyceride Levels of CF_1 Mice (Intraperitoneally) and Sprague-Dawley Rats (Orally) (X ± S.D.)

	Serum cholesterol (mg%)		Serum triglyceride (mg%)	
	Day 9	Day 16	Day 16	
CF_1 Mice (n = 6)				
3-phenyl-piperidine-2,6-dione, mg/kg/day				
10	78 ± 4*	65 ± 5*	74 ± *	
20	70 ± 5*	66 ± 5*	73 ± 5*	
30	67 ± 6*	75 ± 4*	70 ± 5*	
60	104 ± 3*	86 ± 6*	68 ± 5*	
Control: 1% carboxymethylcellulose	128 ± 6	130 ± 6	137 ± 6	
	Day 7	Day 14	Day 7	Day 14
Sprague-Dawley rats (n = 6)				
Treated: 3-Phenyl-piperidine-2,6-dione, 10 mg/kg/day	52 ± 4*	46 ± 3*	100 ± 4*	67 ± 5*
Control: 1% carboxymethylcellulose	75 ± 5	78 ± 6	109 ± 7	110 ± 6

*, $p \leq 0.001$.

TABLE 3

In Vitro Effects of 3-Phenyl-Piperidine-2,6-Dione at 50, 100 and 200 μM on CF_1 Mouse Liver Enzyme Activity (n = 6; X ± S.D.)

	% Mitochondrial citrate exchange to cytoplasma	ATP-dependent citrate lyase (mg of citrate formed/ g wet tissue)	Acetyl CoA synthetase (mg acetyl CoA formed/g wet tissue)	*sn*-Glycerol-3-phosphate acyl transferase (dpm/g wet tissue)	Phosphatidate phosphohydrolase (μg PI/g wet tissue)	Hepatic lipoprotein lyase (dpm/g wet tissue)
Control: 1% CMC	30.8 ± 2.2	30.5 ± 2.1	28.5 ± 1.4	537,800 ± 37546	16.7 ± 1.00	278,128 ± 18070
3-Phenyl-piperidine-2,6-dione						
50 μM	21.2 ± 2.1*	24.1 ± 2.4*	16.8 ± 1.3*	342,578 ± 7894*	5.6 ± 0.94*	197,470 ± 15025*
100 μM	18.8 ± 1.9*	24.0 ± 2.3*	15.9 ± 1.5*	300,092 ± 9261*	2.7 ± 0.51*	219,721 ± 16782*
200 μM	19.7 ± 2.0*	21.3 ± 1.9*	21.6 ± 1.2*	249,001 ± 7062*	2.5 ± 0.71*	241,971 ± 12142

*$p \leq 0.001$.

rats (~300 g) were administered 3-phenyl-piperidine-2,6-dione at 10 mg/kg for 14 days, orally. On day 13, 10 μCi of ^{3}H-cholesterol was administered orally by intubation needle to the rats, according to procedures described previously (5). Some tissue samples were combusted in a Packard tissue oxidizer or plated on filter paper, dried and digested for 24 hr in Hyamine hydroxide (New England Nuclear, Boston, Massachusetts) at 40 C and counted (Fisher scintiverse in a Packard scintillation counter). Results were expressed as disintegration/min (dpm) per total organ.

Serum lipoprotein fractions. Sprague-Dawley male rats (~300 g) were administered 3-phenyl-piperidine-2,6-dione at 10 mg/kg/day orally for 14 days. Blood was collected from the abdominal vein, and lipoprotein fractions were obtained by the methods of Hatch and Lees (24) and Havel et al. (25). Each of the fractions was analyzed for cholesterol (4), triglyceride (Bio-Dynamics/ bmc triglyceride kit), neutral lipids (23), phospholipid (24) and protein levels (19).

Statistical analysis. In Tables 1-7, the data is expressed as mean ± standard deviation. All groups have six animals for each determination. The statistical determination used was the Student's t-test, using a $p \leq 0.001$ for significant difference.

RESULTS

These studies have demonstrated that 3- and 4-substituted piperidine-2,6-dione derivatives possess hypolipidemic activity in rodents (Table 1). The structure activity relationship studies at 20 mg/kg/day IP showed that those compounds not substituted on the nitrogen atom appeared to have the best activity. The 3-phenyl-piperidine-2,6-dione had improved activity over the 4-phenyl-derivative. In the dose response study, the 3-phenyl isomer at 10 mg/kg/day produced a maximum cholesterol decrease in mice, with 50% reduction after 16 days. Serum triglyceride levels were reduced maximally at 60 mg/kg/day by 50% after 16 days' administration (Table 2). The 3-phenyl-piperidine-2,6-dione was also effective in Sprague-Dawley rats, with a 40% reduction of serum cholesterol levels and a 39% reduction in serum triglyceride levels after 14

TABLE 4

In Vivo Effects of 3-Phenyl-Piperidine-2,6-Dione from 10–60 mg/kg/day Intraperitoneally on Lipid de novo Enzyme Activity from Livers of CF_1 Mice (n = 6, X ± S.D.)

3-Phenyl-piperidine-2,6-dione (mg/kg/day)	ATP-dependent citrate lyase (mg citrate hydrolyzed/g wet tissue)	Acetyl CoA synthetase (mg acetyl CoA formed/g wet tissue)	*sn*-Glycerol-3-phosphate acyl transferase (dpm/g wet tissue)	Phosphatidate phosphohydrolase (μg Pi formed/g wet tissue)	Hepatic lipoprotein lipase (dpm/g wet tissue)
10	30.4 ± 2.2	25.9 ± 1.5	306,546 ± 9762*	14.5 ± 0.99	150,224 ± 11202*
20	28.9 ± 1.9	16.5 ± 0.9*	328,058 ± 8899*	8.0 ± 0.97*	166,877 ± 8573*
40	26.1 ± 1.7	7.7 ± 0.5*	290,412 ± 8697*	6.7 ± 0.56*	144,627 ± 7653*
60	8.8 ± 0.8*	9.9 ± 0.7*	317,302 ± 9752*	7.2 ± 0.82*	203,033 ± 19051*
1% CMC	30.5 ± 2.1	28.5 ± 1.4	537,800 ± 37646	16.7 ± 1.00	278,128 ± 18078

*$p \leq 0.001$.

days' administration at 10 mg/kg/day (Table 2). In mice with hyperlipidemia induced by diet, where the serum cholesterol levels were elevated from 125 to 354 mg% above the control value, the drug lowered this level 54% to 163 mg%. In the same mice, the serum triglyceride level was elevated from 137 to 367 mg%, and drug administration lowered this elevated level by 61% to 143 mg% after 14 days. The LD_{50} as a single dose administered IP to CF_1 male mice was ≥1 g/kg. Administration of 3-phenyl-piperidine-2,6-dione at 20 mg/kg/day for 14 days did not alter the daily food consumption. Weights of the treated animals were not altered from the control rat weight, and there appeared to be no change in the weights of the individual organs after drug treatment, including the weight of the adrenal, which would suggest no estrogenic activity or hypertrophy of the adrenal cortex as a compensatory mechanism for steriodiogenesis.

In vitro enzymatic assay demonstrated that 3-phenyl-piperidine-2,6-dione significantly reduced the activity of hepatic mitochondrial citrate exchange, ATP-dependent citrate lyase and acetyl CoA synthetase (Table 3). No inhibition of HMG CoA reductase, cholesterol-7α-hydroxylase or acyl cholesterol acyl transferase was noted when 3-phenyl-piperidine-2,6-dione was present from 50–200 μM. The regulatory enzyme of fatty acid synthesis pathway, acetyl CoA carboxylase, was not affected by the drug. However, the regulatory enzymes of the triglyceride pathway were suppressed by the 3-phenyl isomer. The 200-μM concentration afforded a 50% reduction of *sn*-glycerol-3-phosphate acyl transferase and a 65% reduction of phosphatidylate phosphohydrolase activity. Hepatic lipoprotein lipase was inhibited marginally in vitro 29% at 50 μM and 21% at 100 μM. Examination of the same enzyme activity after in vivo administration demonstrated that ATP-dependent citrate lyase activity was reduced 71% at 60 mg/kg, while acetyl CoA synthetase activity was reduced 73% (Table 4). *sn*-Glycerol-3-phosphate acyl transferase activity was reduced 20% from 20–60 mg/kg. Phosphatidylate phosphohydrolase activity was reduced 50% from 20–60 mg/kg. Hepatic lipoprotein lipase activity was reduced 40% at 20–40 mg/kg. No observable changes in emzymatic activity were noted with HMG CoA reductase, cholesterol-7α-hydroxylase, acyl CoA cholesterol acyl transferase or acetyl CoA carboxylase.

3-Phenyl-piperidine-2,6-dione treatment resulted in reduction of cholesterol, triglyceride and phospholipid, but not protein, content in the livers of CF_1 mice after 16 days' administration (Table 5). In livers of rats, triglyceride and phospholipid levels were reduced (Table 6). There were no significant changes in cholesterol, triglycerides, neutral lipids or phospholipids in the small intestine. Fecal lipid analysis suggested an increase in cholesterol and phospholipid excretion after drug

TABLE 5

Effects of 3-Phenyl-Piperidine-2,6-Dione from 10–60 mg/kg/day Intraperitoneally on CF_1 Mouse Liver Lipid Levels (n = 6, X ± S.D.)

	Mg lipid extracted /g wet liver	mg/g wet tissue				
		Cholesterol	Triglycerides	Neutral lipids	Phospholipids	Protein
1% CMC	95 ± 6.5	12.24 ± 0.73	4.77 ± 0.24	28.35 ± 1.98	4.39 ± 0.35	4.5 ± 0.32
3-Phenyl-piperidine-2,6-dione						
10 mg/kg/day	76 ± 4.5*	11.87 ± 0.56	2.80 ± 0.25*	26.65 ± 1.05	9.82 ± 0.47*	4.35 ± 0.41
20 mg/kg/day	77 ± 4.0*	11.50 ± 0.63	2.48 ± 0.28*	29.20 ± 1.56	3.38 ± 0.63	5.14 ± 0.21
40 mg/kg/day	72 ± 5.5*	9.79 ± 0.81*	2.62 ± 0.36*	28.07 ± 2.04	3.82 ± 0.63	4.61 ± 0.17
60 mg/kg/day	65 ± 5.0*	9.91 ± 0.75*	3.01 ± 0.51*	22.68 ± 1.74*	4.17 ± 0.28	4.32 ± 0.38

*$p \leq 0.001$.

TABLE 6

In Vivo Effects of 3-Pheneyl-Piperidine-2,6-Dione on Lipid Contents of Rat Liver, Small Intestine, Feces and Serum Lipoproteins after 14 Days' Dosing at 10 mg/kg/day Orally (n = 6, X ± S.D.)

3-Phenyl-piperidine-2,6-dione	mg Lipid extracted/g wet tissue	mg/g wet tissue				
		Cholesterol	Triglyceride	Neutral lipids	Phospholipids	Protein
Liver						
Control	58.5 ± 4.5	24.03 ± 1.44	6.37 ± 0.38	44.11 ± 3.96	7.19 ± 0.70	4.51 ± 0.23
Treated	46.0 ± 3.5*	21.38 ± 0.92	4.90 ± 0.44*	49.40 ± 3.15	5.03 ± 0.32*	4.78 ± 0.16
Small intestine						
Control	45.5 ± 3.4	7.82 ± 0.55	1.12 ± 0.09	6.98 ± 0.56	2.06 ± 0.19	42.0 ± 2.9
Treated	43.0 ± 4.4	7.35 ± 0.31	1.38 ± 0.11	7.54 ± 0.83	2.16 ± 0.22	42.0 ± 3.1
Feces						
Control	8.0 ± 0.5	28.47 ± 2.27	1.86 ± 0.13	33.94 ± 2.38	1.39 ± 0.11	6.99 ± 0.56
Treated	11.8 ± 0.9*	39.85 ± 2.56*	0.95 ± 0.08*	35.97 ± 1.64	1.89 ± 0.08*	8.10 ± 0.84
		μg/ml serum				
Chylomicrons						
Control	—	337 ± 20	420 ± 21	67 ± 5	149 ± 10	184 ± 13
Treated	—	246 ± 17*	332 ± 20*	58 ± 9	119 ± 12*	180 ± 3
VLDL						
Control	—	190 ± 13	22 ± 2	98 ± 9	26 ± 2	50 ± 3
Treated	—	153 ± 9*	21 ± 3	55 ± 8*	19 ± 5*	49 ± 6
LDL						
Control	—	210 ± 13	45 ± 3	10 ± 1	41 ± 3	122 ± 7
Treated	—	159 ± 6*	38 ± 3	7 ± 2	36 ± 5*	126 ± 8
HDL						
Control	—	544 ± 43	27 ± 2	620 ± 49	153 ± 12	657 ± 39
Treated	—	1115 ± 28*	19 ± 7*	527 ± 36	301 ± 15*	637 ± 45

*$p \leq 0.001$.

treatment. Examination of the lipid content of the serum lipoproteins indicated that cholesterol content was reduced in the chylomicron, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) fractions, with significant elevation of cholesterol content in the high density lipoprotein (HDL) fraction. The phospholipid changes followed a similar pattern. The triglyceride content of the chylomicron, LDL and HDL fractions was reduced, and neutral lipids were reduced in all four fractions. Protein content was not altered in the lipoprotein fractions after drug treatment (Table 6).

^{3}H-cholesterol distribution studies after 14 days' dosing with 3-phenyl-piperidine-2,6-dione compared to untreated controls demonstrated that the drug reduced the concentration of ^{3}H-cholesterol and its metabolites in the brain (70% of control), heart (63%), lung (59%), liver (75%), kidney (75%) and stomach (70%), whereas ^{3}H-cholesterol content of the spleen was not significantly altered (Table 7). The gastrointestinal tissues as well as the chyme and feces contained more radioactivity than the control, e.g. small intestine (151%), large intestine (173%), chyme (358%) and feces (743%).

TABLE 7

Effects of 3-Phenyl-Piperidine-2,6-dione on ^{3}H-Cholesterol Distribution in Sprague-Dawley Rat after 14 Days' Dosing at 10 mg/kg/day Orally (n = 6)

Tissue	Organ weight (g)		DPM/total organ	
	Control	Treated	Control	Treated
Brain	1.74	1.73	43,020	29,921
Heart	1.10	1.07	25,193	15,979
Lung	2.10	2.05	100,066	58,495
Liver	9.45	9.93	461,487	343,850
Kidney	2.02	2.07	66,245	49,883
Spleen	0.53	0.67	37,669	39,371
Stomach	2.27	2.23	53,847	37,685
Small intestine	7.26	6.43	299,086	452,995
Large intestine	3.17	2.83	68,325	118,357
Chyme	6.1	5.27	59,862	214,551
Feces	4.67	5.47	84,474	627,649
Adrenal	0.0714	0.0829	—	—
Plasma (ml)	—	—	2,102	806

DISCUSSION

The 3- and 4-phenyl-(N-substituted)-piperidine-2,6-dione derivatives did not afford improved hypolipidemic activity over the unsubstituted compounds. Further, the respective 3-phenyl-piperidine-2,6-dione derivatives were more active than the corresponding 4-phenyl derivatives. The 3-phenyl-piperidine-2,6-dione (α-phenyl glutarimide) was more active than glutarimide itself. At a dose of 20 mg/kg/day IP, 3-phenyl-piperidine-2,6-dione lowered serum cholesterol 49% and serum triglyceride 36% in mice. At 10 mg/kg/day, the derivative lowered rat levels of serum

cholesterol 54% and serum triglyceride 33% after 14 days' dosing, which indicated the agent was active orally. Examination of the effect of 3-phenyl-piperidine-2,6-dione on lipid de novo synthetic pathways showed that the agent inhibited enzymatic steps early in the de novo synthetic pathway of cholesterol, e.g., mitochondrial citrate exchange, acetyl CoA synthetase and ATP-dependent citrate lyase. Inhibition at these three sites theoretically leads to reduced levels of cytoplasma acetyl CoA required for cholesterol and fatty acid synthesis. Apparently this is not the only reason for a reduction of cholesterol levels in the liver, since ^{3}H-cholesterol distribution studies demonstrated that after drug treatment less radioactivity was found in the tissue, while higher concentrations of the radiolabeled cholesterol and its metabolites were found in the small and large intestinal tissue and chyme and fecal contents. These data suggest that the drug may bring about a large excretion rate of the cholesterol and its bile acids via the bile or, alternatively, that the drug reduces absorption of orally administered cholesterol from the intestine. The plasma levels of ^{3}H-cholesterol after 24 hr demonstrated a 62% reduction after drug treatment, which would support a more rapid clearance of cholesterol from the body. The observed reduction in triglyceride levels appears to be the result of inhibition by the drug of the two regulatory enzymes of the de novo synthetic pathway, e.g., *sn*-glycerol-3-phosphate acyl transferase and phosphatidylate phosphohydrolase. The latter enzyme appeared to be inhibited to a higher magnitude. The heparin-induced lipoprotein lipase, an enzyme which releases triglycerides from lipoproteins for tissue storage, was also inhibited by the drug.

The effects of the drug on lipid levels of rat serum lipoproteins appear to be encouraging. Whereas it is difficult to draw a direct parallel between rat serum lipoprotein levels and lipoprotein levels in man, it was interesting to observe that 3-phenyl-piperidine-2,6-dione caused favorable changes in lipoprotein lipid levels; for example, the cholesterol content of the HDL fraction was elevated significantly. This supposedly protects against myocardial infarction in man (26). HDL is responsible for the conduction of cholesterol from peripheral cells, e.g., aorta plaques, to the liver for the purpose of excretion. Drug treatment lowered triglyceride, neutral lipid and cholesterol in the LDL fraction. The LDL fraction is responsible for lipid delivery into the aorta plaques. In hyperlipidemic patients, HDL lipids are low and LDL lipids are high. Therapeutically, one would like to reverse these parameters to achieve a better clearance of lipid from the blood compartment and peripheral tissues. Drug therapy with 3-phenyl-piperidine-2,6-dione appears to modulate the lipoprotein lipids in a favorable direction. Thus, it is desirable to investigate this group of agents further for its potential hypolipidemic effects in man.

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Transfer of Arachidonate from Phosphatidylcholine to Phosphatidylethanolamine and Triacylglycerol in Guinea Pig Alveolar Macrophages

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Guinea pig alveolar macrophages were labeled by incubation with either arachidonate or linoleate. Arachidonate labeled phosphatidylcholine (PC), phosphatidylethanolamine (PE) and triglycerides (TG) equally well, with each lipid containing about 30% of total cellular radioactivity. In comparison to arachidonate, linoleate was recovered significantly less in PE (7%) and more in TG (47%). To investigate whether redistributions of acyl chains among lipid classes took place, the macrophages were incubated with 1-acyl-2-[1-^{14}C]arachidonoyl PC or 1-acyl-2-[1-^{14}C]linoleoyl PC. After harvesting, the cells incubated with 1-acyl-2-[1-^{14}C]linoleoyl PC contained 86% of the recovered cellular radioactivity in PC, with only small amounts of label being transferred to PE and TG (3 and 6%, respectively). More extensive redistributions were observed with arachidonate-labeled PC. In this case, only 60% of cellular radioactivity was still associated with PC, while 22 and 12%, respectively, had been transferred to PE and TG. Arachidonate transfer from PC to PE was unaffected by an excess of free arachidonate which inhibited this transfer to TG for over 90%, indicating that different mechanisms or arachidonoyl CoA pools were involved in the transfer of arachidonate from PC to PE and TG. Cells prelabeled with 1-acyl-2-[1-^{14}C]arachidonoyl PC released ^{14}C-label into the medium upon further incubation. This release was slightly stimulated by zymosan and threefold higher in the presence of the Ca^{2+}-ionophore A_{23187}. Labeling of macrophages with intact phospholipid molecules appears to be a suitable method for studying acyl chain redistribution and release reactions.
Lipids 21, 623–628 (1986).

Macrophages are known to release a variety of eicosanoids in reaction to phagocytotic stimulation (1–8). The main products formed are arachidonic acid and derived metabolites synthesized via either the cyclooxygenase pathway, such as prostaglandins and thromboxanes, or the lipoxygenase pathway, such as leukotrienes and hydroxyeicosatetraenoic acid (HETE). The bulk of arachidonate in mammalian cells is esterified to glycerophospholipids, almost exclusively at the *sn*-2-position of the glycerol backbone. It is widely accepted that phospholipases are the key enzymes in the regulation of free arachidonate production, although the occurrence of a phospholipase that can specifically liberate arachidonate from glycerophospholipids has not been unequivocally demonstrated (see 9 and 10 for reviews). It is obvious that the level of free arachidonate in the cell is determined by the balance of hydrolysis, through phospholipase activity and re-esterification of the released fatty acid into other lipids via action of acyltransferases. Normally, the level of free arachidonate in cytoplasma is low. This low level is probably caused by a high affinity of the lysophosphoglyceride acyltransferases for arachidonoyl-CoA (11–16). Whether this preference can be explained by the presence of separate acyltransferases for arachidonoyl-CoA and other long chain acyl-CoA esters is still subject to discussion (12,17–21).

The reaction catalyzed by lysophosphoglyceride:acyl-CoA acyltransferase is reversible, as first demonstrated by Irvine and Dawson (22). The presence of the acyltransferase operating in reverse has since been confirmed in various cells and organelles (23–28). The reverse reaction can lead to a direct ATP-independent formation of acyl-CoA ester. Since the lysophosphoglyceride:acyl-CoA acyltransferase exhibits a high preference for arachidonoyl-CoA, the reverse reaction could provide a means for a specific cleavage of arachidonate from glycerophospholipids and may contribute to the redistribution of arachidonate over lipid classes.

In comparison to phospholipids, neutral lipids have received less attention in contributing to the total store of arachidonate inside the cell, since the main neutral storage lipid, triacylglycerol, is generally low in arachidonate content. Recently, however, some evidence was presented that a neutral lipid lipase is involved in arachidonate release in the renal inner medulla (29). This observation, together with an earlier report of MacDonald et al. on a high turnover of neutral lipid arachidonate in brain (30), may be indicative of an as-yet-unknown role of neutral lipid species in arachidonate liberation.

In this paper we describe the redistribution of arachidonate over both phospholipids and neutral lipids of guinea pig alveolar macrophages. For that purpose, we labeled the macrophages with either [1-^{14}C]arachidonate or vesicles prepared of 1-acyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine (PC). Arachidonate incorporation was demonstrated into phosphatidylethanolamine (PE) as well as triacylglycerol. Evidence is presented that the incorporation of arachidonate into these lipids proceeds through different pathways.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-[1-^{14}C]arachidonoyl PC (58 mCi/mmol), [1-^{14}C]linoleic acid (57 mCi/mmol) and [1-^{14}C]arachidonic acid (57 mCi/mmol) were purchased from Amersham, Buckinghamshire, United Kingdom. 1-Acyl-2-[1-^{14}C]linoleoyl PC was synthesized biochemically from 1-acyl-2-lysoPC and linoleoyl-CoA as described by van den Bosch et al. (31) for the synthesis of 1-acyl-2-[1-^{14}C]linoleoyl PE. Egg yolk PC was purified by high performance liquid chromatography and donated by W.S.M. Geurts van Kessel of this laboratory. Sodium pentabarbitol (Nembutal) was a product of Abbott Laboratories (North Chicago, Illinois). Fetal calf serum (FCS) was obtained from Gibco (United King-

dom). PC exchange protein was purified from bovine liver as described by Westerman et al. (32) and was a gift of Dr. van Loon of this laboratory. Ionophore A_{23187}, zymosan A and lipopolysaccharides from *E. coli* O_{111}:B_4 were purchased from Sigma Chemical Co. (St. Louis, Missouri). All other chemicals were obtained from either Merck (Darmstadt, Federal Republic of Germany) or Fluka (Buchs, Switzerland) and were of standard laboratory grade.

Isolation of guinea pig alveolar macrophages. Male guinea pigs (CPB, TNO, Zeist, The Netherlands) weighing 400–700 g were injected intraperitoneally with an overdose of Nembutal. Lungs were lavaged six times with 20 ml of 2.6 mM EDTA/0.15 M NaCl through a cannula in the trachea. During the lavage a pressure of 20 cm H_2O was applied. The lavage fluids were pooled and subsequently centrifuged at 800 × g for 10 min at 4 C. The cells in the resulting pellet were washed twice with 10 ml modified Hank's balanced salt solution (MHBSS), containing (in mM) NaCl (137.9), KCl (5.4), $CaCl_2$ (5.0), $MgSO_4$ (0.8), K_2HPO_4 (0.3), KH_2PO_4 (0.4) and glucose (5.6; pH 7.4). Cells were resuspended in the same buffer solution and counted in a hemocytometer. After staining according to Giemsa, 90% of cells were identified as alveolar macrophages, the remaining being mainly lymphocytes. Granulocytes could not be detected (<1%). From each animal, up to 20 × 10^6 macrophages were obtained. The viability of the macrophages, as assessed by trypan blue exclusion, always exceeded 98%.

Preparation of labeled PC vesicles. Vesicles were prepared by sonication of 50 μM 1-acyl-2-[1-^{14}C]arachidonoyl PC (12,500 dpm/nmol) or 50 μM 1-acyl-2-[1-^{14}C]linoleoyl PC (12,500 dpm/nmol) in the presence of 2 mol % phosphatidic acid in 3 ml MHBSS for 15 min at 50 W under N_2 at 0 C.

Labeling of alveolar macrophages with radioactive PC. Alveolar macrophages from lung lavage in a concentration of 5 × 10^6 cells/ml MHBSS were incubated with 20 μM of the radiolabeled PC vesicles for 45 min at 37 C. For each incubation, an aliquot of 0.2 ml from this suspension was pipetted into a glass tube and adjusted to 0.5 ml with 5% (v/v) FCS in MHBSS. The labeled macrophages were allowed to attach to the glass by incubation for 2 hr at 37 C. Unincorporated vesicles, dead macrophages and contaminating cells were subsequently removed by washing twice with 0.5 ml MHBSS. The incubation was stopped by addition of 0.5 ml KCl/10% (v/v) glycerol and 1.5 ml chloroform/methanol/acetic acid (50:100:2, v/v/v). To study the time dependency of the incorporation of label from 1-acyl-2-[1-^{14}C]arachidonoyl PC into other lipid classes, alveolar macrophages were allowed to attach to glass tubes and were washed as described, prior to labeling with 20 μM 1-acyl-2-[1-^{14}C]arachidonoyl PC vesicles in FCS medium and washing as described above.

Labeling of alveolar macrophages with radioactive fatty acid. Alveolar macrophages from lung lavage were allowed to attach to glass tubes by incubation for 2 hr at 37 C in 0.5 ml MHBSS containing 2 × 10^6 cells/ml. Nonattached cells were removed by washing twice with 0.5 ml MHBSS. Subsequently, the alveolar macrophages were incubated with either 0.165 nmol [1-^{14}C]arachidonic acid (126,318 dpm/nmol) or 0.176 nmol [1-^{14}C]linoleic acid (122,100 dpm/nmol) for 45 min in a final volume of 0.5 ml 5% (v/v) FCS in MHBSS. At the end of the incubation, the nonincorporated radioactivity was removed by washing twice with 0.5 ml 5% (v/v) FCS in MHBSS. Incubation was stopped by the addition of 0.5 ml 1 MKCl/10% (v/v) glycerol and 1.5 ml chloroform/methanol/acetic acid (50:100:2, v/v/v).

In vitro stimulation of alveolar macrophages labeled with 1-acyl-2-[1-^{14}C]arachidonoyl PC. Alveolar macrophages were labeled with 1-acyl-2-[1-^{14}C]arachidonoyl PC, attached to glass and washed as described. Subsequently, the macrophages were incubated with either 10 μM A_{23187} or 20 μg/ml opsonized zymosan in 0.5 ml 5% (v/v) FCS in MHBSS for 2 hr at 37 C. After the incubation, the cells were washed twice with 0.5 ml 5% (v/v) FCS in MHBSS. The lavage fluids were pooled and radioactive fatty acid released into the medium was determined after heptane extraction and separation on silica gel minicolumns as described previously (31,33).

Extraction and separation of lipids. Lipids were extracted according to the method of Bligh and Dyer (34). Phospholipid and neutral lipid classes were separated on silica gel 60 H thin layer plates using chloroform/methanol/acetic acid/water (90:54:5.5:5.5, v/v/v/v) and petroleum ether/diethyl ether/formic acid (60:40:1.5, v/v/v), respectively, developing systems. Spots were visualized by short exposure to I_2 vapor and subsequently scraped into scintillation vials.

Analytical procedures. Radioactivity was measured after addition of Packard emulsifier scintillation fluid in a Packard 3320-Tricarb liquid scintillation spectrometer.

Statistical analysis. Data are expressed as means ± SEM. Student's t-test was used to determine the significance of the observed differences. Results are regarded as significant when $P < 0.05$.

RESULTS AND DISCUSSION

In this paper we describe the labeling of guinea pig alveolar macrophages with radioactive fatty acid as well as with vesicles of radioactive PC. In suspension, radioactivity from PC vesicles was readily incorporated into the cells. After 2 hr of incubation, 15–20% of the radioactivity could be recovered in macrophagal lipids. Labeling of the macrophages with the free fatty acid resulted in a nearly complete (>90%) recovery of radioactivity in the lipids.

In Figure 1, the relative distribution of radioactivity among lipids is depicted when alveolar macrophages were incubated with [1-^{14}C]linoleic acid or [1-^{14}C]arachidonic acid. The fatty acids are equally well incorporated into PC, but there is a striking preference for arachidonate over linoleate for esterification into PE. On the other hand, linoleate seems to be more readily incorporated than arachidonate into triglycerides (TG), with 47 and 28% being recovered in this neutral lipid, respectively. Lipid classes, other than those indicated in Figure 1, did not contain any detectable radioactivity (data not shown). The preference for the incorporation of arachidonate over linoleate into ethanolamine glycerophospholipids is in agreement with results of similar experiments performed with bone marrow–derived macrophages by Flesh et al. (27) and rabbit alveolar macrophages by Sugiura et al. (35). The latter authors further showed a 11–13% recovery of arachidonate in ino-

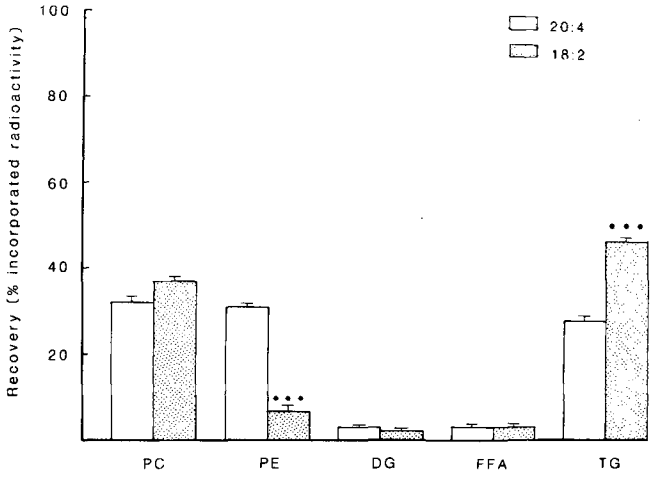

FIG. 1. Labeling of alveolar macrophages with [1-^{14}C]arachidonic acid and [1-^{14}C]linoleic acid. Alveolar macrophages were isolated and labeled with radioactive fatty acid as described in Materials and Methods. At the end of the incubation, cells were washed to remove noncellular radioactivity. Subsequently, lipids were extracted and separated on silica gel thin layer plates. Spots were scraped into scintillation vials and radioactivity was measured as described. Results are given as percentage of recovery of cellular radioactivity and are expressed as means ± SEM from 3–8 experiments. Statistical significance of the difference in incorporation of linoleic acid and arachidonic acid was determined by Student's t-test: *, $p < 0.05$; **, $p < 0.001$. FFA, free fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DG, diglyceride; TG, triglyceride.

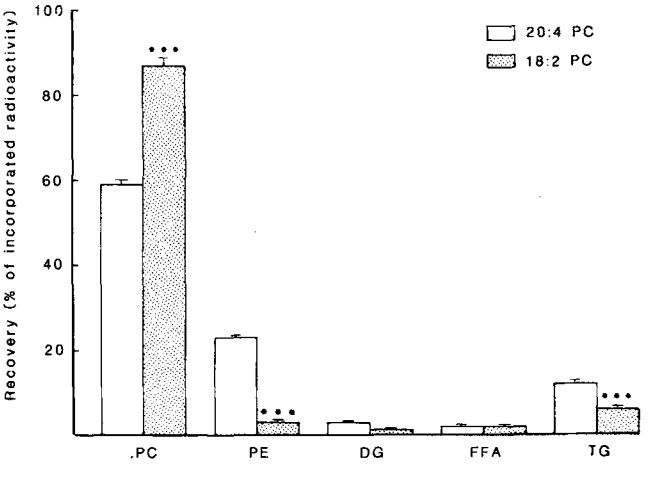

FIG. 2. Labeling of alveolar macrophages with 1-acyl-2-[1-^{14}C] arachidonoyl phosphatidylcholine and 1-acyl-2-[1-^{14}C] linoleoyl phosphatidylcholine. Alveolar macrophages were isolated and labeled in suspension with vesicles of radioactive phosphatidylcholine by incubation for 45 min at 37 C as described. The labeled macrophages were subsequently allowed to attach to glass by incubation for another 2 hr at 37 C in 0.5 ml 5% (v/v) FCS in MHBSS in a concentration of 2×10^6 cells/ml. At the end of the incubation, macrophages were washed to remove excess vesicles and contaminating cells. Lipids were extracted from the macrophages and separated as indicated in the legend of Fig. 1. Radioactivity in the spots was measured as described. Results are expressed as percentage of recovery of cellular radioactivity and are given as means ± SEM from 7–8 experiments. Data were analyzed statistically as described in the legend of Fig. 1. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

sitol glycerophospholipids. For unknown reasons, we were not able to detect any arachidonate incorporation into this phospholipid. The 28% recovery of labeled arachidonate in TG species is much higher than the 2% reported for peritoneal macrophages (36). The reasons for this difference are currently unknown. Fatty acid distributions as observed in Figure 1 may be the result of initial incorporation via acyl-CoA and subsequent redistributions by acyltransfer reactions between lipid classes as recently described for a variety of biological systems. To investigate whether arachidonate became specifically transferred from PC to PE we labeled the macrophages with vesicles of either 1-acyl-2-[1-^{14}C]arachidonoyl PC or 1-acyl-2-[1-^{14}C]linoleoyl PC. As shown in Figure 2, arachidonate is indeed much more efficiently transferred from PC to PE species than linoleate. A preference of arachidonate over linoleate could also be observed for the incorporation into TG, although this was less pronounced than found for transfer to PE. It is noteworthy that the relative proportion of radioactivity in PE and TG is quite different, both for linoleate and arachidonate, from that obtained after labeling the cells with these free fatty acids. This excludes the possibility that labeling of the lipids proceeded by incorporation of fatty acids that were produced outside the macrophages by putative phospholipases from adhering PC.

Figure 3 shows the time-dependent incorporation of arachidonate from 1-acyl-2[1-^{14}C]arachidonoyl PC into other lipid classes. In this experiment, macrophages were allowed to attach to the glass tube prior to incubation with radiolabeled PC vesicles. The incorporation of label into PE and TG increased nearly linearly over the time interval tested. The total amount of radioactivity recovered in the lipids of the macrophages increased to about 8% of added radioactivity after 3 hr incubation. This is significantly lower than the 15–20% recovery found upon labeling of the cells in suspension for 2 hr. Possibly, in the former case, there is a reduced contact area for vesicles and macrophages, resulting in a diminished uptake of radioactive vesicles.

However, it is obvious that the relative proportion of arachidonate in PE and TG is constant in time and of similar magnitude as observed in Figure 2, where the cells were labeled in suspension. In a separate series of experiments, we tried to facilitate and enhance PC uptake from vesicles into macrophages by addition of PC transfer protein from bovine liver to the incubation. However, addition of this protein (12 μg/ml) had no stimulatory effect on the incorporation of label from the PC vesicles into the macrophages, nor did it influence the relative distribution of label among the macrophagal lipid classes (data not shown). These results indicate that PC transfer protein cannot be used for the labeling of alveolar macrophages. It is quite likely that alveolar macrophages are capable of internalizing the PC vesicles by means of phagocytosis. In studies of arachidonate transfer, it can be advantageous to label cells or organelles with intact phospholipid molecules to distinguish between initial incorporation into lipids and subsequent redistribution among lipid classes. The results of Figures 2 and 3 clearly indicate that extensive redistribution

takes place with arachidonate initially confined to PC and suggest that such redistributions would also occur during the time interval required to label the cells with free fatty acids.

In the next series of experiments, we investigated the possible role of phospholipase A activity in the redistribution of arachidonate. For that purpose, we labeled alveolar macrophages in the presence of both 20 μM 1-acyl-2-[1-^{14}C]arachidonoyl PC and 110 μM unlabeled arachidonate. If phospholipases are active in the redistribution process, the labeled arachidonate released upon action of these enzymes is expected to be diluted immediately with excess unlabeled arachidonate. Consequently, lipids that depend for their labeling on a free arachidonate pool previously released by phospholipase action should show reduced labeling. The results of such experiments demonstrate (Fig. 4) that under these conditions arachidonate incorporation into PE is unaffected. Incorporation of label into TG, however, is completely inhibited in the presence of excess unlabeled arachidonate. These results indicate that the transfer of arachidonate from PC to PE and TG proceeds through different pathways. Incorporation of arachidonate into TG is preceded by a phospholipase A–mediated cleavage of PC, whereas labeling of PE most likely can be accounted for by direct arachidonate transfer.

In the next experiments, alveolar macrophages were labeled with 1-acyl-2-[1-^{14}C]arachidonoyl PC and subsequently stimulated with a number of compounds

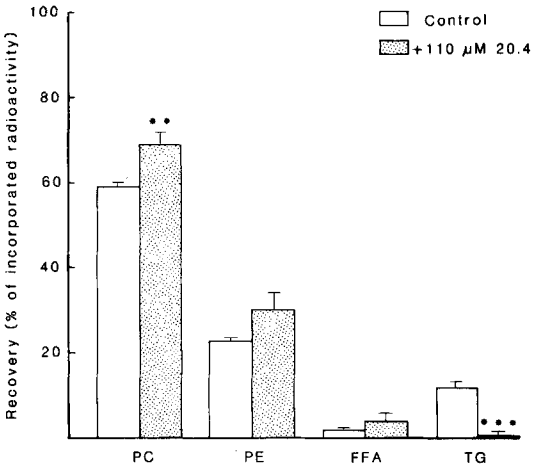

FIG. 4. Labeling of alveolar macrophages with 1-acyl-2-[1^{14}C] arachidonoyl phosphatidylcholine in presence of unlabeled arachidonate. Alveolar macrophages were labeled with 1-acyl-2-[1-^{14}C] arachidonoyl phosphatidylcholine as described in the legend of Fig. 2, with the exception that 110 μM arachidonate was present during the labeling procedure. At the end of the incubation, cells were washed and lipids were extracted from the macrophages. Radioactivity in the various lipid classes was quantitated as described. Results are given as percentage of recovery of cellular radioactivity and are expressed as means ± SEM from 4–8 experiments. Data were analyzed statistically as described in the legend of Fig. 1. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

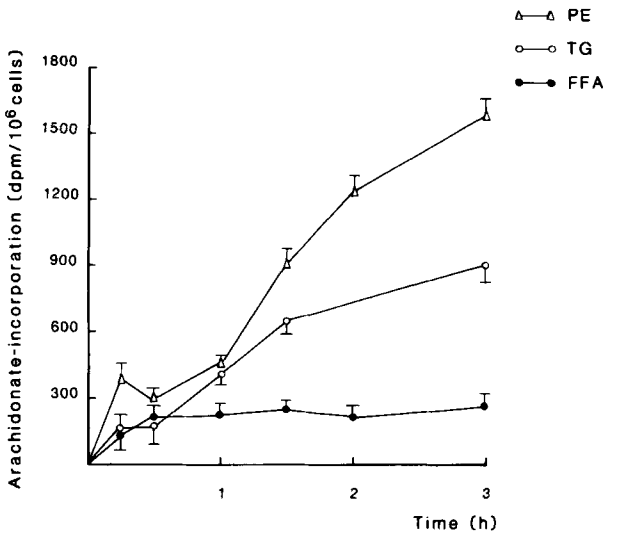

FIG. 3. Time course of arachidonate redistribution upon labeling of alveolar macrophages with 1-acyl-2-[1-^{14}C]-arachidonoyl phosphatidylcholine. Alveolar macrophages were isolated as described in Materials and Methods and attached to glass tubes. Incubations were carried out for the indicated time periods at 37 C in 0.5 ml 5% (v/v) FCS in MHBSS containing 10^6 macrophages and 20 μM 1-acyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine (12,500 dpm/nmol). At the end of the incubation, cells were washed and lipids were extracted from the macrophages as described in the legend of Fig. 1. Subsequently, lipids were separated and radioactivity was quantitated. Results are expressed as means ± SEM from three experiments. After 1, 2 and 3 hr of incubation, 85, 76 and 69% of cellular radioactivity, respectively, was recovered in phosphatidylcholine.

known to trigger release of arachidonate and related metabolites. After stimulation, the radioactivity of fatty acids released into the medium was determined. From the results depicted in Figure 5, it can be concluded that treatment of the cells with either the Ca^+-ionophore A_{23187} or zymosan gave rise to a significant increase of arachidonate label released in the medium. The observed stimulations of released arachidonate label into the medium are comparable to results reported by Albert and Snyder (4) upon incubation of rat alveolar macrophages with A_{23187} and zymosan after labeling of the cells with free fatty acids.

In rabbit alveolar macrophages, treatment with zymosan resulted in a threefold increase in free arachidonate (2). Lipopolysaccharides from bacterial membranes, stimuli of arachidonate release from alveolar macrophages (39), did not increase the release of arachidonate in our experimental set-up. When the relative distribution of arachidonate was determined after labeling of alveolar macrophages with 1-acyl-2-[1-^{14}C] arachidonoyl PC and subsequent treatment with or without 10μM A_{23187}, the distribution of label among the lipid classes remained virtually unchanged. Probably the amount of arachidonate, released upon treatment with ionophore (amounting to 2.3% of total cellular label) (Fig. 5), was too small to detect possible changes in the distribution of arachidonate among the lipid classes of the macrophage in our experiment. In contrast, Hsueh et al. (2) demonstrated a loss of arachidonate from PC, with a concomitant increase of arachidonate in PE after stimulation of rabbit alveolar macrophages. In peritoneal macrophages, there are strong indications of inositol phosphoglycerides being actively

involved in intracellular arachidonate turnover (37,38). In this cell type, it was also shown that TG play only a minor role in arachidonate storage (36). We could show that in guinea pig alveolar macrophages arachidonate is readily incorporated to TG species. The actual significance of TG as an intracellular arachidonate pool is still obscure, but it may be important to the arachidonate metabolism in alveolar macrophages.

In summary, we have shown considerable transfer of arachidonate from PC to PE and TG in guinea pig alveolar macrophages. These transfer processes appear to proceed by different mechanisms. Addition of free arachidonate almost completely abolished transfer to triacylglycerol, suggesting that this transfer proceeds via an intracellular arachidonoyl-CoA pool after phospholipase A–catalyzed release of labeled arachidonate from PC. By contrast, transfer to PE was unaffected by unlabeled arachidonate, and the mechanism of this transfer in intact cells cannot be assessed with certainty. One possibility would be a CoA-independent transfer of arachidonate, as recently described in both platelets (25) and rabbit alveolar macrophages (40). On the other hand, CoA-mediated transfer of fatty acids, which has been described in various systems (22–28), as catalyzed by lysophosphoglyceride:acyl-CoA acyltransferase operating in both backward and forward direction, cannot be completely excluded. Such a mechanism would require that the intermediary, and in this case presumably membrane-associated, acyl-CoA pool is not freely miscible with the total cellular acyl-CoA pool during the time scale of the acyltransfer reaction. Experimental evidence that the product of a membrane-associated enzyme can be used as substrate by another membrane-associated enzyme without prior mixing with the total pool of this compound has been obtained earlier in studies on phosphatidic acid biosynthesis (41).

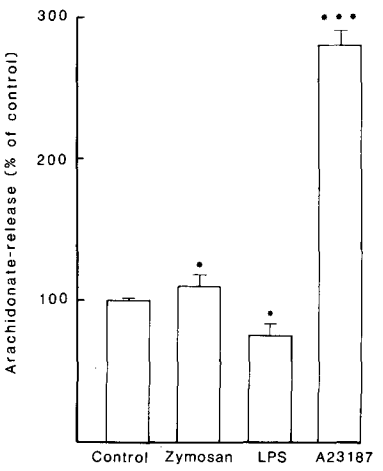

FIG. 5. Release of arachidonate upon stimulation of alveolar macrophages, labeled with 1-acyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine. Alveolar macrophages were isolated and labeled with 1-acyl-2-[1-^{14}C]arachidonoyl phosphatidylcholine as described in the legend of Fig. 2. Labeled macrophages were allowed to attach to glass and were washed. Subsequently, the macrophages (10^6 cells) were incubated for 2 hr at 37 C with either 10 μM A_{23187}, 20 μg/ml bacterial lipopolysaccharides (LPS) or 2 mg/ml zymosan in a final volume of 0.5 ml 5% (v/v) FCS in MHBSS. At the end of the incubation, macrophages were washed twice with 0.5 ml 5% (v/v) FCS in MHBSS. Fatty acids were isolated from the pooled lavage fluids as described in Materials and Methods. Results are expressed as percentage of recovery of arachidonate in the medium, relative to controls. The control value amounted to 0.8% of total cellular radioactivity. Means ± SEM from three experiments are depicted. Statistical significance of the difference in arachidonate release of treated and control macrophages was determined by Student's t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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Regulation of Liver Cell Ganglioside Composition by Extracellular Fluid Viscosity

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The viscosity of plasma and extracellular fluid has been shown to be a regulator of lipoprotein production both in cultured hepatocytes and in vivo. The possibility that this extracellular effect on cell function involves modulation of cell surface membrane components was examined. In the present work, we studied the effect of medium viscosity on liver cell gangliosides known to be involved in various membrane functions and to be located predominantly at the cell surface membrane. Cultivation of isolated hepatocytes as primary cultures markedly reduced the ganglioside content, but this reduction process was attenuated by increasing the viscosity of the culture medium. Elevation of extracellular fluid viscosity inhibited the degradation of the cell gangliosides and secretion of lysosomal enzymes involved in ganglioside degradation. The cellular activity of these enzymes as well as the activity of enzymes involved in ganglioside synthesis, CMP-NANA:GM_1 sialyltransferase, CMP-NANAP:GM_3 sialyltransferase and UDP-galactose:GD_2 galactosyltransferase, were not affected by modulation of the extracellular medium viscosity. It is proposed that the modulation of cell ganglioside content by extracellular fluid viscosity is due to an effect on enzymes involved in ganglioside catabolism. *Lipids 21,* 629–633 (1986).

The viscosity of blood and plasma is elevated in numerous pathological conditions and has been studied mainly in relation to circulation and hemodynamics (1). In recent years, it has been shown that the solvent viscosity is an important determinant in protein dynamics and enzyme-substrate interaction (2,3). The relevance of extracellular fluid viscosity to cell function has been demonstrated by Yedgar et al., who showed that the viscosity is a regulator of lipoprotein metabolism, both in vivo and in cultured hepatocytes. Increasing the plasma viscosity of hyperlipidemic rats markedly reduced plasma triglyceride and cholesterol levels (4). In hepatocyte cultures, increased viscosity of the extracellular fluid linearly inhibited the secretion and synthesis of protein and lipid components of very low density lipoproteins (VLDL), while their cellular levels were not altered (5). Medium viscosity was modulated by the addition of various macromolecules that did not penetrate into cells. This was, therefore, an extracellular effect on cell function, likely to be mediated by an effect on the cell surface membrane.

Gangliosides, involved in various membrane events, are present in cellular membranes, predominantly in the cell surface membrane (6–8). Their metabolism, in which membrane and extracellular enzymes may take part (9–11), is likely to be influenced by changes in properties of the extracellular environment. In accord with this notion, we have recently demonstrated that ganglioside content of rat liver cells is increased in hyperlipidemic state. Similarly, elevated lipid level of extracellular medium increased the ganglioside content of cultured rat hepatocytes (12). Hence, it is likely that the metabolism of gangliosides, present at the cell surface, is influenced by changes in hydrodynamic properties of the extracellular environment. The effect of extracellular fluid viscosity on gangliosides of cultured hepatocytes was explored in this study.

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MATERIALS AND METHODS

Rat liver cells were isolated and cultivated as primary monolayers on fibronectin-coated plates in serum-free Dulbeco Modified Eagle (DME) medium (Gibco, New York), as previously described (5).

Viscosity (η_r) of the culture medium was raised from 1.04 to about 4 (relative to water at 37 C) by the addition of 4% Dextran-500 (Dex; Pharmacia, Uppsala, Sweden) or 0.2% methylcellulose MX880 (MeC; Matheson, Coleman and Bell, Norwood, Ohio). These viscosity values were chosen because they are in the range of changes in plasma viscosity occurring in physiological and pathological states (1). As previously discussed (5), the use of these macromolecules, which differ widely in chemical characteristics and their capacities to increase medium viscosity, makes it possible to differentiate the viscosity from other variables such as osmolarity or chemical interaction. Relative viscosities were determined by measuring flow times of the media through a capillary viscometer at 37 C (Cannon Instrument Co., State College, Pennsylvania).

Cell gangliosides were extracted from lyophilized hepatocytes and determined by measurement of lipid-bound sialic acid (LBSA) and chromatographed according to Den et al. (13) after purification on DEAE-Sephadex (A25) columns (Pharmacia) and hydrolysis with 0.1 N KOH in methanol at 37 C for 3 hr.

Labeling of cell gangliosides was performed by incubation of cultured hepatocytes with N-acetyl-D-[$U^{14}C$]-mannosamine (sp act 231 mCi/mmol, Amersham, Buckinghamshire, England), 1 μCi in 2 ml of culture medium in a plate of 2×10^6 cells.

Activity of CMP-NANA:GM_1 sialyltransferase (GD_{1a} synthase) and CMP-NANA:GM_3 sialyltransferase (GD_3 synthase) was determined by incorporation of N-acetyl-[4-^{14}C]neuraminic acid (NANA) (Amersham) to GD_{1a} and GD_3, respectively, and that of UDP-galactose:GD_2 galactosyl transferase (GD_{1b} synthase) was determined by incorporation of ^{14}C-galactose (Amersham) to GD_{1b} (14,15).

Cell gangliosides were radioactively labeled by incubation with ^{14}C-N-acetyl-mannosamine (Amersham).

Activities of β-D-galactosidase and β-D-N-acetyl-glucosaminidase (β-hexosaminidase) were determined by measurement of the formation of 4-methylumbelliferone (4MU) from 4MU-β-D-galactoside and 4MU-N-acetyl-β-D-glucosaminide (Sigma Chemical Co., St.

Louis, Missouri), respectively (16).

Activity of lactate dehydrogenase (LDH) was determined by the UV-method with reagents and procedures of Boehringer (Mannheim, Federal Republic of Germany) using a centrifichem analyzer (17).

Cells in suspension were counted under the microscope on a hemocytometer. Cultured cells were suspended after being detached from their substratum by trypsinization.

Determination of liposome-bound ganglioside hydrolysis by condition medium of cultured hepatocytes: pyrene dodecanoyl GM_1 ($P_{12}GM_1$; GM_1 labeled with pyrene dodecanoic acid on the ceramide amido group) was provided by Dr. S. Gatt. Liposomes of egg phosphatidylcholine (PC) (Sigma) were prepared by sonication of 2 mg lipid in 2 ml acetate buffer (0.1 M, pH = 4.4) added to 40 nmol of dry $P_{12}GM_1$, vortexed thoroughly and left overnight in the cold room. The mixture was then applied to a column of Sephadex G-25 (Pharmacia) and eluted with the same acetate buffer. The elution was monitored by evaluation of the pyrene fluorescence and the phosphate content. The pyrene was associated almost exclusively with the liposome fraction, which was eluted in the void volume as expected.

The liposomal $P_{12}GM_1$ was incubated for the desired time with conditioned serum-free minimum essential medium (MEM) (collected after incubation with liver cells) at a final volume of 0.5 ml. The reaction was terminated by the addition of 2 ml chloroform/methanol (1:1, v/v) and dried under nitrogen. The gangliosides were extracted as described above, chromatographed on silica thin layer plate in chloroform/methanol/0.25% $CaCl_2$ (65:30:8, v/v/v), then in chloroform/methanol (1:2, v/v), and their fluorescence intensity was measured.

RESULTS

Effect of culture condition on liver cell gangliosides. Isolated liver cells were incubated for 4 hr after plating in DME medium supplemented with 1% fetal calf serum and 1 mu/ml insulin. The cell ganglioside content was then determined and compared to that of freshly isolated liver cells. The ganglioside level of freshly isolated hepatocytes, which was 0.46 nmol/mg cell protein, was reduced by about tenfold upon cultivation in control medium, but increasing the viscosity of the culture medium diminished the reduction of ganglioside content, as shown in Figure 1. It should be noted that the cell protein content was the same for freshly isolated or cultured liver cells and was not significantly altered by incubation in viscous medium. Thin layer chromatography, depicted in Figure 2, shows that while most of the ganglioside species practically disappeared during a day of cultivation in control medium, GD_3 and GD_{1a} were not affected. This selectivity is not yet understood, but the results in general suggest that upon cultivation of isolated hepatocytes a process is initiated that causes reduction of ganglioside content, and this process is attenuated by increasing the extracellular fluid viscosity. Subsequently, possible effects of the viscosity on synthesis, degradation or shedding of gangliosides were considered.

Activity of enzymes involved in ganglioside synthesis. The changes in the ganglioside pattern shown in Figure 2 suggest that cultivation of hepatocytes as a primary

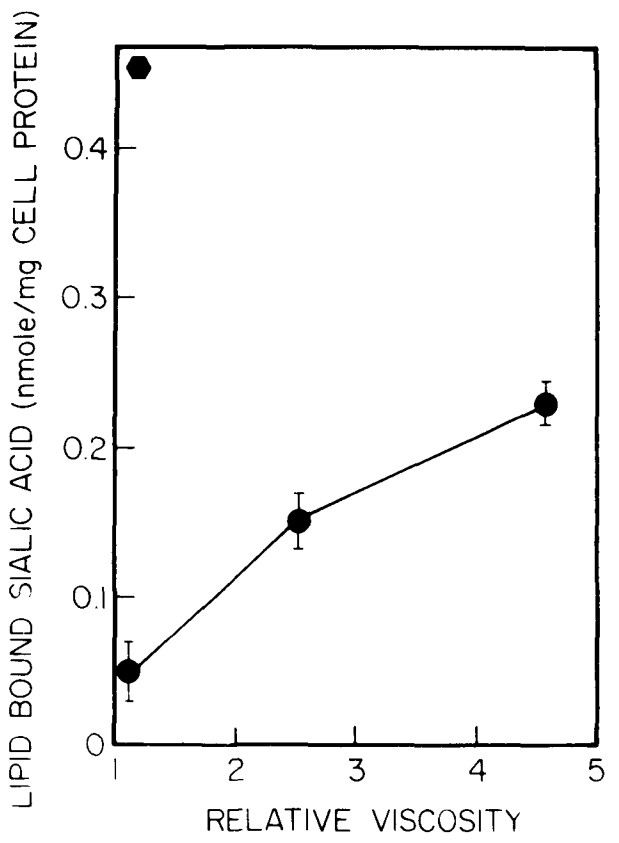

FIG. 1. Effect of medium viscosity on ganglioside content of cultured hepatocytes. Following 20 hr of incubation in control or viscous medium, cell-associated gangliosides were extracted and lipid-bound sialic acid was determined. Each datum is mean ± S.D. of results of two experiments each including 10 culture dishes (20×10^6 cells), where Dex or MeC was used to increase the viscosity. Relative viscosity of 2.4 was obtained by 2% Dex or 0.15% MeC and that of 4.6 by 4% Dex or 0.2% MeC (●), ganglioside content of freshly isolated hepatocytes.

monolayer modulates the activity of enzymes related to the metabolism of several ganglioside species, of which GM_3, GM_1, and GD_{1b} are prominent. Correspondingly, the activities of GD_3 synthase (which transforms GM_3 to GD_3), GD_{1a} synthase (which transforms GM_1 to GD_{1a}) and GD_{1b} synthase were determined following the various treatments. The activity of these enzymes in cultured hepatocytes was not significantly different from that of the culture medium (not shown).

Ganglioside degradation. The effect of culture medium viscosity on liver cell ganglioside degradation was examined by using ^{14}C-N-acetylmannosamine. Gangliosides were labeled by incubation of the hepatocytes with this precursor for one day. The cells were then washed and incubated with control or viscous culture medium for 20 hr. Gangliosides from the cells and the culture medium were separately extracted and their radioactivity was determined. The results, presented in Figure 3, clearly show that when the medium viscosity was elevated, after the cell gangliosides had been labeled, the cell-associated radioactivity increased. At the same time, a negligible amount of labeled gangliosides was detected in the culture medium. This

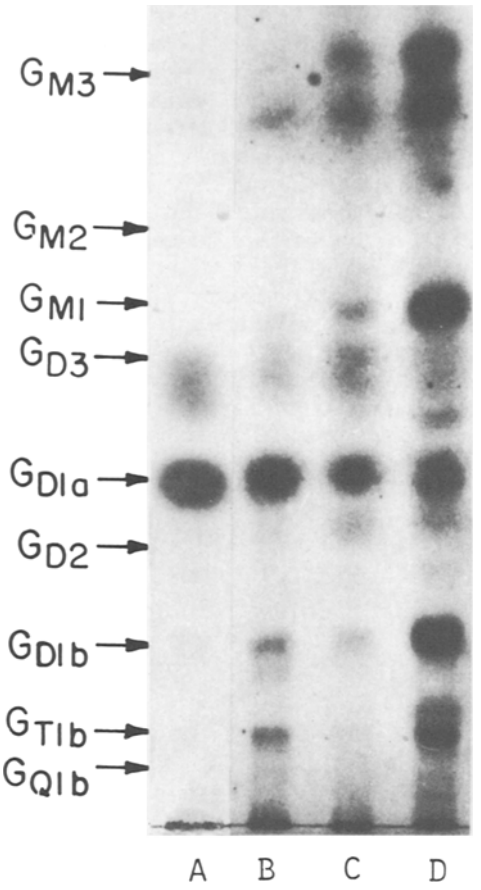

FIG. 2. Thin layer chromatogram of gangliosides of liver cells, either freshly isolated or cultured in medium with increasing viscosity. A, η_r = 1.04 (control DME medium); B, η_r = 2.4; C, η_r = 4.6; D, freshly isolated cells. Experimental procedure as described in legend to Fig. 1. Cell-associated gangliosides were extracted, chromatographed on HPTLC and identified by simultaneous chromatography of known markers, which were visualized as purple bands by resorcinol-HCl spray. The chromatogram represents three independent experiments, in which cells corresponding with 20 mg cell protein (about 20 × 10^6 cells) were used for each experiment.

suggests that increased viscosity of the extracellular fluid reduces the cell membrane ganglioside degradation.

Shedding of surface gangliosides (18). The finding noted above, that following the labeling of the cell gangliosides with ^{14}C-N-acetylmannosamine no detectable amount of labeled gangliosides accumulated in the culture medium, already indicates that their shedding was insignificant. This was further examined by measurement of LBSA that accumulated in the culture medium during a day of cultivation of cultured hepatocytes in control or viscous medium. The amount of gangliosides in the medium was negligible (practically undetectable) under all treatment. This further suggests that shedding of cell membrane gangliosides in this system is insignificant.

Secretion of lysosomal enzymes. Degradation of cell membrane gangliosides may involve membrane or extracellular enzymes (10,11). A satisfactory method for studying the activity of surface sialidases in intact cells in culture is not available (see Discussion), hence this aspect was not studied in the present work. The effect was examined of the extracellular viscosity on secretion of lysosomal enzymes which are involved in ganglioside degradation and are secreted in active form (19,20). β-D-galactosidase and β-D-N-acetylglucosaminidase, which have been studied extensively, were chosen for this purpose. Following 20 hr of incubation of cultured hepatocytes in control DME medium, the cells were washed with MEM and incubated for up to 2 hr in control or viscous MEM. The medium was then collected, and detached cells were removed by centrifugation. The activity of β-galactosidase and β-hexosaminidase was assayed in each batch of collected medium at acidic (4.4) and physiological (7.4) pH. The results clearly demonstrated that upon incubation of the cultured hepatocytes in viscous medium, the level of secreted lysosomal enzymes was considerably decreased, as shown in Figure 4, which depicts the data obtained at the acidic pH. The same effect was observed also when the extracellular enzyme activities were determined at neutral pH, except that the activities were about 7 and 10% that of acidic pH for β-galactosidase and β-hexosaminidase, respectively. It should be noted that prior to interacting the collected medium (containing the secreted enzymes) with the synthetic substrates, the viscosity of the control media was

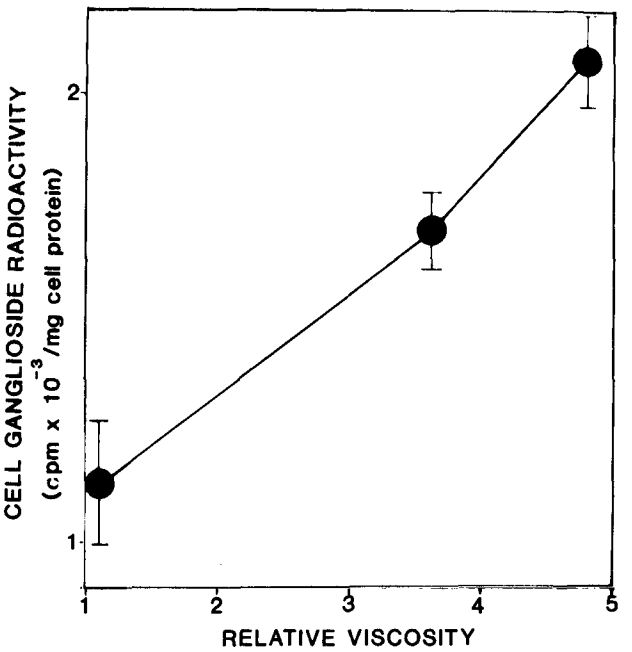

FIG. 3. Effect of medium viscosity on liver cell ganglioside degradation. Cells were incubated for one day with ^{14}C-N-acetylmannosamine, then washed and incubated for 20 hr in either control or viscous medium as in the Fig. 1 experiment. Cell-associated gangliosides were extracted, chromatographed on HPTLC and identified using known markers which were visualized by resorcinol-HCl spray. The corresponding areas were then scraped off, pooled and subjected to extraction, and the total radioactivity of the extracted gangliosides was measured. Cells corresponding to 10 mg cell protein (about 10^7 cells) were used in each experiment. The cell radioactivity following the pulse (before the chase start) was 4.2 × 10^3 cpm/mg cell protein.

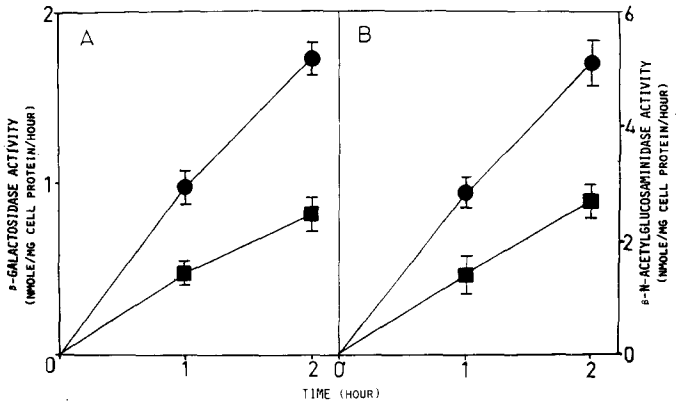

FIG. 4. Activity of β-D-galactosidase and β-D-N-acetylglucosaminidase secreted into the culture medium of hepatocytes incubated in control MEM (●) or viscous (■) medium. 4% Dex and 0.2% MeC were used to increase the viscosity to the same level. Four culture dishes were used for each treatment. Culture medium was collected after 1 or 2 hr of incubation, acidified to pH = 4.4 and incubated with 4MU-galactoside or 4MU-N-acetylhexosaminide for 1 hr at 37 C. Each datum represents mean ± S.D. for eight separate culture dishes (four for each treatment with a viscous macromolecule).

compared to that of the viscous media by addition of Dex or MeC. Modulation of the viscosity of the reaction mixture did not affect the rate of hydrolysis. This suggests that the changes in enzymatic activities presented in Figure 4 are not due to an effect on the enzyme-substrate interaction but reflect decreased levels of the secreted enzymes due to increased viscosity of the culture medium.

The activity of lysosomal enzymes described above was determined using a water-soluble synthetic substrate. It has already been shown that liver β-galactosidase acts upon natural GM_1 in micellar structure (21). We examined the hydrolysis of liposome-bound $P_{12}GM_1$ by medium collected from liver cell culture, as described in Materials and Methods. Cultured liver cells were incubated in serum-free MEM for 2 hr, and the medium was then collected. A 0.5-ml quantity of this conditioned medium was interacted with 2 nmole of liposome-bound $P_{12}GM_1$ and incubated at 37 C for 1 hr. The reaction mixture was then subjected to lipid extraction and chromatographed on a thin layer plate, in parallel with GM_1, $P_{12}GM_1$ and GM_2 markers, as described in Materials and Methods. In this system $P_{12}GM_1$ migrated in front of GM_1, closer to GM_2. The extract of the reaction mixture showed two fluorescent bands in addition to that of $P_{12}GM_1$. One moved in front of the GM_2 marker. Since $P_{12}GM_1$ migrates ahead of the unconjugated ganglioside, we assume that this band, which precedes GM_2, is $P_{12}GM_2$, produced by the action of β-galactosidase upon $P_{12}GM_1$. This product was extracted from the silica and its fluorescence intensity corresponded to 0.3 nmol pyrene (out of a total of 2 nmol in the reaction mixture). The second band, additional to $P_{12}GM_1$, was much closer to the front, but since other fluorescent gangliosides were not available this was not identified. Yet it is clear that the liposome-bound $P_{12}GM_1$ is utilized by lysosomal enzymes secreted by cultured liver cells.

The cellular level of the lysosomal enzymes was determined after 20 hr of incubation, as well as after 2 hr of incubation in control or viscous medium, in parallel with determination of the secreted activity. The cellular activities of β-galactosidase and β-hexosaminidase were practically constant at about 4.1 mu/mg cell protein and 11.7 mu/mg cell protein, respectively, and were not affected by the incubation with viscous medium. These values resemble those reported for human liver by Van Hoof (22). Comparison with the activities accumulated in the extracellular medium suggests that the rate of secretion was about 0.4%/hr of the total activity. This ratio is close to that reported by Von Figura and Weber (19) for fibroblasts, although the total activity in fibroblasts was markedly higher than that in liver cells.

To examine the possible effect of the viscosity on cell lysis, the activity of LDH, a cytosolic enzyme, was determined in the collected MEM medium, in which the lysosomal enzymes were determined. The LDH activity in the cultured medium after 1 hr of incubation was 0.6 mu/mg cell protein, a negligible amount (0.02%) compared to the cellular level of 3000 mu/mg cell protein. Both the cellular and extracellular levels of LDH were unaffected by the viscosity of the culture medium. Cell lysis, therefore, could not account for the extracellular content of lysosomal enzymes observed here.

DISCUSSION

Our data demonstrate that upon cultivation of isolated hepatocytes as primary cultures, a process is initiated which rapidly reduces the level of the cell gangliosides. This process is hindered as the viscosity of the culture medium is increased. The viscosity did not affect the shedding of membrane gangliosides or the levels of enzymes participating in ganglioside synthesis. The results (Fig. 3) clearly suggest that extracellular fluid viscosity influences the cell ganglioside degradation. The cellular contents of enzymes participating in ganglioside metabolism were not affected by the extracellular viscosity. This is in accord with the observation that the cellular content of protein and lipid components of VLDL is not altered, while the VLDL secretion is inhibited by increased viscosity of the culture medium (5). It is therefore plausible to assume that this is an effect of an extracellular parameter on secretion and membrane composition.

The modulation of the cell membrane gangliosides by extracellular fluid viscosity might be attributed to an effect on activity of cell membrane enzymes, or on the content of secreted enzymes which participate in ganglioside degradation (10,11,23,24). The activity of cell membrane sialidases is likely to be dependent on dynamic properties (25), which are affected by changes in the topological state of the cell; they are altered when the cell is detached from its substratum or when the membrane is isolated (26). Henceforth it becomes desirable to study membrane sialidase activity in the intact cultured cell; however, a satisfactory method is not yet available. Lysosomal hydrolases involved in ganglioside metabolism are secreted into the medium in active forms (19,20), and their activity in physiological pH is sufficient to account for changes in ganglioside content observed here. Yet other enzymes, especially sialidases, ought to be affected by the viscosity to account for the modulation of cell ganglioside composition observed in this study.

Hydrodynamic properties of the extracellular fluid might influence membrane recycling or endocytosis, which are related to exocytosis. It is possible that medium viscosity inhibits endocytosis and subsequently ganglioside degradation in lysosomes. However, the ganglioside degradation process observed here must involve the action of ganglioside sialidases, which are not intracellular, but found in the plasma membrane (24). It is therefore not likely that the inhibition of ganglioside degradation by extracellular fluid viscosity is due to an effect on membrane endocytosis, although this possibility, which was not examined in this study, is not ruled out. The mechanism and extent of lysosomal enzyme uptake by liver cells (27), which is different than that of the phosphomanosyl recognition pathway (20,28), are not yet known. However, it should be noted that VLDL of the extracellular medium is not taken up by cultured hepatocytes (5,29), which makes it a definite secretory system. This observation, together with the immediate effect of the medium viscosity on the level of secreted enzymes observed here, suggests that the extracellular fluid viscosity affects primarily secretion. Of special interest is the previously reported finding (5) that medium viscosity did not influence the secretion or synthesis of albumin by cultured hepatocytes. Considering that albumin is not a glycoprotein, this might suggest that the extracellular fluid viscosity regulates the exocytosis of glycoproteins such as the lysosomal enzymes studied here and VLDL apoproteins (30). This finding is in agreement with other evidence that the secretion mechanism of albumin is not identical with that of glycoproteins (31).

Changes in fluid viscosity similar to those applied in this study occur in vivo in various diseases (1) and in vitro, when culture media are supplemented with serum, albumin or other additives. The regulation of secretion and cell ganglioside composition by the viscosity of the extracellular fluid might be pertinent to various cell functions and to physiological states accompanied by increased viscosity of body fluids (1).

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Effect of Clofibric Acid on the Molecular Species Composition of Diacyl Glycerophosphocholine of Rat Liver Microsomes

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The effect of administering *p*-chlorophenoxyisobutyric (clofibric) acid to rats on the molecular species composition of diacyl-glycerophosphocholine (GPC) of rat liver microsomes was studied. Microsomal choline glycerophospholipids were converted to 1,2-diradyl-3-acetylglycerol and were separated into molecular species by reverse-phase high performance liquid chromatography. Diacyl-GPC consisted of 17 different molecular species. The predominant species were arachidonoyl derivatives, such as 18:0–20:4 (22.2% of the total) and 16:0–20:4 (22.0%). Administration of clofibric acid to rats caused a marked increase in 16:0–18:1 species of diacyl-GPC from 8% to 30%, making these the predominant species of diacyl-GPC in clofibric acid–fed rats. Also, a significant decrease (50% of controls) in 18:0–18:2 and 18:0–20:4 species was observed, whereas the decrease in molecular species containing 16:0 at the 1-position such as 16:0–18:2 and 16:0–20:4 was small (approximately 85% of control). The results show that clofibric acid caused marked changes in the molecular species composition of diacyl-GPC. The participation of 1-acyl-GPC acyltransferase and stearoyl-CoA desaturase in the regulation of the molecular species composition of diacyl-GPC is discussed.
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p-Chlorophenoxyisobutyric (clofibric) acid is known as a hypolipidemic drug capable of causing peroxisome proliferation in liver (1,2) and of inducing numerous enzymes that participate in lipid degradation (3–6). Recently, it has been found that clofibric acid induces several liver microsomal enzymes that are involved in lipid biosynthesis (7–10). In a previous paper, we reported that clofibric acid caused considerable changes in the fatty acyl moieties of choline glycerophospholipid of rat liver microsomes (9). There have been several reports on the changes in the fatty acid compositions of glycerophospholipids caused by agents such as ethanol (11,12), 1-(*O*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT) (13) and CCl_4 (14). However, these earlier studies generally involved analysis of glycerophospholipids consisting of various heterogeneous molecular species. It is well known that all types of glycerophospholipids are composed of populations of well-defined molecular species that occur in characteristic proportions in various mammalian tissues (15). Little information is available on pure molecular species of glycerophospholipids. To obtain more precise information on changes in the fatty acid compositions of glycerophospholipids induced by drugs, it is necessary to achieve complete resolution of a complex mixture of various kinds of molecular species having a common structure and to analyze their molecular species.

Recently, we developed a high performance liquid chromatography (HPLC) system for the separation of individual molecular species of the diacyl, alkylacyl and alkenylacyl subclasses derived from glycerophospholipids (16). We found that this method was useful for determining the unique distribution of the molecular species of glycerophospholipids in bovine brain (16) and rabbit alveolar macrophages (17).

The present study was undertaken to investigate changes in the molecular species composition of diacylglycerophosphocholine (GPC) of rat liver microsomes due to clofibric acid administration.

MATERIALS AND METHODS

HPLC-grade organic solvents and distilled water were purchased from Wako Pure Chemical Ind. (Osaka, Japan), except for methyl-*t*-butyl ether which was from Burdick and Jackson Lab. (Muskegon, Michigan). Clofibric acid, palmitoyl-CoA, oleoyl-CoA and stearoyl-CoA were from Sigma Chemical Co. (St. Louis, Missouri).

Treatment of animals with clofibric acid and preparation of hepatic microsomes. Male rats of the Wistar/s strain (140–160 g) (Sankyo Lab. Service Co., Tokyo, Japan) were used in the study. The rats, three in each group, were fed ad libitum a control diet or a diet containing 0.5% clofibric acid (w/w) for seven days as described previously (18). All animals were exposed to an alternating light-dark cycle (light from 0800 to 1800 hr), and rats were killed between 1000 and 1100 hr. Control and clofibric acid–fed rats were killed by decapitation, and their livers were perfused with ice-cold saline and then rapidly excised. All subsequent operations were performed at 4 C. Microsomes from the rat livers were prepared by differential centrifugation as described previously (10). Livers were homogenized in 3 vol of 0.25 M sucrose, followed by centrifugation at 20,000 × g for 15 min. The supernatants were further centrifuged at 105,000 × g for 60 min. The pellets were suspended in a sucrose solution and then recentrifuged. The pellets were resuspended in 0.25 M sucrose and used as the microsome fractions for analysis of the molecular species of glycerophospholipids and for enzyme assays.

Extraction and fractionation of diacyl-GPC. Lipids were extracted from hepatic microsomes (30 mg) by the method of Bligh and Dyer (19). CGP was purified by thin layer chromatography (TLC) as described previously (9). CGP (2 μmol) was treated with phospholipase C (Bacillus cereus) in 1.0 ml of 0.1 M Tris-HCl buffer (pH 7.2) and 3.0 ml of diethyl ether for 3 hr to remove the polar head groups (20). The products were extracted and then acetylated with acetic anhydride in the presence of pyridine at 37 C for 2 hr. The 1,2-diradyl-3-acetylglycerol obtained was separated into the diacyl, alkylacyl and alkenylacyl subclasses by TLC as described previously (20).

Separation of 1,2-diradyl-3-acetylglycerol by reverse-phase HPLC. 1,2-Diacyl-3-acetylglycerol (1 μmol) dissolved in 20 μl of methanol was fractionated into individual molecular species by HPLC with a Model 655 liquid chromatograph (Hitachi Co., Tokyo, Japan) equipped

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with a reverse-phase column (4.6 mm × 25 cm, Zorbax ODS, Dupont Co., Wilmington, Delaware). The analytical conditions were essentially the same as described in the previous report (17). The solvent system was acetonitrile/2-propanol/methyl-*t*-butyl ether/water (72:18:8:2, v/v/v/v). The flow rate was 1 ml/min, and the column temperature was controlled at 35 C with a heating block (Model CH-20-C, Scientific System Inc., State College, Pennsylvania). Fractions from the column were collected and transmethylated with 0.5 N sodium methoxide. Fatty acid methyl esters were analyzed at 195 C with a Hitachi 163 gas liquid chromatograph for identification and quantitation of the molecular species separated by HPLC (20). The methyl ester of heptadecanoate was used as an internal standard for the quantitation.

Enzyme assay. 1-Acyl-GP acyltransferase was assayed with oleoyl-CoA as a substrate by the method of Eible et al. (21). The 1-acyl-GPC and 2-acyl-GPC acyltransferase were assayed with oleoyl-CoA and palmitoyl-CoA, respectively, as substrates by the method of Lands and Hart (22). Stearoyl-CoA desaturase was measured according to the method of Oshino et al. (23).

RESULTS

Clofibric acid greatly affected the fatty acid composition of diacyl-GPC of rat liver microsomes (Table 1). The relative percentage of 16:0 of microsomal diacyl-GPC was considerably increased, from 23% to 33%, upon treatment of rats with clofibric acid, whereas that of 18:0 was decreased from 20% to 12%. The sum of two saturated fatty acids in diacyl-GPC remained constant after the clofibric acid treatment. The proportions of 18:1 and 20:3 of diacyl-GPC in clofibric acid-fed rats were three times higher than those in control rats. The percentage of all polyunsaturated fatty acids in diacyl-GPC, except for 20:3, was decreased by clofibric acid treatment.

The 1,2-diacyl-3-acetyl-glycerol derived from CGP was fractionated into molecular species by reverse-phase HPLC (Fig. 1). The 1,2-diacyl-3-acetyl-glycerol was resolved into 13 separate peaks by reverse-phase HPLC. Each peak was collected, and the individual molecular species were identified and quantitated by GLC after transmethylation (Table 2). Diacyl-GPC of hepatic microsomes consisted of 17 different molecular species. CGP extracted from the liver microsomes of both control and clofibric acid-fed rats was composed of almost the same kind of molecular species. However, the quantitative distributions of the molecular species were quite different. The molecular species composition of diacyl-GPC reflects the overall fatty acid composition of this glycerophospholipid. Thus, the predominant molecular species in diacyl-GPC of control rats contained 20:4 at the 2-position, such as 18:0-20:4 (22.2% of the total) and 16:0-20:4 (22.0%). The total proportion of the arachidonoyl molecular species comprised ca. 50% of the total diacyl-GPC. The high proportion of the arachidonoyl molecular species of CGP of rat liver is in good agreement with the earlier finding using the combination of $AgNO_3$ TLC and GLC techniques (24). The treatment of rats with clofibric acid caused marked changes in the molecular species composition of microsomal diacyl-GPC, as reflected by the changes in the overall fatty acid composition of diacyl-GPC. The 16:0-18:1 species were markedly increased, from 8% to 30%, upon treatment of the rats with clofibric acid and became the most predominant species of diacyl-GPC in clofibric acid-fed rats. The proportion of the molecular species with the combination of a polyunsaturated fatty acid at the 2-position, except for 20:3, and stearate at the 1-position, such as the 18:0-20:4 and 18:0-18:2 species, was markedly decreased. The proportions of these molecular species were decreased by 50% compared to the control values. On the other hand, the extent of the decrease in the molecular species containing 16:0 at the 1-position, such as the 16:0-20:4 and 16:0-18:2 species, was much smaller than in the case of

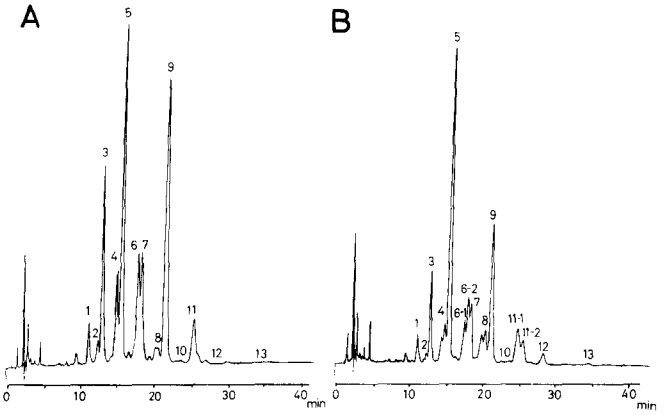

FIG. 1. HPLC separation of the molecular species of 1,2-diacylglycerol compounds of choline glycerophospholipid (CGP) of control (A) and clofibric acid-fed rats (B). 1,2-Diacyl-3-acetyl-glycerol derived from CGP (1 μmol) was dissolved in 20 μl of methanol and then injected into and chromatographed on a Zorbax ODS column at a flow rate of 1.0 ml/min. The solvent system was acetonitrile/2-propanol/methyl-*t*-butyl ether/water (72:18:8:2, v/v/v/v). Each peak was monitored by ultraviolet absorption at 205 nm. The molecular species were identified and quantified by GLC analysis. The peak numbers correspond to those in Table 2.

TABLE 1

Fatty Acid Composition (%) of Diacyl-GPC of Liver Microsomes after Clofibric Acid Administration to Rats[a]

	16:0	18:0	18:1	18:2	20:3[b]	20:4	22:6
Control	23.3 ± 1.1	20.3 ± 1.0	8.7 ± 0.5	16.3 ± 1.6	2.5 ± 0.3	23.6 ± 2.5	5.1 ± 0.5
Clofibric acid	32.7 ± 1.8	12.3 ± 0.4	20.6 ± 1.7	10.3 ± 1.3	7.3 ± 0.6	16.0 ± 1.9	1.4 ± 0.5

[a]All the values are the means (wt %) ± SD for three separate experiments.

[b]20:3 is a mixture of two isomers (n-6 and n-9).

TABLE 2

Molecular Species Composition of Diacyl-GPC of Liver Microsomes after Clofibric Acid Administration to Rats[a]

Peak number[b]	Molecular species	Control (wt %)	Clofibric acid (wt %)	%[c]
1	18:2-20:4	1.3 ± 0.2	1.1 ± 0.2	−0.2
2	18:1-22:6	0.8 ± 0.1	0.1 ± 0.1	−0.7
3[d]	16:0-22:6	4.6 ± 0.7	2.5 ± 0.3	−2.1
	18:2-18:2	0.9 ± 0.2	0.8 ± 0.1	−0.1
4	18:1-20:4	4.5 ± 0.3	1.9 ± 0.2	−2.6
5	16:0-20:4	22.0 ± 1.0	18.7 ± 3.0	−3.3
6-1	18:1-18:2	3.9 ± 0.6	2.8 ± 0.4	−1.1
6-2	16:0-18:2	11.6 ± 1.2	9.2 ± 1.2	−2.4
7	16:0-20:3 n-6	0.8 ± 0.2	4.9 ± 0.6	+4.1
8	16:0-20:3 n-9	nd[e]	1.7 ± 0.4	+1.7
9	18:0-20:4	22.2 ± 0.5	10.4 ± 1.4	−11.8
10	18:1-18:1	0.6 ± 0.1	1.6 ± 0.5	+1.0
11-1[f]	16:0-18:1	8.6 ± 0.6	30.2 ± 2.8	+21.6
	18:0-18:2	13.8 ± 1.5	6.5 ± 1.1	−7.3
11-2	18:0-20:3 n-6	0.7 ± 0.1	2.9 ± 0.3	+2.2
12	18:0-20:3 n-9	nd[e]	1.4 ± 0.3	+1.4
13[f]	18:0-18:1	1.7 ± 0.3	4.5 ± 1.0	+2.8

[a]The mean percentages ± SD are for separate samples of liver microsomes (n = 3).

[b]The peak numbers correspond to the numbers of peaks in Figure 1.

[c]Percentages were calculated by subtracting the control values from the corresponding clofibric acid treatment values.

[d]This peak is a mixture of the 16:0-22:6 and 18:2-18:2 species.

[e]These molecular species could not be detected.

[f]The positional distribution of fatty acids was not determined for these compounds. Therefore, the 18:1-16:0 and 18:1-18:0 species are probably also present in peaks 11-1 and 13, respectively.

TABLE 3

Changes in the Activities of the Liver Microsomal Enzymes of Rats after Clofibric Acid Administration

	1-Acyl-GPC acyltransferase (nmol/min/mg protein)	2-Acyl-GPC acyltransferase (nmol/min/mg protein)	1-Acyl-GP acyltransferase (nmol/min/mg protein)	Stearoyl-CoA desaturase K^+ (min^{-1})
Control	34.80 ± 4.73	57.35 ± 8.35	80.15 ± 13.42	1.04 ± 0.42
Clofibric acid	227.20 ± 16.00	43.87 ± 2.25	174.10 ± 13.23	5.30 ± 0.62

All values are means ± SD for three experiments.

the corresponding molecular species having 18:0 at the 1-position.

To investigate the contribution of acyltransferase to the modification of the fatty acid composition of diacyl-GPC caused by clofibric acid, we examined the effects of clofibric acid on the activities of the 1-acyl-GP, 1-acyl-GPC and 2-acyl-GPC acyltransferase of hepatic microsomes (Table 3). Clofibric acid induced a marked increase in the activity of 1-acyl-GPC acyltransferase, the activity in clofibric acid-fed rats being ca. seven times greater than the control value. The activity of 2-acyl-GPC acyltransferase was hardly changed by clofibric acid. The activity of 1-acyl-GP acyltransferase in clofibric acid-fed rats was higher than that in control rats; however, the extent of the change was small compared to that in 1-acyl-GPC acyltransferase. Clofibric acid also influenced the activity of stearoyl-CoA desaturase, which affects the composition of free fatty acid in hepatic microsomes. The activity of desaturase was increased five times following the administration of clofibric acid.

DISCUSSION

The present study showed the effect of clofibric acid on the proportions of the individual molecular species of diacyl-GPC in rat liver microsomes. HPLC analysis revealed that clofibric acid caused marked alterations in the molecular species composition of diacyl-GPC of rat liver (Table 2). A significant increase in the proportion of the 16:0-18:1 species was observed after the administration of clofibric acid (from 9% to 30%), whereas the increase in the 18:0-18:1 species was small (from 2% to 4%). A marked decrease in the 18:0-20:4 species (22% to 10%) was found in the clofibric acid-fed rats as compared to the 16:0-20:4 species (from 23% to 19%). This was the case in the 18:0-18:2 and the 16:0-18:2 species.

These results posed an interesting question as to the regulation of the composition of the molecular species containing 16:0 and 18:0 at the 1-position of diacyl-GPC in rat liver microsomes. The observed changes in the molecular species composition of CGP may be concerned with several enzymatic steps in the regulation of the fatty acid composition of CGP (25): (i) the stepwise acylation of glycerol-3-phosphate by acyltransferase; (ii) the formation of CGP from 1,2-diacylglycerol by cholinephosphotransferase; and (iii) the reacylation of 1-acyl-2-lyso-GPC by 1-acyl-GPC acyltransferase. It has been reported that the treatment of rat with clofibrate causes an induction of glycerol-3-phosphate acyltransferase (7), suggesting that the acylation of glycerol-3-phosphate is involved in the changes in the molecular species containing 16:0 and 18:0 at the 1-position of CGP. In addition, clofibric acid caused an increase in the concentration of free 16:0, but not 18:0 (10), which may increase the formation of 1-16:0-2-lyso-GP by glycerol-3-phosphate acyltransferase. Although clofibric acid caused an induction of 1-acyl-GP acyltransferase, the extent of the induction was much smaller than that of 1-acyl-GPC acyltransferase (Table 3). Moreover, clofibric acid hardly affected the activities of cholinephosphotransferase (8) and 2-acyl-GPC acyltransferase (Table 3). These facts suggest that 1-acyl-GP acyltransferase and cholinephosphotransferase may not contribute greatly to the selective increase in the proportion of the 1-16:0 species of hepatic diacyl-GPC due to clofibric acid. The significant changes of composition of these molecular species may be the result of the induction of 1-acyl-GPC acyltransferase. Holub et al. (26) demonstrated in in vitro experiments that 1-acyl-GPC acyltransferase exhibits strong selectivity (3.5-fold) toward the molecular species of 1-16:0-2-lyso-GPC over the 1-18:0 species of 1-acyl-GPC. This finding may explain our results showing that 1-acyl-GPC acyltransferase induced by clofibric acid facilitated the reacylation of the 1-16:0 GPC in preference to the 1-18:0 GPC, resulting in a preferential increase in the proportion of the 1-16:0 species.

Clofibric acid also induced changes in the fatty acids at the 2-position of diacyl-GPC (Table 2). An increase in the proportion of the molecular species containing 18:1 and a concomitant decrease in the species containing 18:2 and 20:4 were observed in clofibric acid-fed rats. In previous work, we examined the relationship between the proportional changes of free fatty acids in rat liver microsomes and those of fatty acids at the 2-position of CGP in rats in different physiological states: clofibric acid-fed rats, diabetic rats, insulin-treated diabetic rats, starved rats and starved-refed rats (27). We concluded that the alteration of the fatty acyl composition of hepatic CGP of rats in physiological states other than clofibric acid-fed can be interpreted as the result of the compositional changes of microsomal free fatty acids, which are caused by changes in the activity of stearoyl-CoA desaturase. On the other hand, the changes in the fatty acyl moieties of CGP in clofibric acid-fed rats may be the combined result of two factors: an increase in the activity of 1-acyl-GPC acyltransferase that is enhanced only in clofibric acid-fed rats and changes in fatty acid composition in the free fatty acid pool in microsomes. This hypothesis may explain our present results because our previous study (10) showed that the administration of clofibric acid to rats increases the relative concentration of microsomal free 18:1 by induction of stearoyl-CoA desaturase. There is some discrepancy between the increase in the activity of 1-acyl-GPC acyltransferase and the compositional changes of the arachidonoyl molecular species. It is generally accepted that 1-acyl-GPC acyltransferase exhibits marked selectivity toward 20:4-CoA (28,29). However, little increase in the proportion of the molecular species containing 20:4 was observed in clofibric acid-fed rats, even though clofibric acid significantly increased the activity of 1-acyl-GPC acyltransferase. Although the reason for this discrepancy has not yet been clarified, one explanation may be the existence of separate acyltransferases (30) or an acyl-CoA synthetase (31,32) showing specificity for 20:4, which was not induced by clofibric acid.

From these results, it would appear that 1-acyl-GPC acyltransferase induced by clofibric acid shows selectivity for the 1-16:0-GPC over the 1-18:0 species and facilitates the acylation of 18:1 to 1-acyl-GPC as compared to 18:2, because stearoyl-CoA desaturase induced by clofibric acid increases free 18:1 concentration and decreases the relative concentration of free 18:2. Consequently, clofibric acid accelerates the increase in the proportion of the 16:0-18:1 species of diacyl-GPC, while the proportion of the 18:0-18:2 species significantly decreases. These results suggest that 1-acyl-GPC acyltransferase, in concert with stearoyl-CoA desaturase, may play an important role in the regulation of the molecular species composition of CGP in rat liver.

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New Intermediates in the Conversion of Stigmasterol to Cholestanol in the Mexican Bean Beetle[1]

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Three intermediates involved in the conversion of stigmasterol to cholestanol in the Mexican bean beetle, *Epilachna varivestis*, were isolated and identified from an insect for the first time. The three new insect sterols, 22-stigmastenol, 22,24-cholestadienol and 24-cholestenol, were shown to be metabolites of [^{3}H]stigmasterol and accumulated in insect tissues when larvae were fed lima bean leaves coated with azasteroid inhibitors. The side chain metabolism of the ubiquitous phytosterol stigmasterol in the Mexican bean beetle is similar to that in other species that produce cholesterol from C_{28} and C_{29} phytosterols; however, the Mexican bean beetle initially reduces the Δ^5-bond and produces mainly cholestanol and lesser amounts of lathosterol as dealkylation products.
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The Mexican bean beetle, *Epilachna varivestis,* is unique among insects because it can metabolize the common dietary C_{28} and C_{29} phytosterols such as sitosterol, stigmasterol and campesterol to produce mostly stanols (>50% of total sterols) such as cholestanol (1,2). In addition, it produces more lathosterol (>10% of total) than any other phytophagous insect to date (1,2). In these earlier studies, it was determined that the Mexican bean beetle reduces the Δ^5-bond of the steroid nucleus prior to dealkylation of the 24-alkyl substituent from the side chain. Then cholestanol is produced by side chain dealkylation and, in addition, the Δ^7-bond is introduced to produce lesser amounts of lathosterol. Studies with several radiolabeled sterols coated on soybean leaves verified that this insect was able to dealkylate 24-alkyl sterols (2) and, significantly, that cholesterol was not produced from this dealkylation and conversion, as is the case with most other phytophagous insects (3). However, in these previous studies we obtained no information on the intermediates involved in these unusual pathways of sterol metabolism in the Mexican bean beetle. Therefore, in a recent examination of the metabolism of [^{3}H]stigmasterol in the Mexican bean beetle, we used azasteroid inhibitors, which cause an accumulation of certain intermediates involved in the conversion of C_{28} and C_{29} phytosterols to C_{27} sterol, and have isolated and identified three new intermediates involved in the conversion of stigmasterol to cholestanol in this insect. We report here the details of the isolation and identification of these three sterols, which have not been previously reported to occur in an insect.

[1]Nomenclature: 22-stigmastenol: 24α-ethyl-5α-cholest-22-en-3βol; 22,24-cholestadienol: 5α-cholesta-22,24-dien-3β-ol; 24-cholestenol: 5α-cholest-24-en-3β-ol; cholestanol: 5α-cholestan-3β-ol; lathosterol: 5α-cholest-7-en-3β-ol; cholesterol: cholest-5-en-3β-ol; stigmasterol: 24α-ethylcholesta-5,22E-dien-3β-ol; 25-azacoprostane: 5β-cholan-24-dimethylamine; 25-azacholesterol: 3β-hydroxy-chol-5-en-24-dimethylamine; campestanol: 24α-methyl-5α-cholestan-3β-ol; stigmastanol: 24α-ethyl-5α-cholestan-3β-ol; 7-stigmastenol: 24α-ethyl-5α-cholest-7-en-3β-ol; desmosterol: cholesta-5,24-dien-3β-ol; sitosterol: 24α-ethyl-cholest-5-en-3β-ol; isofucosterol: 24Z-ethylidenecholest-5-en-3β-ol.

MATERIALS AND METHODS

Biological material. Since an artificial diet for rearing the Mexican bean beetle is not available, the larvae were reared on lima bean leaves *(Phaseolus lunatus);* leaves were treated with radiolabeled sterol and/or azasteroids as needed in the various experiments. The azasteroids, either 25-azacoprostane or 25-azacholesterol, synthesized as previously described (4), were coated with a camel's-hair brush on lima bean leaves at a concentration of 50 ppm in acetone. Preliminary studies had revealed that the sterols of interest accumulated to satisfactory levels when these azasteroids were fed at this concentration. The plants were dried (15 min in a hood) before insects were placed on the leaves. Control larvae were reared on plants treated with acetone only or untreated. The insects were collected as prepupae or pupae to avoid diet material in the gut and were weighed and stored frozen until analysis.

For metabolic studies, [2,4-^{3}H]stigmasterol (sp act 5000 cpm/μg) (5) at a concentration of 1 μg/μl in acetone was coated on the leaves in combination with 25-azacoprostane (50 ppm), and last instar larvae were placed on the leaves after complete solvent evaporation. These insects were also collected as prepupae or pupae and stored frozen prior to analysis.

Sterol isolation, purification and identification. Insect samples were homogenized in $CHCl_3$/MeOH (2:1, v/v) in a Virtis homogenizer, the homogenates were partitioned against distilled water and the aqueous-MeOH phase was washed twice with fresh $CHCl_3$. The combined $CHCl_3$ phases were dried over Na_2SO_4 and filtered, and the solvent was removed on a rotoevaporator. The crude lipids were saponified in 4% KOH in MeOH, and the sterols from the nonsaponifiable fraction were isolated by column chromatography on acid grade 1.5 and neutral grade II alumina (Woelm, ICN Pharmaceuticals, Cleveland, Ohio) columns as previously described (6). The alumina column fractions were monitored by thin layer chromatography. The sterol fractions were subjected to digitonide precipitation to remove accompanying fatty alcohols. The purified sterols were acetylated in pyridine/acetic anhydride (2:1, v/v), and the resulting acetates were fractionated by argentation chromatography on 3-g columns of 20% $AgNO_3$-impregnated Unisil (Clarkson Chemical Company, Williamsport, Pennsylvania). The steryl acetate fractions were eluted with 20 ml each of *n*-hexane; 1, 2, 3 and 4% diethyl ether in *n*-hexane; and diethyl ether. The column fractions were monitored by argentation TLC on Anasil H $AgNO_3$-impregnated chromatoplates (toluene/hexane, 2:3, v/v).

Sterols and steryl acetate fractions were quantitatively and qualitatively analyzed by gas liquid chromatography (GLC) on a J&W DB-1 fused silica capillary column, 15 m × 0.25 mm i.d. (0.25 μm film), at 235 C, helium carrier gas at 25 cm/sec linear velocity, 23:1 split ratio, in a varian model 3700 gas chromatograph interfaced with a Shimadzu C-R1B Chromatopac data processor. Radiolabeled samples were fractionated on a 15 m × 0.53 mm

i.d. J&W DB-1 Megabore GLC column (1.5 μm film), and fractions were trapped from the effluent for counting. GLC identifications were based on comparisons of retention times (RRTs) relative to cholestane as an internal standard. Unknown sterols were also analyzed by gas chromatography-mass spectrometry (GC-MS) of appropriate steryl acetate fractions with a Finnigan model 4510 automated instrument equipped with a J&W DB-1 fused silica capillary column.

RESULTS

The major sterols isolated and identified from samples of mixed Mexican bean beetle prepupae and pupae and their relative concentrations are listed in Table 1. The major sterols of lima bean leaves are listed in Table 2. The sterol compositions of samples from insects reared on control (untreated) and acetone-treated leaves are very similar and are comparable to values published previously when the insects were reared on soybean leaves (1). Cholestanol, lathosterol, campestanol, stigmastanol and 7-stigmastenol were among the most abundant sterols of the samples. In addition, 22-stigmastenol (8.8 and 9.1% of total) was identified by GC-MS (described later) as a major component in both samples, and 22,24-cholestadienol was also shown by GC-MS to be present at lower levels. All of these sterols except the latter two were easily identified by comparison of GLC RRTs with those of standards and by GC-MS and had been previously isolated and identified from the Mexican bean beetle (1). Very little of the major dietary lima bean sterols (stigmasterol and sitosterol) were present in either sample.

The sterols from the bean beetles that were reared on leaves coated with azasteroids reflected the effects of the inhibitors on sterol metabolism. There were notable reductions in the levels of cholestanol and lathosterol but sizeable increases in campestanol with both azasteroid treatments. The stigmastanol level did not change appreciably in the sterols from 25-azacoprostane-fed insects, but did increase >50% over the control value in the sterols from 25-azacholesterol-fed insects. The level of 7-stigmastenol was not altered to any appreciable extent by the presence of either azasteroid in the diet. There was a considerable accumulation of 22,24-cholestadienol (24.0%) in the sterols from 25-azacoprostane-fed insects, but 25-azacholesterol had little effect on the level of this sterol. On the other hand, 22-stigmastenol accumulated to a greater extent (21.1%) with the 25-azacholesterol treatment than with 25-azacoprostane treatment (11.3%). Small but identifiable quantities of 24-cholestenol accumulated in each of the samples from inhibitor-fed insects (verified by GC-MS) particularly with 25-azacoprostane treatment. There were also identifiable (<1%) amounts of cholesterol in the two samples from azasteroid-fed insects. Comparative GLC RRTs of cholesterol, 24-cholestenol, 22,24-cholestadienol and 22-stigmastenol were 1.83, 2.05, 2.23 and 2.65, respectively.

The three sterols not previously reported from insects, 24-cholestenol, 22,24-cholestadienol and 22-stigmastenol, were identified by GC-MS of fractions from argentation column chromatography of the acetates of the sterols from the 25-azacoprostane-fed insects. A sample containing fractions 1 and 2 from argentation chromatography combined provided adequate material for a strong mass spectrum of 22-stigmastenyl acetate (GLC RRT = 3.54; cholesteryl acetate RRT = 2.49). An aliquot of fractions 3 and 4 combined was adequate for spectra of both 24-cholestenyl acetate (RRT = 2.76) and 22,24-cholestadienyl acetate (RRT = 3.00). MS data for these three steryl acetates include the following: for 24-cholestenyl acetate, m/e (relative intensity), 428 (M^+, 13%), prominent fragments at 413 (M-CH_3, 12), 353 (M-CH_3-CH_3COOH, 5), 315 (M-C_8H_{15}-2H, 7..), 255 (M-CH_3COOH-C_8H_{15}-2H, 45) and 69 (C_5H_9, 100) (cf. ref. 7); for 22,24-cholestadienyl acetate, 426 (M^+, 4%), with fragments at 344 (M-C_6H_9-H, 6), 329 (M-C_6H_9-H-CH_3, 4), 315 (M-C_8H_{13}-2H, 55), 257 (M-C_8H_{13}-CH_3COOH, 16), 109 (C_8H_{13}, 100) and 82 (C_6H_9-H, 86); and for 22-stigmastenyl acetate, 456 (M^+, 22%) and fragments at 353 (M-CH_3COOH-C_3H_7, 16), 344 (C_8H_{15}-H, 21), 315 (M-$C_{10}H_{19}$-2H, 23), 285 (M-CH_3COOH-C_8H_{15}, 5), 257 (M-CH_3COOH-$C_{10}H_{19}$, 43), 55 (C_4H_7, 100) (cf. ref. 8).

To verify that these intermediates were synthesized from [^{3}H]stigmasterol we isolated the sterols from Mexican bean beetle prepupae from larvae reared on leaves coated with [^{3}H]stigmasterol plus 50 ppm 25-aza-

TABLE 1

Relative Percentages of Major Sterols of Mexican Bean Beetles Reared on Lima Bean Leaves[a]

Sterol	Control (2.17 g)[b]	Acetone (2.08 g)	ASA-6, 50 ppm (1.64 g)	25-Aza, 50 ppm (2.07 g)
Cholesterol	—	—	0.8	0.7
Cholestanol	31.5	29.7	2.0	1.2
24-Cholestenol	—	—	} 4.6	} 1.5
Lathosterol	22.8	20.8		
22,24-Cholestadienol	0.3	2.1	24.0	1.5
Campestanol[c]	10.1	11.4	22.9	22.3
Stigmasterol	0.6	T[d]	T	T
22-Stigmastenol	8.8	9.1	11.3	21.1
Sitosterol	1.2	1.0	3.7	5.5
Stigmastanol	17.7	20.0	21.5	30.5
7-Stigmastenol	6.0	5.5	6.4	8.0

[a]Lima bean leaves treated as follows: control, no treatment; acetone, leaves coated with acetone; ASA-6, leaves coated with acetone solution of 25-azacoprostane; 25-Aza, leaves coated with acetone solution of 25-azacholesterol.

[b]Fresh weight of insect samples.

[c]Identifiable levels of campesterol present as indicated by GC-MS.

[d]Trace.

TABLE 2

Major Sterols Isolated from Lima Beans

Sterol	Relative percentage
Cholesterol	0.4
Campesterol	6.9
Stigmasterol	43.5
Sitosterol	32.1
Isofucosterol	2.9
7-Stigmastenol	7.9

coprostane, which provided a satisfactory accumulation of the desired metabolites. The sterols isolated from the inhibited insects (Table 3) contained 112,500 cpm. These radiolabeled sterols were acetylated and fractionated on an $AgNO_3$-impregnated Unisil column. Pairs of the column fractions were collected from the column eluant as follows: fractions 1 and 2, fractions 3 and 4, and fractions 5 and 6. Combined fractions 1 and 2 contained the bulk of the acetates of the saturated and singly unsaturated sterols, fractions 3 and 4, largely $\Delta^{22,24}$- and singly unsaturated steryl acetates and fractions 5 and 6, more polar steryl acetates. These fractions were chromatographed on $AgNO_3$-impregnated Anasil H plates, and appropriate fractions were recovered by scraping the plates and counting the adsorbent by liquid scintillation spectrometry.

Fractions 1 and 2 combined contained about 24% of the total radioactivity recovered from the $AgNO_3$-Unisil column; fractions 3 and 4 combined contained about 67% of the recovered radioactivity. When an aliquot of combined fractions 1 and 2 was run on $AgNO_3$-TLC and appropriate areas were scraped and counted, it was determined that ca. 68% of the recovered radioactivity was associated with the area corresponding to the saturated steryl acetates (cholestanol and stigmastanol) and ca. 30% was associated with the area corresponding to acetates of Δ^7- and Δ^{22}-sterols (7-stigmastenol, lathosterol and 22-stigmastenol). By GLC analysis, combined fractions 3 and 4 showed three major components, of which 22,24-cholestadienyl acetate was the major one (56.9%) and 24-cholestenyl acetate (with some lathosteryl acetate) was the third most abundant (9.4%). When an aliquot of combined fractions 3 and 4 was run on $AgNO_3$-TLC (developed three times) and areas were scraped and counted, about 30% of the radioactivity was recovered from the area corresponding to 22,24-cholestadienyl acetate, and about 15% occurred in the area corresponding to 24-cholestenyl acetate. It was necessary to develop the plate three times in this system to obtain 24-cholestenyl acetate free of lathosteryl acetate. The bulk of the remaining radioactivity was associated with the Δ^5- and Δ^7-steryl acetate area.

The fate of [^{3}H]stigmasterol was also determined by trapping fractions from the effluent of GLC separations of the components of the above $AgNO_3$-Unisil column chromatography fractions and counting the trapped fractions by liquid scintillation spectrometry. This provided further evidence for association of radioactivity with the 22,24-cholestadienyl acetate (38% of recovered radioactivity from fractions 3 and 4) and 22-stigmastenyl acetate (30% of recovered radioactivity from fractions 1 and 2) peaks and further verified their formation from [^{3}H]stigmasterol. It was not possible to correlate sufficient radioactivity from GLC trapping exclusively with 24-cholestenyl acetate, as this GLC peak also included some lathosteryl acetate, but presence of the Δ^{24}-steryl acetate was verified by GC-MS. In addition, the components of other fractions from $AgNO_3$ column chromatography were further identified by GC-MS.

TABLE 3

Major Sterols of Mexican Bean Beetles Reared on Lima Bean Leaves Coated with ^{3}H-Stigmasterol plus 25-Azacoprostane (50 ppm)

Sterol	Relative percentage
Cholesterol	0.8
Cholestanol	6.2
24-Cholestenol	6.4
Lathosterol	
22,24-Cholestadienol	26.1
Campestanol[a]	19.3
22-Stigmastenol	6.9
Sitosterol	1.9
Stigmastanol	14.9
Isofucosterol	1.9
7-Stigmastenol	10.7

[a]Identifiable levels of campesterol present as indicated by GC-MS.

DISCUSSION

Although previous metabolic studies (2) verified that 24-alkyl groups were dealkylated from radiolabeled sterols by the Mexican bean beetle to form primarily saturated sterols and the Δ^7-sterol (lathosterol), we obtained no information on intermediates involved in these pathways. In the present studies with bean beetles reared on lima bean leaves coated with 25-azacoprostane or 25-azacholesterol, we noted a considerable change in the mixture of sterols isolated from these insects, including accumulation of 22,24-cholestadienol in the 25-azacoprostane–treated insects and 22-stigmastenol, particularly with 25-azacholesterol treatment (Table 1). Interestingly, it appears that 25-azacholesterol affects the initial dealkylation process, resulting in formation of lesser quantities of C_{27}-sterols, particularly 22,24-cholestadienol, more than does 25-azacoprostane. The 22-stigmastenol and 22,24-cholestadienol most likely would result from stigmasterol metabolism, after reduction of the Δ^5-bond and dealkylation of the C_2 unit on C-24 of the side chain. These experiments were repeated with different concentrations of the azasteroids, but the effects on the sterol composition were essentially the same. That these sterols were metabolites of stigmasterol (a 24α-ethylsterol) was verified in the experiments with [^{3}H]stigmasterol, in which radiolabeling was found to be associated with 22,24-cholestadienol and 22-stigmastenol after $AgNO_3$-TLC and GLC fractionation. In addition, 24-cholestenol was implicated as a metabolite of [^{3}H]stigmasterol (by $AgNO_3$-TLC and GC-MS) and an intermediate in the overall conversion of stigmasterol to cholestanol (Fig. 1).

Thus, although cholestanol is the major product of metabolism of stigmasterol in the Mexican bean beetle, the sequence of side chain alterations is similar to the side chain metabolism of stigmasterol in other insects such as *Manduca sexta* (3), which routinely form cholesterol from stigmasterol. Cholesta-5,22,24-trien-3β-ol was found to be an intermediate in the conversion of stigmasterol to cholesterol in *Manduca* (9), and it was determined that the Δ^{24}-bond was essential for enzyme specificity in order to reduce the Δ^{22}-bond (9). In addition, desmosterol was demonstrated to be the terminal intermediate in the formation of cholesterol from several C_{28} and C_{29} phytosterols, including stigmasterol (10). Thus, following reduction of the Δ^5-bond of stigmasterol to form 22-stigmastenol, the sequence of side chain alterations to form cholestanol in the Mexican bean beetle is the same as that

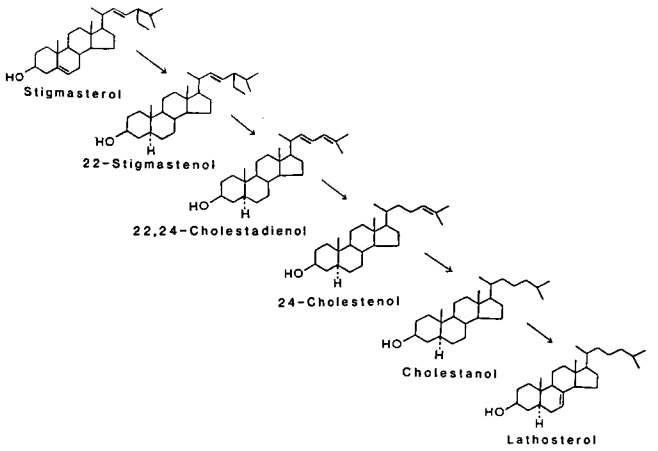

FIG. 1. Metabolism of stigmasterol in the Mexican bean beetle, *Epilachna varivestis*.

involved in the conversion of stigmasterol to cholesterol in *Manduca* and a number of other insect species. A scheme similar to the *Manduca* pathway has also been reported from studies with the silkworm *Bombyx mori* (11,12). In addition, a similar pathway of side chain alteration has recently been reported to occur in the nematode *Caenorhabditis elegans* (13). It is notable that the tissues of the Mexican bean beetle, even in inhibited insects, contain little stigmasterol or sitosterol, which are the major lima bean leaf sterols. Both of these Δ^5-phytosterols are very efficiently metabolized by this insect, whereas Δ^7-stigmastenol apparently is not metabolized, since it occurs in the insect tissue sterols at about the same level as it occurs in lima bean sterols.

The present study describes the first identification of three intermediates involved in the formation of cholestanol and lathosterol from stigmasterol in *Epilachna varivestis* that occurs under normal conditions (1). It is also the first report of these three sterols occurring in an insect. This species is still the only known phytophagous insect that dealkylates the 24-alkyl sterol side chain of dietary C_{28} and C_{29} phytosterols but produces sterols other than cholesterol as the final product. This unique system is apparently an adaptive mechanism since the Mexican bean beetle is secondarily phytophagous. We have no explanation as to why these insects developed the ability to dealkylate the sterol side chain only after saturating the Δ^5-bond. It will be of interest to determine whether this unusual aspect of neutral sterol metabolism will be reflected by differences in ecdysteroid metabolism, as has been recently discovered in phytophagous insects such as certain Hemiptera (14) and the honey bee *Apis mellifera* (15), which are unable to alter the 24-alkyl side chain and have acquired the capability to synthesize a C_{28} ecdysteroid, makisterone A.

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Altered Metabolism and Cell Surface Expression of Glycosphingolipids Caused by Vitamin E in Cultured Murine (K3T3) Reticulum Sarcoma Cells[1]

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Vitamin E caused a generalized reduction in the metabolism and cell surface expression of glycosphingolipids (GSL) in cultured Kirsten murine sarcoma virus-transformed nonproducer (K3T3) cells. Metabolism of gangliosides was decreased two- to fourfold in cells treated for 72 hr with 1 and 2 μg/ml but not with 12 μg/ml vitamin E compared to control cultures. This was demonstrated by a quantitative reduction in precursor ^{3}H-galactose label incorporated in ganglioside fraction and further substantiated by thin layer chromatography of colorimetrically and radiochemically detected GSL homologues. The composition of neutral GSL homologues was only slightly changed. The cell surface expressions of sialoglycoconjugates, analyzed by selective periodate-borotritide labeling, were also diminished quantitatively. These results are discussed in light of a previously demonstrated increase in antigenicity of K3T3 cells treated with vitamin E and the reduced tumorigenicity of these cells when transplanted into mice fed vitamin E-supplemented diets.
Lipids 21, 643–647 (1986).

Balb/c mice fed a vitamin E-supplemented diet were significantly protected against transplantation of Kirsten murine sarcoma virus-transformed Balb/3T3 (K3T3) tumor cells (1). The extent of tumor inhibition was dependent on the degree of unsaturation of dietary fat. Antitumor activity of vitamin E was due to the stimulation of the host's immune response (1). An enhanced antibody synthesis in mice fed a vitamin E-supplemented diet was shown to require macrophages that exhibit increased Fc- and C_{3b}-receptor mediated phagocytosis and the expression of Ia antigen (2). The latter finding indicates that vitamin E can modulate the expression of membrane molecules and their functions.

We hypothesize that vitamin E enhances the display of tumor cell surface antigens either by prevention of peroxidation of membrane polyunsaturated fatty acids (PUFA), thereby preserving the overall integrity and stability of the tumor cell (3), or by inducing changes in the expression of cell surface glycoconjugate antigens, suggested by the observation of an enhanced in vitro immunization against vitamin E-pretreated K3T3 cells (1), which also showed increased binding of antitumor antibodies to K3T3 cells (4). Vitamin E has also been reported to reverse the morphological expression of the transformed phenotype in cultured K3T3 cells (5). In previous reports, the association of altered glycolipid patterns with antigenic activities in these murine reticulum sarcoma cells (6) and the importance of cell surface glycoconjugates in the tumorigenic and metastatic potential of tumor cells have been well documented (7–9). Because K3T3 cells injected in vitamin E-fed mice tend to sequester the vitamin relative to other host tissues (10), the present report examines the effect of vitamin E on the expression and biosynthesis of cell surface glycosphingolipids (GSL) in cultured K3T3 cells.

MATERIALS AND METHODS

Cell culture. K3T3 cells (11) obtained from G. Todaro of the National Institutes of Health were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Grand Island Biological Co., Grand Island, New York), supplemented with 10% heat-inactivated fetal calf serum (FCS, Sterile Systems, Logan, Connecticut), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Grand Island Biological Co.). Cells were grown and maintained at 37 C in a 100% humidified incubator with 95% air and 5% CO_2 and subcultured once every five days from confluent cultures following removal by trypsin.

Preparation of vitamin E-supplemented medium and treatment of cells. DL-α-tocopherol oil (Hoffman-La Roche, Nutley, New Jersey) was dissolved in ethanol/propylene glycol (1:9, v/v) to make a final concentration of 10 mg/ml. Appropriate amounts of stock DL-α-tocopherol were added to FCS and incubated for 45 min at 37 C to allow vitamin E to bind serum lipoproteins. DMEM was then added to make 10% FCS and desired tocopherol concentration. Control medium consisted of very low levels of vitamin E (30 ng/ml) present in 10% FCS and supplemented with traces of ethanol/propylene glycol (1:9, v/v). Cells were cultured for 72 to 96 hr in vitamin E-supplemented or control medium.

^{3}H-Galactose incorporation. Ca. 1×10^6 control or vitamin E-treated K3T3 cells were cultured in duplicate 100-mm tissue culture plates for 72 hr. These cultured cells were terminally labeled for an additional 24 hr in the same medium with 1 μCi/ml galactose-D[4,5-^{3}H(N)] (51.7 Ci/mM, New England Nuclear Corp., Boston, Massachusetts). Labeled cells harvested by gentle scraping with a rubber policeman were precipitated in 10% trichloroacetic acid (TCA) overnight at 0–4 C. TCA-precipitable glycoconjugate was washed three times with ice-cold 10% TCA and finally with 0.5% TCA before lipid extraction.

Selective periodate-borotritide labeling of cell surface sialoglycoconjugates. Cell surface sialyl components were labeled selectively at sialic acid residues by mild periodate oxidation at 0–4 C, followed by reduction with sodium borotritide according to the procedure of Gahmberg and

[1]Glycosphingolipid and ganglioside nomenclature and abbreviations are as recommended by IUPAC/IUB (*Lipids 12,* 445–468, 1977).

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Andersson (12) with the following modifications: ca. 2×10^6 cells per duplicate 150-mm tissue culture plates were cultivated in 25 ml control or vitamin E-supplemented medium for 72 hr. Cells were rinsed twice in situ with phosphate-buffered saline (PBS), harvested by gentle scraping with a rubber policeman, resuspended in 1 ml PBS and distributed equally (0.5 ml) to two 10×75-mm glass culture tubes. Sodium metaperiodate (0.25 mM) was added, oxidized at 0-4 C in the dark for 15 min and washed twice with PBS by centrifugation. Periodate-treated (P) and untreated (C) samples were resuspended in 50 ml PBS and immediately tritiated with 500 μCi sodium borotritide (6.0 Ci/mM, Amersham/Searle Corp., Arlington Heights, Illinois) for 5 min. Free borotritide in labeled cell suspensions was removed by washing the cells four times with PBS. The cell pellet was resuspended in PBS and aliquoted in duplicates and was TCA-precipitated as described above; specific radioactivity incorporated in glycoconjugates was determined by liquid scintillation counting and was expressed per mg total cell protein (13). Specific label incorporated into cell surface sialylated components was calculated by subtracting the radioactivity in the untreated cells from that in the periodate-treated cells (P − C).

GSL analysis. GSL, in TCA-precipitated cells labeled with ^{3}H-galactose or surface-labeled by periodate-borotritide as described above, were extracted with 4 ml chloroform/methanol (2:1, v/v) by probe sonication using 50 W for 30 sec. A second extraction was performed as described above with 4 ml methanol plus 5% water for each sample. After centrifugation of the homogenate, the clear extract was collected and dried. The nonlipid residue which remained as a precipitate after extraction contained nucleic acids, proteins, glycoproteins and glycosaminoglycans. The nonlipid residue was solubilized in 1% sodium dodecyl sulfate in 0.1 N NaOH, and protein content (13) and incorporated radioactivity were determined. The protein content of the nonlipid residue was used in the calculation of specific radioactivity (cpm/mg protein) of various lipid and nonlipid residue fractions.

Gangliosides were extracted (14) from the neutral GSL and other lipids in chloroform/methanol (2:1, v/v). The neutral GSL present in the lower phase after ganglioside isolation were purified from the phospholipids and neutral lipids by the saponification technique of Ledeen et al. (15).

Each fraction of ganglioside corresponding to an amount of 750 μg total cell protein equivalent was spotted on two 0.20-mm silica gel gypsum-coated high performance-thin layer chromatography (HPTLC) plates (Kiesegel 60, EM Science, Gibbstown, New Jersey) previously activated at 100 C for 90 min. Plates spotted with ganglioside fractions were developed in chloroform/methanol/0.2% calcium chloride (60:40:10, v/v/v), dried, sprayed with resorcinol and heated with a cover glass at 150 C for 10-15 min. Separated ganglioside classes were detected as purple bands (16,17).

Neutral GSL fractions of ^{3}H-galactose labeled cells were developed in chloroform/methanol/water (65:25:4, v/v/v), dried, sprayed with orcinol reagent and heated at 150 C with a cover glass for 5 min. Neutral GSL homologues were visualized as purple or brown spots on the chromatograms (18). A second TLC plate of GSL separated for each fraction was also analyzed by fluorography according to the procedure of Bonner and Stedman (19).

TABLE 1

Quantitative Analyses of ^{3}H-Galactose Incorporation in Nonlipid Residue and GSL of K3T3 Cells Treated with Vitamin E[a]

Treatment (μg/ml Vitamin E)	Glycoconjugate fraction[b] (dpm/μg cell protein)			
	Nonlipid residue	Ganglioside	Neutral GSL	Neutral GSL + ganglioside
Control	713 ± 11	64 ± 7	264 ± 18	328 ± 23
1	708 ± 22	20 ± 6[c]	229 ± 14[d]	250 ± 17[d]
2	678 ± 9	14 ± 2[c]	247 ± 26[d]	262 ± 13[d]
12	780 ± 31	68 ± 13	223 ± 20	301 ± 27

[a]Duplicate cultures of K3T3 cells were treated with vitamin E for 72 hr and then pulsed with 1 μCi/ml ^{3}H-galactose for an additional 24 hr. Results are mean ± SEM of four experiments.

[b]Nonlipid residues include glycoproteins and glycosaminoglycans; GSL, glycosphingolipids.

[c,d]Values were significantly different from control cultures by student's T-test ($p < .001$ and $p < .05$, respectively).

RESULTS

GSL metabolism. A quantitative comparison of neutral GSL and gangliosides of control and vitamin E-treated cultured cells studied by metabolic labeling with ^{3}H-galactose is shown in Table 1. Vitamin E treatment had no effect on ^{3}H-galactose incorporation into the nonlipid residue (i.e., glycoproteins and glycosaminoglycans) and neutral GSL fractions. However, incorporation of ^{3}H-galactose into the gangliosides was decreased two- to fourfold in cells treated with 1 and 2 μg/ml ($p < 0.001$), but not with 12 μg/ml, vitamin E compared to control cultures. The quantitative reduction in label incorporated in the ganglioside fraction was further substantiated by thin layer chromatograms of colorimetrically and radiochemically detected gangliosides (Figs. 1B and 2B). The ganglioside extracts of K3T3 cells were found to have chromatographic mobilities identical with the standards G_{M3} and G_{M2}, which are the principal gangliosides of K3T3 cells and resolve as a double band on TLC (7,20). Resorcinol positive gangliosides were markedly reduced in cells treated with 1 and 2 μg/ml vitamin E (Fig. 1B, channels 3 and 4, respectively) compared with control cells or those treated with 12 μg/ml vitamin E (Fig. 1B, channels 2 and 5, respectively). Similar quantitative changes in ganglioside patterns were shown by TLC of labeled gangliosides detected radiochemically by autofluorography (Fig. 2B; compare channels 2 and 3 with channels 1 and 4, respectively). Turnover and release of gangliosides in tissue culture medium did not show an appreciable change (data not shown), suggesting that the observed cellular alterations are not due to increased turnover and shedding.

Vitamin E caused only a slight reduction ($p < 0.01$) in incorporation of ^{3}H-galactose in neutral GSL (Table 1). Chemical and radiochemical analyses of the neutral GSL patterns are shown in Figures 1A and 2A, respectively. K3T3 cells possess two major neutral GSL with chromatographic migration of ceramide-glucose (CM)and ceramide-glucose-galactose (CD) and minor asialo ceramide-galactose-N-acetyl galactosamine (G_{M2}) band (Fig. 2A). Vitamin E did not alter the levels of major neutral GSL bands (Fig. 1A) as previously illustrated in Table 1. However, a small

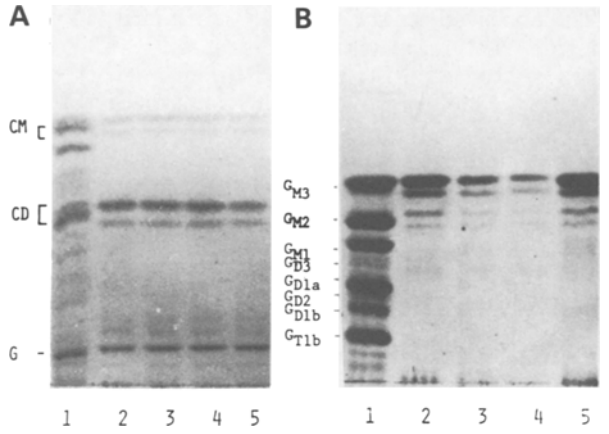

FIG. 1. Thin layer chromatography (TLC) patterns of colorimetrically detected gangliosides (A) and neutral GSL (B) patterns of control and vitamin E-treated K3T3 cells. Neutral GSL fractions (resulting lower phase) and ganglioside fractions corresponding to 750 μg cell protein equivalent were separated on silica gel-gypsum high performance TLC plates with the use of chloroform/methanol/water (65:25:4, v/v/v) and chloroform/methanol/0.2% calcium chloride (60:40:10, v/v/v), respectively. Neutral GSL and gangliosides were visualized by orcinol spray (17) and resorcinol reagent (16), respectively. Neutral GSL standard mixture containing CM, CD, asialo G_{M1} and asialo G_{M2} was obtained by acid hydrolysis and purification of human brain ganglioside mixture. Authentic ganglioside standards consisted of canine erythrocyte hematoside (G_{M3}), Tay Sachs' brain (G_{M2}) and normal human adult brain (G_{M1}, G_{D1a}, G_{D1b} and G_T). Sample order: 1, standard mixture; 2, control; 3-5, 1, 2 and 12 μg/ml vitamin E-treated, respectively.

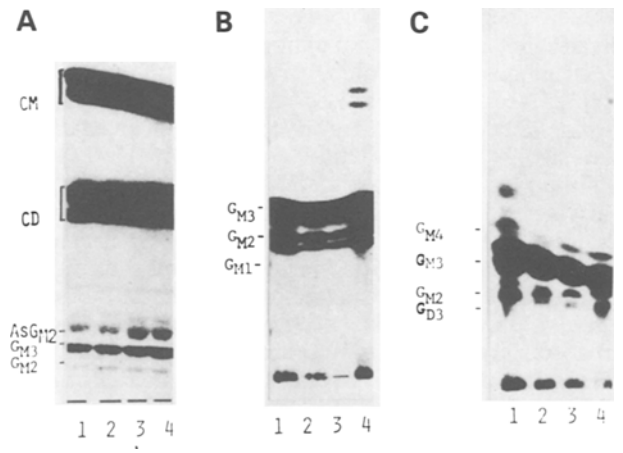

FIG. 2. Thin layer chromatography (TLC) patterns of autofluorographically detected gangliosides and neutral GSL in K3T3 cells labeled by ^{3}H-galactose and periodate-borotritide. Seven-hundred-fifty μg cell protein equivalent of neutral GSL (lower phase, ca. 442,000 dpm) and ganglioside (upper phase, 154,000 dpm) of ^{3}H-galactose labeled GSL were separated on silica gel-gypsum high performance TLC in appropriate solvents. A and B: neutral GSL and ganglioside of ^{3}H-galactose labeled cells, respectively: 1, control; 2-4, 1, 2 and 12 μg/ml vitamin E, respectively. C: Ganglioside of periodate-borotritide labeled cells, respectively. Sample order as in A and B.

increase in ^{3}H-galactose incorporation into CD and asialo G_{M2} (Fig. 2A) was seen. Analyses of total GSL (gangliosides and neutral GSL) synthesized in control and vitamin E-treated cultures are shown in Table 1. As seen, vitamin E treatment caused a significant reduction ($p < 0.01$) in total GSL synthesized.

TABLE 2

Cell Surface Sialoglyconjugates Determined by Periodate Borotritide Labeling[a]

Treatment (μg/ml Vitamin E)	Glycoconjugate fraction[b] (dpm/μg Cell protein)		
	Nonlipid residue	Gangliosides	Total (nonlipid residue + gangliosides)
Control	605 ± 25	220 ± 7	826 ± 30
1	238 ± 10[c]	84 ± 2[c]	322 ± 20[c]
2	238 ± 7[c]	103 ± 3[c]	342 ± 10[c]
12	270 ± 7[c]	107 ± 3[c]	376 ± 9[c]

[a]Vitamin E-treated K3T3 cells were surface labeled in duplicate by periodate borotritiation.

[b]Each value was subtracted from cells treated with borotritide alone and represents the mean ± SEM of three separate experiments.

[c]Values were significantly different from control cultures to the $p <$ .005 level by student's T-test.

Cell surface GSL. The cell surface labeling technique involved mild periodate oxidation of vicinyl hydroxyl groups of sialic acid residues of sialyl glycoproteins and gangliosides at 0-4 C (a condition that limited periodate anion transport into the cell) followed by borotritiation. This study was designed to determine if vitamin E, which is localized predominantly in the mitochondrial and endoplasmic reticulum membranes, had an effect on cell surface sialoglycoconjugates. Vitamin E at all concentrations markedly decreased ($p < 0.005$) gangliosides in the upper phases of lipid extract (Table 2). Note that 2 μg/ml vitamin E caused the highest reductions. TLC autofluorographic detection of gangliosides (present in the upper glycolipid fraction) showed the presence of bands with the chromatographic mobilities of G_{M4}, G_{M3}, G_{M2} and G_{D3} in K3T3 cells. All concentrations of vitamin E markedly decreased several gangliosides relative to control cultures (Fig. 2C). Cells treated with vitamin E continuously for 6-8 months exhibited very similar results in all the above studies (data not shown). Study of periodate-borotritide labeled cell surface sialoglycoproteins present in the nonlipid residue, which accounted for 60-70% of total label, also showed a marked reduction in vitamin E-treated cultures ($p < 0.001$) compared to control cultures not treated with vitamin E (Table 2). Like the gangliosides of vitamin E-treated cultures, sialoglycoproteins of these cells were reduced without any dose response. These results suggest that vitamin E primarily affected the synthesis and expression of cell membrane gangliosides and sialoglycoproteins.

DISCUSSION

Ninety percent of all cellular tocopherol is localized in the membranous subcellular organelles such as mitochondria, endoplasmic reticulum, Golgi apparatus and lysosomes (21), where it is proposed to interact in a unique way with arachidonic acid residues of major phospholipids, conferring stability and protection from peroxidative damage by intracellular free radicals (21,22). This hypothesis is further supported by human and animal red blood cells that show increased resistance to hemolysis in vitamin

E-supplemented states and a high degree of hemolysis in vitamin E deficiency conditions (23). Furthermore, studies by Catignani (24) showed that a number of membrane-bound enzymes are altered in their activities in vitamin E-deficient animals.

Our present data show that the chemical content, synthesis and cell surface expression of GSL are altered by the incorporation of vitamin E in cultured cells. These vitamin E-dependent alterations in GSL have been documented here by three sets of experimental approaches: i) study of chemical composition of GSL homologues using colorimetric reaction of GSL glycan groups with resorcinol and orcinol reagents; ii) radiochemical labeling of glycan groups through incorporation of labeled galactose in whole cells; and iii) surface labeling of gangliosides by the periodate-borotritiation method. These three independent but complementary studies clearly demonstrate a generalized reduction of GSL syntheses and their cellular expression at lower (1, 2 and 4 μgE/ml) but not higher (12 μgE/ml) levels in vitamin E-treated K3T3 cells. It is not clear why 12 μg/ml vitamin E had no effect on GSL synthesis. In other studies (Mbawuike, I., Corwin, L. M., and Yogeeswaran, G., submitted for publication), vitamin E at concentrations similar to those used in the present study induced a dose-dependent reduction in cell surface sialic acid and sialoglycoconjugates. It is possible that lower concentrations of vitamin E are required to show dose-dependent effects. Moreover, pretreatment of tumor cells with 1 or 3 μg/ml vitamin E resulted in a dose-dependent stimulation of antitumor cytotoxic responses in mouse spleen cells, while 6 μg/ml vitamin E inhibited this response (1). These results suggest that high concentrations of vitamin E depress antigen exposure by increasing cellular sialoglycoconjugates.

GSL are exclusively localized in endoplasmic reticulum, Golgi and surface membrane organelles in which vitamin E is also enriched. Biosynthesis of GSL takes place in the endoplasmic reticulum, after which they are predominantly transported to the cell surface membranes. The observed alterations of GSL in K3T3 cells may result from a generalized pleiotropic change in membrane-bound glycosyl transferase activity accompanied by a reduction of glycolipid substrates for additional glycosylation reactions. Future studies on the activities of glycosyl transferases and glycosyl hydrolases in vitamin E-treated vs. untreated cultures could establish the metabolic basis of vitamin E-dependent GSL changes in these cells. Another mechanism to consider for future studies for the vitamin E-dependent GSL changes may include an alteration in the maintenance of sulfahydryl groups of membrane-bound thiols, which might regulate glycosylation and GSL synthesis.

It is not known how general these vitamin E-mediated changes in GSL may be in other cell systems, but similar results were obtained using two clones of K3T3 and a variant line grown in medium supplemented with delipidized serum (unpublished observations; 4,5).

Removal of vitamin E from cells did result in a slow shift of GSL patterns similar to the control after 1 wk in culture (unpublished results). These observations are consistent with the relatively slow turnover and lipophilic nature of vitamin E, which has to be diluted during subsequent cell divisions in K3T3 cells every 18 hr following the removal of supplemented vitamin E.

The observed reduction in GSL, primarily in the ganglioside fraction, in vitamin E-treated K3T3 cells may be correlated with the reduced tumorigenicity of these cells in mice fed supranormal levels of dietary α-tocopherol (1). These observations are further supported by the findings that vitamin E-treated cultured K3T3 cells are more antigenic as measured by increased humoral antibodies produced in Balb/c mice (4). More recently we have demonstrated that pretreatment of K3T3 cells with vitamin E increased their binding to tumor-specific antisera as measured in enzyme-linked immunosorbent assay (4). Certain GSL are considered to be tumor-associated markers or antigens in various cancers (8). Notably ceramide dihexoside and asialo G_{M2} that appear in K3T3 cells have been identified as tumor markers in K3T3 tumors (6). We observed that these neutral GSL show a minor vitamin E-dependent increase in K3T3 cells. Presumably such increase in antigenic neutral GSL caused by the vitamin may mediate increased tumor rejection response in the animal.

In a related study, we have shown that vitamin E-treated K3T3 cells showed a decrease in chemical content, biosynthesis and cell surface expression of sialoglycoprotein (4). In the present study, we have observed similar decreases in the biosynthesis and cell surface expression of sialoglycoproteins present in nonlipid residue fractions, using biosynthetic labeling with galactose and cell surface labeling with periodate borotritiation, respectively. Likewise, a generalized reduction in major and minor gangliosides has been observed in vitamin E-treated cultures compared to control in the present study.

Such decrease in sialoglycoconjugates has been implicated in the expression of new antigenic sites and cryptoantigens by Prat et al. (25) and Rogentine and Plocinick (26). These findings are further supported by reduced transplantability of neuraminidase-treated (disialyzed) tumor cells by Currie and Bagshawe (27) and Rios and Simmons (28). A direct demonstration of the relationship between sialylation of glycoconjugates and tumor cell antigenicity has also been demonstrated by Shearer et al. (29). They showed that a variant of L-cell selected by immunoselection of heterogenous population by antibody against parental cells and complement resulted in increased sialylation in the variant cell line. Neuraminidase treatment of the L-cell variant restored increased susceptibility to killing with antibody and complement.

The reduction in synthesis and expression of GSL, especially gangliosides, caused by vitamin E treatment of tumor cells reported here may be important in either directly or indirectly increasing tumor cell antigenicity and facilitating a tumor rejection response by mice fed vitamin E in the diet (1). Further studies designed to elucidate the mechanism of vitamin E in the regulation of glycoconjugate metabolism may be of value in increased understanding of the antitumor chemopreventive potential of the vitamin.

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Oxidation of Ethyl Hexadec-1-enyl Ether, A Plasmalogen Model, in the Presence of Unsaturated Esters[1]

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To test the susceptibility of plasmalogen lipids to oxidation, a series of experiments was run on the model compound ethyl hexadec-1-enyl ether, which was prepared from hexadecanol by a multistep synthesis. Oxidation experiments were carried out on neat mixtures of the model ether with a variety of fatty esters in sealed vials under air. Disappearance of the ether, the fatty esters and, in one set of experiments, oxygen, was followed by gas chromatography. Ethyl stearate, which was inert to oxidation under the experimental conditions, served as internal standard. In the absence of polyunsaturate (linoleate or linolenate), alk-1-enyl ether underwent slow but measurable oxidation. In the presence of polyunsaturate, however, disappearance of the ether was greatly accelerated and proceeded at a rate comparable to that of the polyunsaturate. Reactions did not proceed in the absence of oxygen and were inhibited by antioxidant. The results suggest that oxidation of the alk-1-enyl ether functionality of plasmalogens should not be ignored as a factor that contributes to the oxidative instability of animal tissue or the development of rancidity in meat products.
Lipids 21, 648–651 (1986).

Plasmalogen phospholipids are found in high concentration among the membrane lipids of meat. For example, 20–30% of the choline glycerophosphatides and over 60% of the ethanolamine glycerophosphatides of bovine muscle are plasmalogen (1). Studies on lipid oxidation in foods typically focus on the reactivity of the polyunsaturated acyl functionality (2–5), but the presence of the alk-1-enyl ether functionality of plasmalogens has been ignored as a substrate for such oxidation. Nevertheless, plasmalogen oxidation has been cited in past physiological studies. In rat brain homogenates, for example, plasmalogens were found to undergo Fe^{2+}/ascorbate-catalyzed oxidation at the double bond site to yield, the authors speculated, an α,β-diol that further degraded to α-hydroxyaldehyde and (n-1)-aldehyde (6,7). Plasmalogens in erythrocytes were found to be prone to peroxidation catalyzed by glucose oxidase-glucose or dialuric acid (8). Plasmalogens in spermatozoa were found to be susceptible to oxidation under aerobic conditions (9). Curiously, studies that implicated lipid peroxidation as a mechanism of injury in cardiac tissue failed to consider plasmalogen oxidation (10–13), despite the abnormally high concentrations of this lipid class in cardiac tissue (14). Other studies have shown that the double bonds of alk-1-enyl ethers are subject to attack by singlet oxygen (15–17). One study focused on alkyl ether glycerols, which even without any unsaturation are prone to autoxidation (18). The present study was done to investigate whether plasmalogens, as represented by a model long chain alk-1-enyl ether, are susceptible to autoxidation under conditions that lead to such oxidation of long chain polyunsaturated esters.

EXPERIMENTAL

Synthesis of hexadecanal diethylacetal. Hexadecanal was synthesized from hexadecanol (Aldrich Chemical Co., Milwaukee, Wisconsin) by oxidation using dimethylsulfoxide (DMSO) and dicyclohexylcarbodiimide (Sigma Chemical Co., St. Louis, Missouri), according to the method of Fenselau and Moffatt (19). The crude aldehyde was converted to its diethylacetal using ethanolic benzene (NOTE: benzene is toxic and must be used with care and proper ventilation) with methanesulfonic acid according to the method of Gigg and Gigg (20). The acetal was purified by column chromatography using neutral alumina (80–200 mesh, Fisher Scientific Co., Fairlawn, New Jersey) and eluting the acetal with ether/petroleum ether (bp 30–60 C, 1:5, v/v). The column could be reused after eluting residual hexadecanol and hexadecanal with ether/methanol (1:1, v/v). The eluate stream was monitored by thin layer chromatographic (TLC) analysis.

Synthesis of ethyl hexadec-1-enyl ether. The alk-1-enyl ether was prepared from hexadecanal diethylacetal by preparing the chloroacetal with phosphorus pentachloride according to the method of Chebyshev et al. (21) and then inducing elimination of hydrogen chloride from the unisolated chloroacetal with triethylamine according to the method of Gigg and Gigg (20). Triethylamine hydrochloride was removed by passage through a small column of silica gel according to the Gigg and Gigg method, but with dichloromethane instead of ether. Purification of alk-1-enyl ether product was accomplished by column chromatography using silica gel (grade 60, 230–400 mesh, Aldrich). Elution, by hexane/benzene (9:1, v/v), was monitored by TLC. *Cis/trans* ratios were determined by gas chromatographic (GC) analysis (Fig. 1). The synthesized mixture of isomers (typically 70–80% *cis*) was used in subsequent oxidation studies. Separation of isomers could be achieved on silica gel by use of medium pressure liquid chromatography (Michel-Miller apparatus, Ace Glass Co., Vineland, New Jersey) with monitoring of the eluate by differential refractive index detection (Model R-401, Millipore/Waters Chromatography Division, Milford, Massachusetts). The *cis* isomer eluted before the *trans* (infrared λ_{max} 929 cm^{-1}), with some degree of overlap between the two species.

Commercial ethyl ether contains trace amounts of BHT antioxidant. Use of such ether in these syntheses will lead to product contaminated with BHT. Such product will show inhibition toward oxidation in subsequent experiments. BHT may be removed from any of these products by dissolving the material in hexane and extracting the solution with DMSO (22).

Ethyl esters of fatty acids. Ethyl esters were prepared from the corresponding methyl esters (methyl stearate and methyl linolenate, Nu-Chek Prep, Elysian, Minnesota;

[1]Presented in part at the AOCS 75th annual meeting, Philadelphia, May 1985.

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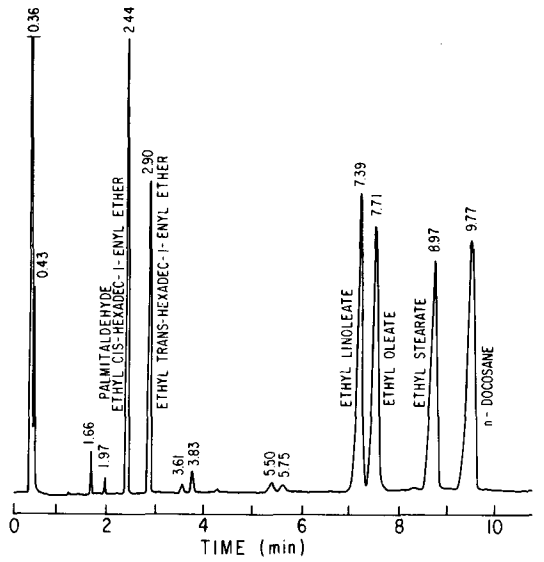

FIG. 1. GC trace of organic component separation. Conditions: 155 C isothermal, 2 mg/ml each component in isooctane; 0.5 μl injection, split ratio 100:1; capillary column, OV-101 bonded phase on fused silica. See Experimental section for further details.

TABLE 1

Composition of Reaction Mixtures Prior to Oxidation, in μmol

Component	Formula wt	Run 1	Run 2	Run 3	Run 4	Run 5
Cis-ether	268.5	9.9		2.4	2.2	2.3
Trans-ether	268.5	2.5		.97	.92	1.0
n-Docosane	310.6	7.1				
18:0	312.5	7.6	2.0	1.8	2.0	1.9
18:1	310.5	7.1				
18:2	308.5	7.4	1.8		1.9	
Oxygen	32.0	80	1100	1100	1100	1100
Vial volume, ml		9	120	120	120	120

methyl oleate, Applied Science, State College, Pennsylvania; methyl linoleate, Hormel Institute, Austin, Minnesota) by rapid transesterification at ambient temperature using ethanolic KOH (23).

Oxidation procedure. Quantitative mixtures of hexadec-1-enyl ether and appropriate ethyl esters, in amounts listed in Table 1, were prepared in 500 μl pentane. Aliquots of 10 μl then were transferred to each of nine "100-ml" capacity (actual capacity 120 ml) serum vials (Supelco, Bellefonte, Pennsylvania) and the pentane was removed under vacuum at ambient temperature. The vacuum was broken with room air and the vials were securely sealed with crimpable aluminum caps containing PTFE-lined butyl rubber septa (Perkin-Elmer, Norwalk, Connecticut). For each study, a set of such sealed vials was immersed in a controlled-temperature water bath at 86 ± 1 C. Vials then were withdrawn at designated times and cooled to ambient temperature. Samples of 20 μl of headspace gas were withdrawn at this time from the vials of run 1 to determine oxygen consumption. For all runs, internal surfaces of the vials were washed with 1 ml injected isooctane and the resultant solution was sampled for GC analysis.

Aldehyde generation from oxidized mixture. Aliquots of 500 μl of the above solution of reaction products from each of the set of vials from a repeat of run 4 (Table 1) were freed of solvent by evaporation under nitrogen and then subjected to mild hydrolysis by treatment with 15 mg ground Amberlyst 15 sulfonic acid resin (Rohm and Haas, Philadelphia, Pennsylvania) in 100 μl acetone and 0.15 μl water for 6 min at ambient temperature. The mixture was taken up in ether and filtered through glass wool. Amounts of hexadecanal so formed were determined by GC analysis.

TLC. Separation of fatty alcohol, aldehyde, diethylacetal and alk-1-enyl ether was achieved on plates of Silica Gel G by developing in toluene. Visualization of aldehyde could be accomplished by fuchsin-bisulfite spray; aldehyde so treated was detected as a purple-to-red spot. Acetal and alk-1-enyl ether spots were visualized in the same way after conversion to aldehyde by 1 min exposure to the vapors of concentrated hydrochloric acid. All species were visualized by spraying the plates with copper sulfate/phosphoric acid, followed by charring.

GC. Oxygen was determined on a Hewlett-Packard 7620A gas chromatograph using a stainless steel column, 6′ × 1/8″, packed with molecular sieve 13X. Determinations were run at ambient temperature using helium as carrier gas and thermal conductivity detection; samples of 20 μl were injected from a gas-tight locking syringe. Signal analysis was accomplished by routing the detector output to the integrating terminal of a Hewlett-Packard 5880A gas chromatograph.

Organic substrates were determined on a Perkin-Elmer Sigma-3 gas chromatograph using a fused silica wall-coated open-tubular column (Hewlett-Packard, Avondale, Pennsylvania) of 0.33 μ thick, cross-linked OV-101 methyl silicone, 12 m × 0.2 mm i.d. Determinations were made isothermally at 155 C using helium as carrier gas and a split ratio of 100:1. n-Docosane (Supelco) served as initial internal standard (run 1, Table 1), followed by ethyl stearate (18:0) once the latter was shown to be stable to oxidation under the experimental conditions. Signal analysis was accomplished in the same manner as described above. A GC trace that shows the separation of all components of interest is shown in Figure 1.

RESULTS AND DISCUSSION

Results are shown graphically (Figs. 2–5) for a series of runs at 86 C, a temperature that allowed for convenient measurement of substrate depletion. Data are graphed in normalized fashion to indicate the percentage of each remaining component at specified reaction times. Table 1 lists the amounts of starting materials for each run. Ethyl esters were selected over methyl esters to more closely parallel the ethyl alk-1-enyl ether. Oxygen content is estimated from the volume of a standard gas at standard temperature and pressure (22.4 l/mol) and the oxygen content of air (21%) (24).

Run 1 was carried out in a series of small vials (9-ml capacity) to allow a measurable depletion of oxygen. Results are depicted in Figure 2. Relative to the extent of oxidation over the 4.5-hr duration, oxygen consumption was minimal (18%). Further runs were done in much

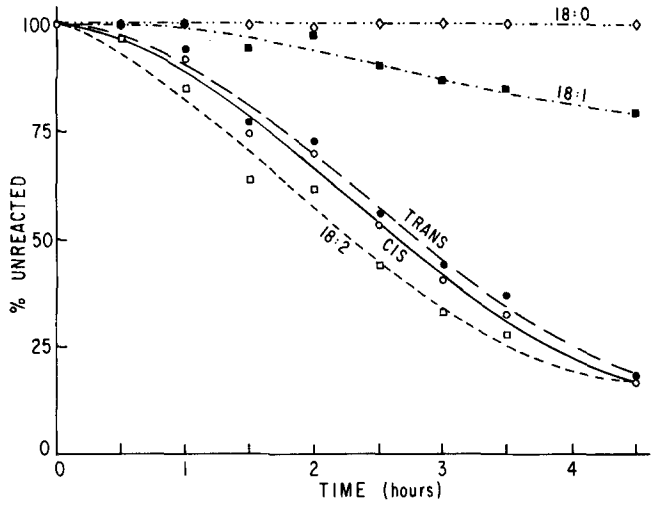

FIG. 2. Course of reaction at 86 C of run 1 (ethyl *cis*- and *trans*-hexadec-1-enyl ethers and 18:0, 18:1 and 18:2 esters; internal standard, n-docosane).

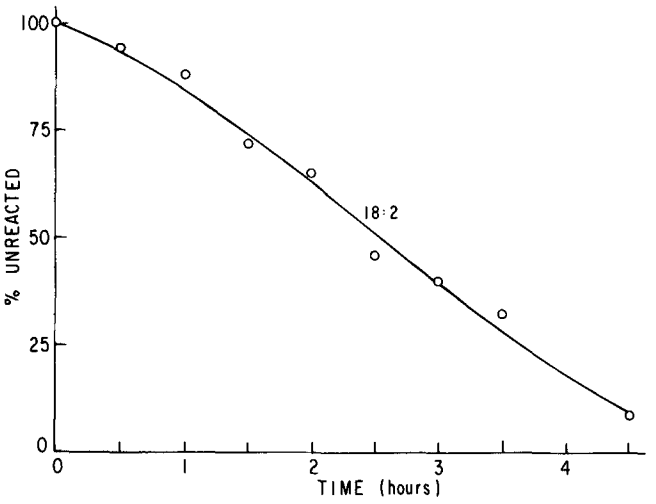

FIG. 3. Course of reaction at 86 C of run 2 (18:2; internal standard, 18:0).

larger vials (120-ml capacity) to eliminate oxygen content as a reaction variable. Run 1 also was carried out in the presence of n-docosane as internal standard to establish the inertness of 18:0. Later runs used 18:0 as internal standard. The results of this run show that 18:0 is stable under the conditions of oxidation and that ethyl oleate (18:1) reacts slowly. Depletion of ethyl linoleate (18:2) is considerable, and the isomers of alk-1-enyl ether are also highly reactive. The ethyl *cis*-hexadec-1-enyl ether (*cis*-ether), whose double bond configuration is the same as that of natural plasmalogen (25), is somewhat more reactive than the ethyl *trans*-hexadec-1-enyl ether (*trans*-ether).

Run 2 (Fig. 3) was carried out to demonstrate the autoxidation of 18:2 in the absence of alk-1-enyl ether. Run 3 (Fig. 4) was carried out analogously to investigate autoxidation of the alk-1-enyl ethers in the absence of polyunsaturate. Run 4 (Fig. 5) then was carried out on a combination of components of runs 2 and 3 to investigate the influence of polyunsaturate on alk-1-enyl ether oxidation. Finally, run 5 (Fig. 6) repeated conditions of run 4, but with ethyl linolenate (18:3) in place of 18:2. Runs 2 and 4 (Figs. 3 and 5) demonstrate that 18:2 autoxidation essentially is independent of the presence of alk-1-enyl ether, but, on the contrary, the extent of autoxidation of alk-1-enyl ether is dependent on the presence of 18:2 (runs 3 and 4, Figs. 4 and 5). Autoxidation of 18:3 is not unexpectedly faster than that of 18:2, and alk-1-enyl ether oxidation is accelerated by substitution of 18:2 and 18:3 (runs 4 and 5, Figs. 5 and 6).

The oxidative nature of the alk-1-enyl ether decomposition was established by repeating runs 1 and 3 with 0.1% antioxidant, w/w (BHA/BHT; Tennox 5; Kodak, Rochester, New York). The presence of antioxidant led to complete inhibition of decomposition over the 4.5-hr duration of the experiments. Moreover, reactions failed to proceed when nitrogen was substituted for air in the vial headspace. Thus, loss of alk-1-enyl ether cannot be attributed simply to hydrolysis by trace amounts of water.

To estimate what proportion of the oxidation of the alk-1-enyl ether occurred on the unsaturated side of the

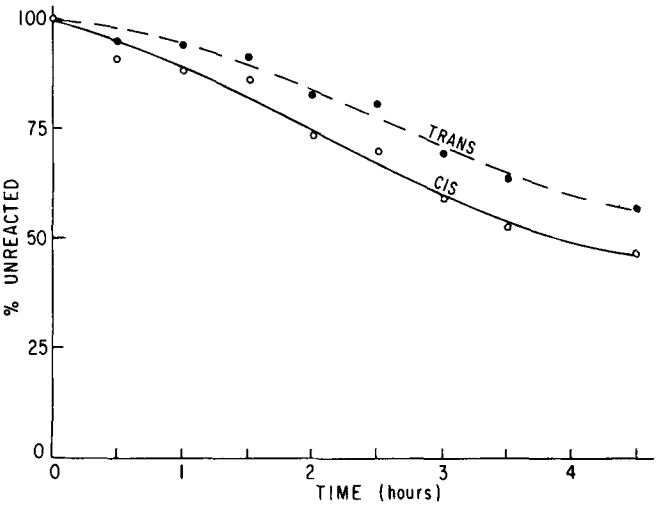

FIG. 4. Course of reaction at 86 C of run 3 (ethyl *cis*- and *trans*-hexadec-1-enyl ethers; internal standard, 18:0).

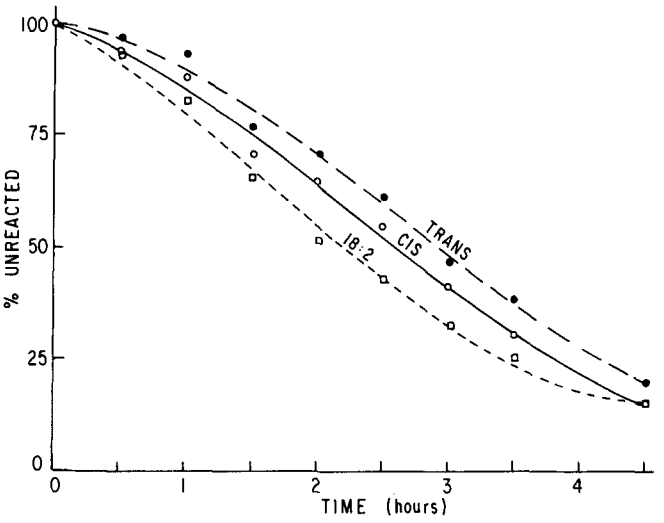

FIG. 5. Course of reaction at 86 C of run 4 (ethyl *cis*- and *trans*-hexadec-1-enyl ethers and 18:2; internal standard, 18:0).

TABLE 2

Hexadecanal Liberated from Aliquots of Run 4 by Hydrolysis

	Run time (hr)						
	0	1.0	1.5	2.0	3.0	3.5	4.5
Remaining total alkenyl ether (%)	100	82.5	54.7	52.1	37.5	21.2	19.8
Generated hexadecanal relative to 0 time (%)	100	89.0	68.4	73.5	53.4	36.1	40.2
Oxidized alkenyl ether oxidized on ethyl group[a] (%)	—	37[b]	30	45	25	19	25

[a]Average 30 ± 9%.

[b][(89.0 − 82.5)/(100 − 82.5)] × 100.

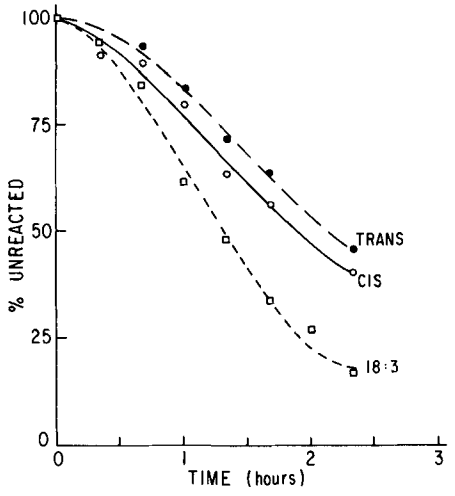

FIG. 6. Course of reaction at 86 C of run 5 (ethyl *cis*- and *trans*-hexadec-1-enyl ethers and 18:3; internal standard, 18:0).

molecule, run 4 was repeated. Following GC analysis of unreacted starting materials, aliquots of 100 μl from each vial of the set were acidified with Amberlyst 15 resin to liberate hexadecanal by hydrolysis. This aldehyde had been seen by GC analysis to be only a negligible product of oxidation (Fig. 1). The amount of hexadecanal liberated by hydrolysis was determined by GC analysis and was found to exceed the amount of remaining alk-1-enyl ether throughout the course of the reaction. Results, listed in Table 2, give insight on the extent of oxidation on the alk-1-enyl portion of the molecule, as opposed to the ethyl portion. The aldehyde is a hydrolysis product of the intact alk-1-enyl portion of the molecule, be it from the original ethyl hexadec-1-enyl ether or from an oxidation product that has the intact alk-1-enyl functionality. Results show that ca. 30% of the oxidation is not on the alk-1-enyl portion of the molecule. This demonstrates that both sides of the molecule are subject to oxidation, but that the unsaturated side is much more prone to oxidation than the saturated side.

These experiments suggest that the easily oxidized polyunsaturated esters accelerate autoxidation of the alk-1-enyl ether. This is highly significant because natural plasmalogens typically are also highly polyunsaturated (26). A future report will present results of more complex model plasmalogens to investigate whether the influence of polyunsaturation on alk-1-enyl ether oxidation might be an intramolecular process in the natural plasmalogen structure.

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The Influence of Dietary Manipulation with n-3 and n-6 Fatty Acids on Liver and Plasma Phospholipid Fatty Acids in Rats

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The interrelations between linoleic acid (LA) metabolites and fish oil fatty acids were studied. Sprague-Dawley rats (200–220 g) were fed a fat-free semisynthetic diet supplemented with 10% (by weight) of different combinations of evening primrose oil (EPO), a rich source of LA and γ-linolenic acid, and polepa (POL), a marine oil rich in eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. The combinations of supplement were as follows: 9% EPO–1% POL, 8% EPO–2% POL, 7% EPO–3% POL, 6% EPO–4% POL and 5% EPO–5% POL. After two weeks on the respective diets, the animals were killed, and the fatty acid compositions of liver and plasma phospholipids were examined. The results showed that animals fed higher proportions of POL consistently contained higher levels of dihomo-γ-linolenic acid (DGLA) ($p < 0.05$), a metabolite of LA and GLA, and lower levels of arachidonic acid (AA) ($p < 0.01$), a metabolite of DGLA through Δ-5-desaturation. Thus, an inverse relationship between AA/DGLA ratio and EPA levels was found to exist ($r = -0.765$ in plasma and -0.792 in liver). However, there was no such relationship between AA/DGLA ratio and DHA levels. This result suggested that EPA but not DHA in fish oil exerts an inhibitory effect on the conversion of DGLA to AA.
Lipids 21, 652–656 (1986).

In mammals, linoleic acid [LA, 18:2(n-6)] is metabolized along a variety of pathways, one of which is its conversion to γ-linolenic acid [GLA, 18:3(n-6)] by Δ-6-desaturase. GLA is rapidly elongated to dihomo-γ-linolenic acid [DGLA, 20:3(n-6)] and subsequently desaturated by Δ-5-desaturase (D5D) to arachidonic acid [AA, 20:4(n-6)] (1–3). There is substantial evidence that polyunsaturated fatty acids, mainly LA and more effectively its metabolites GLA and AA, are capable of lowering plasma cholesterol levels (4–9). Since a high plasma cholesterol level has been implicated by some workers as a risk factor for coronary heart disease (CHD) (10–12), LA and its n-6 metabolites may therefore play an important role in the prevention of CHD. It has been shown in prospective studies and studies of survivors of CHD that plasma, red blood cells and adipose tissue levels of LA correlate inversely with the incidence of cardiovascular disease (13–16). A stronger inverse correlation was also found with DGLA (13).

Recent reports have suggested that a high dietary intake of fish oil, which is rich in n-3 fatty acids, namely eicosapentaenoic [EPA, 20:5(n-3)] and docosahexaenoic acid [DHA, 22:6(n-3)], may be a contributory factor to the low incidence of CHD among Greenland Eskimos on a traditional diet (17–19). Indeed, evidence has shown that n-3 fatty acids lower both plasma cholesterol and triglycerides. The n-3 fatty acids also inhibit platelet aggregation either by competing with AA and subsequently reducing production of thromboxane A_2 (TxA_2) or by increasing production of prostacyclin I_3 (PGI_3) (20,21). The n-3 fatty acids are also known to inhibit the metabolism of n-6 fatty acids, possibly through competition at the desaturation steps (22,23). α-Linolenic acid and DHA have been shown to substantially inhibit the desaturation of n-6 essential fatty acids (EFA), namely LA, while EPA was shown ineffective (24–26).

DGLA is converted to AA by D5D, a regulating step which determines the balance between DGLA and AA. These two acids are metabolized to different groups of compounds with diverse and often contrasting effects. DGLA products, such as prostaglandin E_1 (PGE_1), either are neutral or have a wide range of favorable actions (27–29). Apart from PGI_2, which also has favorable actions, AA is the precursor of TxA_2, $PGF_{2\alpha}$ and the leukotrienes. These substances are implicated in various disease entities ranging from thromboembolic phenomena to inflammation. Thus, control of D5D activity, toward DGLA, should produce more desirable eicosanoid metabolites. The importance of a low DGLA level in predicting risk of CHD suggests that control of D5D activity might have relevance to human medicine (13).

In this study, we fed rats EPA-rich fish oil (polepa; POL) in various ratios with GLA-rich evening primrose oil (EPO) and examined the effects of fish oil fatty acids on the levels of AA and DGLA in plasma and liver phospholipids.

MATERIALS AND METHODS

Thirty male Sprague-Dawley rats, weighing 200–220 g, were randomly divided into five groups and housed in groups of three. All animals had free access to food and water. The animals were maintained on a fat-free, semisynthetic diet supplemented with 10% (by weight) of different combinations of EPO and POL: 9% EPO–1% POL, 8% EPO–2% POL, 7% EPO–3% POL, 6% EPO–4% POL or 5% EPO–5% POL. Both EPO and POL were supplied by Efamol Limited (Guildford, United Kingdom). The composition of the basal diet (prepared by Teklad Test Diets, Madison, Wisconsin) is shown in Table 1. The fatty acid compositions of the different dietary regimens are shown in Table 2.

The metabolism of EFA in rats has been shown to be rapid, and changes could be demonstrated within five days (1). In this study, feeding was continued for two weeks to enable a dietary effect to be observed. At the end of the experiment, the animals were killed by exsanguination under light ether anesthesia between 8 and 10 a.m. Blood was collected from the inferior vena cava into a test tube containing EDTA (1 mg/ml of blood), and plasma was separated by centrifugation. Livers were rapidly excised, rinsed in cold saline, blotted and frozen at −20 C for later analysis (within one week).

Lipid analysis. Plasma and liver lipids were extracted as described by Folch et al. (30) and separated into different lipid classes by thin layer chromatography. Fatty acids in the phospholipid fraction were methylated and

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analyzed by gas liquid chromatography (GLC) as previously described (31).

Statistical analysis. The results were expressed as mean ± SEM. Least squares analysis was performed to determine the linear regression. The Student-Newman-Keuls multiple range test was also used for comparison of different groups.

RESULTS AND DISCUSSION

The effects of EPO and POL supplementation on liver and plasma phospholipid fatty acid composition are shown in Tables 3 and 4, respectively. The phospholipid concentration (mg/g) was not significantly different among the groups (data not shown). Thus the percentage of fatty acid composition reflects the actual change in fatty acid levels in the tissue.

Saturated and monosaturated fatty acids, e.g., 16:0, 16:1, 18:0 and 18:1, and certain liver polyunsaturated fatty acids, e.g., LA, GLA and α-linolenic acid [ALA, 18:3(n-3)], were not significantly different among the groups. However, animals fed higher concentrations of marine oil contained higher proportions of DGLA (in liver, F ratio = 2.93, $p < 0.05$; in plasma, F ratio = 4.49, $p < 0.01$) and lower concentrations of AA (in liver, F ratio = 43.65, $p < 0.01$; in plasma, F ratio = 11.87, $p < 0.01$) and consequently had lower AA/DGLA ratios. The levels of EPA and DHA were also increased in response to the increasing POL supplementation. Inverse correlations between the levels of EPA and AA/DGLA ratio in liver and plasma are presented in Figures 1A and 1B, respectively. However, no significant correlation was seen with DHA ($r = -0.045$ and -0.255 for liver and plasma, respectively).

In this study, DGLA levels in liver and plasma phospholipid were progressively elevated while AA levels were progressively decreased in response to increased marine oil supplementation. This resulted in a progressively reduced ratio of AA to DGLA. Hill et al. (32) have previously demonstrated that liver phospholipid fatty acid composition reflects effectively and reliably the in vivo activity of EFA desaturation. Thus, a reduced ratio of AA/DGLA observed in this study suggests a decreased D5D activity.

EFA deficiency is known to lower the activity of D5D (33). However, the decreased AA/DGLA ratio in the present study could not be attributed to an EFA-deficient status in those animals who received increased marine oil supplements, since 20:3(n-9), a marker for EFA deficiency (33,34), was not detected. LA is known to promote desaturation of DGLA to AA (35), but the decrease in D5D activity could not be due to decreased LA intake since tissue levels of LA were not significantly different among the different groups. It is possible that the increased levels of DGLA were due to enhanced elongation of GLA.

TABLE 1

Composition of the Basal Diet

	g/kg
Casein, vitamin-free	200
Sucrose	602
Fat[a]	100
Cellulose	50
Mineral mix[b]	35
Vitamin mix[c]	10
DL-methionine	3

[a]Wholly evening primrose oil or partially replaced (10, 20, 30, 40 or 50%) by fish oil.

[b]#AIN-76, providing (g/kg mix) $CaHPO_4$, 500; NaCl, 74; K_2SO_4, 52; MgO, 24; KIO_4, 0.01; $CrK(SO_4)\cdot 12H_2O$, 0.55; $MnCO_3$, 3.5; $ZnCO_3$, 1.6; potassium citrate, 220; ferric citrate, 6.0; and sucrose, 118.03.

[c]Providing (g/kg mix): choline dihydrogen citrate, 349.7; ascorbic acid, 101.7; vitamin E acetate, 24.2; inositol, 11.0; p-aminobenzoic acid, 11.0; niacin, 9.9; calcium pentothenate, 6.6; menadione, 5.0; vitamin A palmitate, 4.0; vitamin B_{12}, 3.0; pyridoxine, 2.2; riboflavin, 2.2; thiamine HCl, 2.2; vitamin D_2, 0.4; folic acid, 0.2; biotin, 0.044; and cornstarch, 466.7.

TABLE 2

Fatty Acid Composition (%) of the Evening Primrose (EPO) and Fish (POL) Oils and the Combinations Supplemented to the Fat-Free Semisynthetic Diet

Fatty acid	EPO	9% EPO-1% POL	8% EPO-2% POL	7% EPO-3% POL	6% EPO-4% POL	5% EPO-5% POL	POL
14:0	—	0.7	1.4	2.2	2.9	3.7	8.2
16:0	6.4	7.0	8.2	9.1	10.4	11.2	18.7
16:1(n-7)	—	—	1.7	2.3	3.6	3.9	9.4
18:0	1.7	1.9	2.1	2.3	2.5	2.7	4.3
18:1(n-9)	7.5	8.3	9.1	9.8	10.5	11.3	14.6
18:2(n-6)	74.9	68.8	61.9	54.9	47.9	41.5	2.1
18:3(n-6)	9.0	9.0	8.0	7.0	6.2	5.3	—
18:3(n-3)	—	0.6	0.8	1.0	1.2	1.5	2.4
20:1(n-9)[a]	—	—	0.4	0.7	0.9	1.2	1.1
20:4(n-6)	—	—	—	0.9	1.1	1.4	1.8
20:5(n-3)	—	1.4	3.1	4.8	6.4	8.1	16.5
22:4(n-6)	—	0.5	0.3	0.4	0.3	0.4	—
22:5(n-3)	—	—	0.5	0.7	0.9	1.2	2.5
22:6(n-3)	—	1.0	2.1	3.3	4.5	5.6	11.1

[a]Includes 20:1(n-11) and 20:1(n-7).

Normally, the rate of chain elongation is more rapid in comparison to either Δ-6 or D5D rates (25). However, this possibility is refuted by the fact that the levels of GLA in plasma and liver were not significantly different among the groups despite the decreased intake of GLA.

Evidence has shown that DHA inhibits the Δ-6-desaturation of n-6 fatty acids (24). In this study, the sum of 20:5(n-3) and 22:6(n-3) correlated with the ratio of AA/DGLA ($r = -0.671$ and -0.582 for plasma and liver, respectively; data not shown). However, close examination indicated a distinct correlation between the decreasing ratio of AA and DGLA and the increasing concentration of EPA in both plasma and liver phospholipids ($r = -0.765$ and -0.792, respectively). On the other hand, the correlation between DHA and the ratio of AA to DGLA was not statistically significant, although proportions of DHA were also increased inversely to the decrease in AA. This result suggests a possible inhibitory effect of EPA on the conversion of DGLA to AA. The 22:5(n-3), a metabolite of EPA, also correlated well with the ratio of AA to DGLA ($r = -0.799$ and 0.811 for plasma and liver, respectively; data not shown). As the levels of 22:5(n-3) in plasma and liver were higher than those present in the diet, this result indicated that

TABLE 3

Fatty Acid Composition (mg/100 mg) of Liver Phospholipid Fraction in Rats Fed a Fat-Free Semisynthetic Diet Supplemented with Different Combinations of Evening Primrose (EPO) and Fish (POL) Oils

Fatty acid	9% EPO-1% POL	8% EPO-2% POL	7% EPO-3% POL	6% EPO-4% POL	5% EPO-5% POL	F ratio
16:0	17.7 ± 0.5	17.7 ± 0.2	17.6 ± 0.3	17.3 ± 0.3	18.2 ± 0.2	1.14
16:1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	0.13
18:0	22.0 ± 0.5	22.8 ± 0.5	22.4 ± 0.6	22.9 ± 0.3	23.1 ± 0.9	0.95
18:1	6.1 ± 0.2	5.8 ± 0.1	5.9 ± 0.3	5.7 ± 0.2	6.3 ± 0.1	1.51
18:2(n-6)	10.5 ± 0.5	10.2 ± 0.6	10.7 ± 0.4	10.7 ± 0.3	9.7 ± 0.2	0.68
18:3(n-6)	0.3 ± 0.02	0.3 ± 0.02	0.3 ± 0.03	0.3 ± 0.01	0.2 ± 0.02	0.98
20:2[a]	0.5 ± 0.05	0.4 ± 0.05	0.4 ± 0.02	0.3 ± 0.01	0.2 ± 0.05	8.33*
20:3(n-6)	1.8 ± 0.01	1.8 ± 0.2	2.4 ± 0.2	2.4 ± 0.1	2.3 ± 0.1	2.93**
20:4(n-6)	25.9 ± 0.3	25.1 ± 0.4	23.4 ± 0.4	21.5 ± 0.6	18.2 ± 0.5	43.65*
22:4(n-6)	0.5 ± 0.02	0.3 ± 0.06	0.3 ± 0.01	0.3 ± 0.07	0.2 ± 0.06	7.17*
22:5(n-6)	0.5 ± 0.06	0.2 ± 0.04	0.3 ± 0.02	0.3 ± 0.01	0.2 ± 0.05	5.04*
18:3(n-3)	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.03	0.04 ± 0.01	0.1 ± 0.04	0.54
20:5(n-3)	0.3 ± 0.03	0.6 ± 0.03	1.1 ± 0.1	1.6 ± 0.05	2.3 ± 0.2	40.82*
22:5(n-3)	1.2 ± 0.05	1.3 ± 0.07	1.7 ± 0.1	1.9 ± 0.08	2.0 ± 0.1	13.91*
22:6(n-3)	10.5 ± 0.4	11.5 ± 0.5	11.3 ± 0.4	12.4 ± 0.3	14.8 ± 0.6	13.05*
20:4(n-6)/20:3(n-6)	14.7	13.6	9.9	9.1	8.1	

Results are expressed as mean ± SEM of six observations; * = $p < 0.01$, ** = $p < 0.05$. F ratio was determined by Student-Newman-Keuls multiple range test with equal sample sizes.

[a]Including both n-6 and n-9 isomers.

TABLE 4

Fatty Acid Composition (mg/100 mg) of Plasma Phospholipid Fraction in Rats Fed a Fat-Free Semisynthetic Diet Supplemented with Different Combinations of Evening Primrose (EPO) and Fish (POL) Oils

Fatty acid	9% EPO-1% POL	8% EPO-2% POL	7% EPO-3% POL	6% EPO-4% POL	5% EPO-5% POL	F ratio
16:0	20.9 ± 0.7	21.3 ± 0.6	21.1 ± 1.0	20.6 ± 0.8	21.7 ± 0.4	0.32
16:1	0.9 ± 0.2	0.7 ± 0.7	1.1 ± 0.4	1.0 ± 0.2	1.0 ± 0.2	0.27
18:0	14.9 ± 0.8	19.2 ± 0.7	16.9 ± 1.2	18.3 ± 1.2	17.4 ± 1.5	1.92
18:1	6.7 ± 0.2	6.5 ± 0.3	6.9 ± 0.7	7.8 ± 0.6	7.6 ± 0.3	1.66
18:2(n-6)	22.4 ± 0.7	19.1 ± 0.6	21.0 ± 1.2	19.6 ± 1.2	18.6 ± 0.7	2.58
18:3(n-6)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	1.97
20:3(n-6)	1.7 ± 0.05	1.8 ± 0.2	2.0 ± 0.2	2.1 ± 0.1	2.3 ± 0.1	4.49*
20:4(n-6)	23.2 ± 0.8	21.3 ± 1.2	19.8 ± 1.0	17.8 ± 0.5	16.9 ± 0.3	11.87*
20:5(n-3)	0.7 ± 0.07	1.0 ± 0.09	2.1 ± 0.3	2.5 ± 0.3	3.1 ± 0.2	22.11
22:5(n-3)	0.8 ± 0.01	1.4 ± 0.1	1.4 ± 0.2	1.8 ± 0.1	1.8 ± 0.1	15.28*
22:6(n-3)	6.1 ± 0.2	6.9 ± 0.6	6.7 ± 0.2	7.3 ± 0.4	8.8 ± 0.4	10.72*
20:4(n-6)/20:3(n-6)	13.4	11.8	10.1	8.3	7.3	

Results are expressed as mean ± SEM of six observations, * = $p < 0.01$. F ratio was determined by Student-Newman-Keuls multiple range test with equal sample sizes.

22:5(n-3) was converted from EPA and supported the role of EPA on the inhibition of the conversion of DGLA to AA. EPA, like AA, is readily incorporated into tissue phospholipids (18,36,37), possibly by competing for the incorporation sites, which might explain the reduction in AA. However, accumulation of DGLA, despite the decreasing intake of its immediate precursor GLA, indicates reduced conversion of DGLA to AA.

Eskimos on a traditional diet, who reportedly have a low incidence of CHD, have an apparent increase in DGLA in their plasma triglycerides associated with decreased AA (38,39). Earlier reports have shown n-3 fatty acids to inhibit the metabolism of n-6 acids, especially at the desaturation steps (22,23). Theoretically, accumulation of DGLA may enhance the production of the 1-series prostaglandins. PGE_1 has a wide range of desirable actions, such as lowering of blood pressure and inhibition of platelet aggregation and smooth muscle proliferation (40–42); it also stimulates formation of cyclic AMP, a known inhibitor of AA mobilization and cholesterol biosynthesis (43). The lipoxygenase product of DGLA, a 15-hydroxy derivative, also inhibits the conversion of AA to leukotrienes (43,44). On the other hand, a decreased AA level would reduce the metabolites of AA, including TxA_2, $PGF_{2\alpha}$ and the leukotrienes, which are vasoconstrictors, proaggregatory and abundantly present in various inflammatory conditions. Steroids and nonsteroidal anti-inflammatory drugs are widely used to treat inflammation. Their main line of action is believed to be indiscriminate inhibition of PG production, irrespective of the target organ sought. Hence, they block production of PG with desirable actions, such as PGE_1, PGI_2 and (to

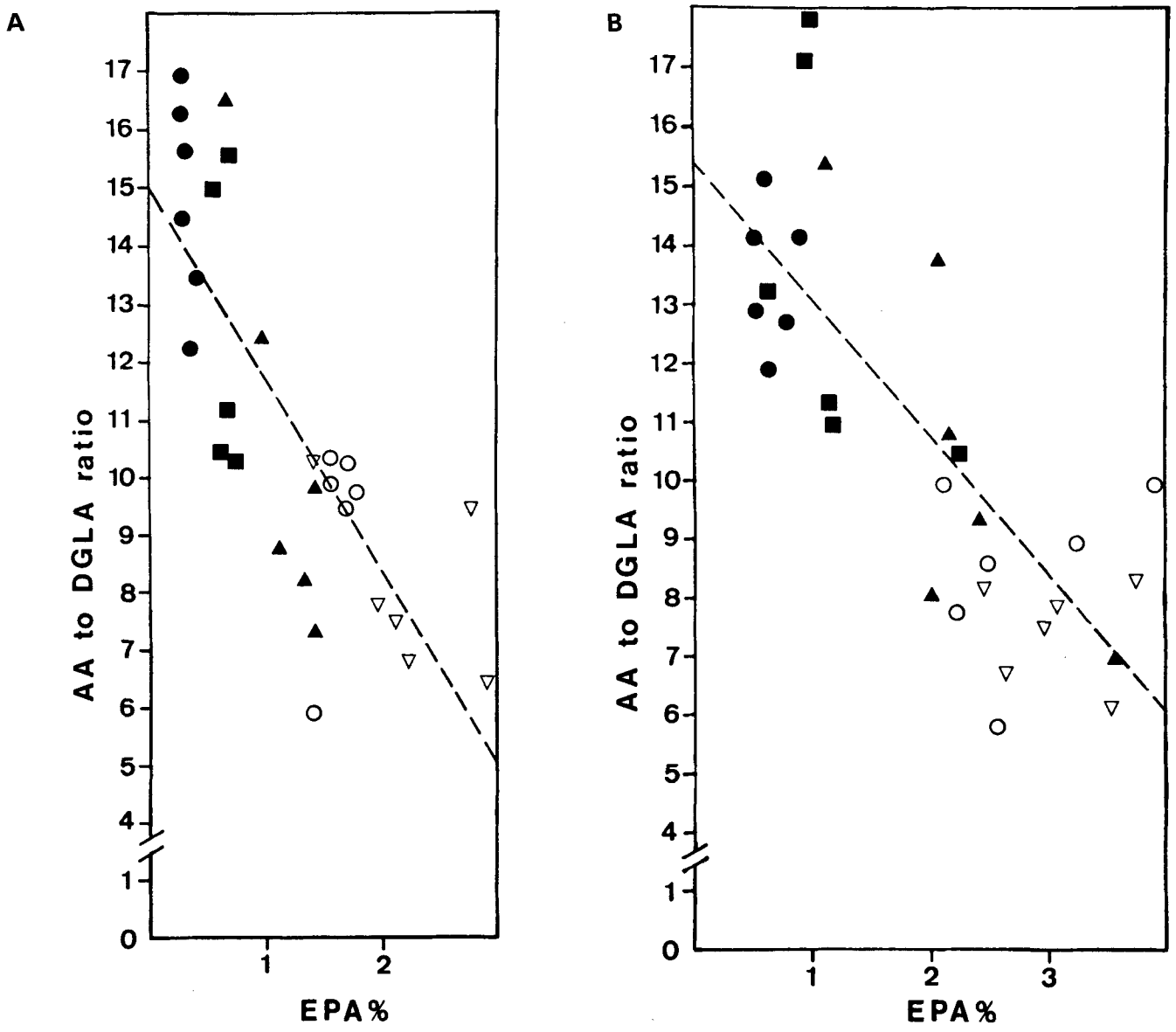

FIG. 1. Correlation of the concentration of EPA [C20:5(n-3)] to the ratio of AA [20:4(n-6)] to DGLA [20:3(n-6)] in liver (A) and plasma (B) in rats receiving five different combinations of evening primrose oil (EPO) and fish oil (POL): ●, 9% EPO–1% POL; ■, 8% EPO–2% POL; ▲, 7% EPO–3% POL; ○, 6% EPO–4% POL; ▽, 5% EPO–5% POL. Correlation coefficient: r = −0.792 (A) and −0.765 (B). Y-intercept: Y = 15.080−3.343X and 15.282−2.347X for A and B, respectively.

a lesser extent) PGE_2, as well as of ones with undesirable effects. Thus, manipulation of the DGLA/AA balance may be of vital importance in producing more selective regulation of eicosanoid biosynthesis (29).

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METHODS

A Comparison of Pyrrolidide and Picolinyl Ester Derivatives for the Identification of Fatty Acids in Natural Samples by Gas Chromatography-Mass Spectrometry

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The pyrrolidide and picolinyl ester derivatives of the fatty acids in two natural lipid samples rich in unsaturated fatty acids, pig testis lipids and cod liver oil were satisfactorily resolved on capillary columns of fused silica coated with stationary phases of varying polarity. The picolinyl esters, in particular, when subjected to gas chromatography-mass spectrometry on a column containing a cross-linked methyl silicone, gave distinctive mass spectra, which could be interpreted in terms of both the numbers and positions of the double bonds.
Lipids 21, 657-661 (1986).

There have been two approaches to the determination of the structures of the common range of saturated and unsaturated fatty acids by gas chromatography-mass spectrometry (GC-MS) (reviewed comprehensively elsewhere [1,2]). The first approach consisted of the reaction of the double bonds in fatty acids or their methyl ester derivatives to form compounds containing substituent groups that produced distinctive mass spectrometric fragmentation patterns. Such methods generally involved an appreciable addition to the molecular weight of the derivative and often increased its polarity. The procedure was thus less suitable for polyunsaturated compounds. The second approach used amide and related derivatives of the carboxyl group of fatty acids, which appeared to stabilize the ions containing double bonds in the mass spectrometer. In consequence, spectra were obtained that were frequently characteristic of particular unsaturated fatty acids. Often there were also diagnostic ions that permitted unequivocal identifications to be made. Pyrrolidide derivatives have been studied most often (reviewed by Andersson [3]), but Harvey (4-6) has shown that picolinyl esters may be more useful. Virtually all the published work with compounds of this type has been with model compounds, although there have been a few applications of GC-MS to pyrrolidide derivatives of natural fatty acid mixtures (7-11).

Although both pyrrolidides and picolinyl esters add to the molecular weight and polarity of a fatty acid in comparison to the methyl ester derivatives, which have generally been preferred for chromatographic analysis, there appeared to be no reason why they should not be successfully subjected to GC analysis, especially on modern capillary columns of fused silica. Therefore, the nature of the gas chromatographic separation of pyrrolidide and picolinyl ester derivatives of fatty acids from two natural sources, i.e., pig testis lipids and cod liver oil, on different stationary phases of varying polarity has now been investigated. The relative value of the two types of derivatives for the elucidation of fatty acid structure has also been determined.

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MATERIALS AND METHODS

Materials. Fatty acid standards, trifluoroacetic anhydride, pyrrolidine and 3-(hydroxymethyl)pyridine were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). Pig testes were obtained from the Hormel Co. (Austin, Minnesota), and the lipids were extracted with chloroform/methanol (2:1, v/v). Cod liver oil was obtained from a local pharmacy. All solvents were of Analar grade and were purchased from Fisons Ltd. (Loughborough, United Kingdom).

Pyrrolidide preparation. Pyrrolidides were prepared from fatty acid standards and from lipid extracts by the method of Andersson and Holman (12). Prior to gas liquid chromatography (GLC), they were purified by elution from a short column of Florisil™ (3 cm) with hexane/diethyl ether (4:1, v/v; 10 ml).

Picolinyl ester preparation. Lipid extracts were hydrolyzed and the nonsaponifiable constituents were removed as described elsewhere (13). The free fatty acids were dissolved in diethyl ether (1 ml) and converted to the mixed anhydride derivatives by reaction with trifluoroacetic anhydride (0.5 ml) at 50 C for 1 hr. The excess reagent was evaporated in a stream of nitrogen; a 10% solution of 3-(hydroxymethyl)-pyridine in tetrahydrofuran (0.5 ml) was added and the mixture was left at 50 C for 1 hr. Diethyl ether (10 ml) and hexane (2 ml) were added, and the mixture was washed with water (2 ml), 1M hydrochloric acid (2 ml; three times) and water (2 ml; three times). The solvents were evaporated in a stream of nitrogen at 40 C, and excess water was removed with isopropanol. The picolinyl esters were purified by Florisil chromatography as above, but with hexane/diethyl ether (1:1, v/v; 10 ml) as the eluting solvent.

Gas chromatography. Coated fused silica capillary columns were obtained from SGE Ltd. (Burke Electronics, Glasgow, United Kingdom). The following were used: FFAP (30 m × 0.22 mm), BP 5 (5% phenylmethyl silicone, equivalent to SE 52) (12 m × 0.22 mm), BP 20 (polyethylene glycol, equivalent to carbowax 20M) (12 m × 0.22 mm) and OV 101 (50 m × 0.22 mm). Helium was the carrier gas at a flow rate of 0.9 to 1.1 ml/min. A Packard Model 428 gas chromatograph (Packard Instrument Co., Downers Grove, Illinois) equipped with a split/splitless injection system was used. Satisfactory separations of pyrrolidide derivatives of fatty acids were obtained on the BP 5 and BP 20 columns by injecting onto the column at 190 C; they were held for 3 min, temperature-programmed at 1 C to 230 C and held for a further 5 min.

With the OV 101 column, the oven temperature limits were 230 to 270 C. For the picolinyl ester derivatives, similar temperature programming schemes were adopted, but the oven temperature was always 5 C higher than for pyrrolidides to obtain elution of a given component in a similar time.

Gas chromatography-mass spectrometry. A Hewlett Packard model 5890A, equipped with on-column capillary injection, was used with a 5970 Series Mass Selective Detector (Hewlett Packard Ltd., Wokingham, Berks, United Kingdom). The column outlet was connected directly to the ion source. A fused silica capillary column (25 m × 0.2 mm) coated with a cross-linked methyl silicone (0.11 micron film thickness) was obtained from the same company. Helium was the carrier gas at a flow rate of about 1 ml/min. For pyrrolidide and picolinyl ester derivatives of fatty acids, the samples were injected onto the column at 60 C; the oven temperature was maintained at this point for 0.5 min, then was raised at 50 C/min to 220 C, where it was held for 2 min. The temperature was then programmed at 1 C/min to 260 C and was held for 1 min. Spectra were recorded at an ionization energy of 70 eV.

RESULTS

Pig testis lipids were chosen for this study because the fatty acid composition has been investigated in great detail (14). A wide range of unsaturated fatty acids of the ω6 series was known to be present. Similarly, cod liver oil has been well characterized and was known to contain a number of different monoenoic constituents together with polyunsaturated fatty acids of the ω3 series (15). Many artifact peaks were seen initially in the chromatograms when Harvey's method for the preparation of picolinyl esters (6) was tried, but these disappeared when the milder method described above was used.

Methyl ester derivatives have been preferred for fatty acid analysis by GC, and highly polar stationary phases have most often been selected. A fused-silica column coated with FFAP gave excellent results with methyl esters prepared from the two lipid samples. Forty-five components were present at levels above 0.1% in the pig testis lipids, for example. Pyrrolidide and picolinyl esters were not eluted below the maximum temperature recommended for this phase. On the other hand, with a stationary phase at the other extreme of polarity, i.e., OV 101, the inherent resolution of the fused silica capillary was sufficient to permit useful separations of the fatty acid derivatives at acceptable temperatures, i.e., with negligible baseline drift, though approximately 50 C higher than for methyl esters. The separation of the picolinyl ester derivatives from the pig testis lipids on OV 101 is shown in Figure 1. Unsaturated components were eluted before the corresponding saturated derivatives, and some resolution according to the position of the double bonds as well as by their number was evident. The position of the double bonds appeared to be the major factor controlling separation, and the 22:5ω6 fatty acid eluted before 22:6ω3, for example. Some separations were not attainable with this column; in particular, 18:2ω6 and 18:3ω3 were not completely resolved. A similar separation was achieved with the BP 5 column. In general, the pyrrolidide derivatives gave slightly better separations

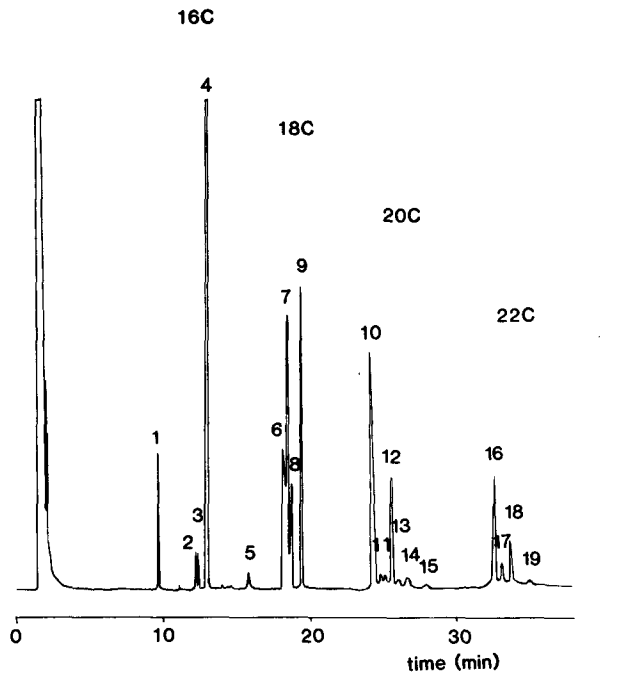

FIG. 1. Separation of the picolinyl ester derivatives of fatty acids from pig testis lipids by gas liquid chromatography on a capillary column of fused silica coated with OV 101. See Materials and Methods for the chromatographic conditions. The fatty acids were as follows: 1, 14:0; 2, 16:1ω9; 3, 16:1ω7; 4, 16:0, 5, 17:0; 6, 18:2ω6; 7, 18:1ω9; 8, 18:1ω7; 9, 18:0; 10, 20:4ω6; 11, 20:3ω9; 12, 20:3ω6; 13, 20:2ω9; 14, 20:2ω6; 15, 20:1ω9; 16, 22:5ω6; 17, 22:6ω3; 18, 22:4ω6; and 19, 22:3ω9. Only those components greater than 0.5% of the total were identified.

than did the more polar picolinyl esters. The relative proportions of each component in both samples were determined with the flame ionization detector for the methyl esters, pyrrolidides and picolinyl esters. The results were very similar, but differed somewhat from those obtained from the total ion current in the mass spectrometer, probably because of lower yields of ions from the polyunsaturated components. With our equipment, mass spectrometric detection was about 10 times as sensitive as flame ionization detection.

Acceptable separations of pyrrolidides and picolinyl esters were also achieved on BP 20, a stationary phase of intermediate polarity, but with more baseline drift. The separation obtained with the pyrrolidide derivatives from pig testis fatty acids is shown in Figure 2. Unsaturated fatty acids eluted after the saturated component of the same chain length. Positional isomers were often clearly resolved, although one major anomaly was observed, i.e., the 20:3ω6 fatty acid eluted after 20:4ω6. The reverse order was found, as expected, with the corresponding picolinyl ester derivatives.

Because better resolution and baseline stability (low column bleed) was obtained with the lowest polarity phases, a similar type of column was used in the GC-MS system to assess the utility of the two types of derivative for purposes of fatty acid identification. The separation achieved with the Hewlett-Packard methyl silicone stationary phase was almost identical to that obtained with the SGE OV 101 phase, except that lower elution temperatures were possible, due in part only to the former column being shorter.

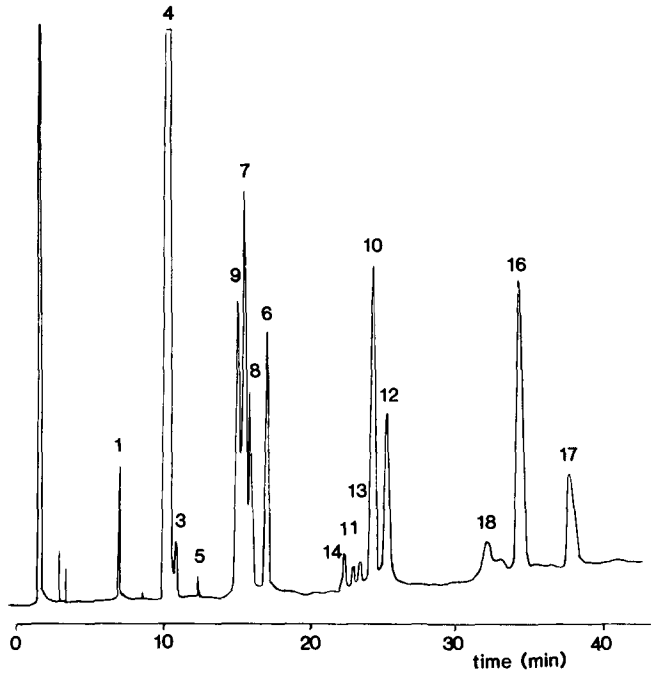

FIG. 2. Separation of the pyrrolidide derivatives of fatty acids from pig testis lipids by gas liquid chromatography on a capillary column of fused silica coated with BP 20. See Materials and Methods for the chromatographic conditions. Fatty acid designations are given in the legend to Fig. 1.

Good molecular ions were obtained in general for the pyrrolidide derivatives of fatty acids, permitting the chain length and degree of unsaturation to be determined unequivocally. Saturated derivatives gave clear spectra of immediate diagnostic value. The positions of double bonds in monoenoic derivatives could be recognized by locating a gap of 12 atomic mass units (amu) in the spectra, as described elsewhere (12). On the other hand, as Andersson (3) has implied, the spectra of individual polyunsaturated pyrrolidides were often distinctive but lacked ions of immediate diagnostic value. The spectra could rarely be used for identification of double bond positions, other than by reference to spectra from authentic samples, and these are not always available.

The picolinyl ester derivatives also gave excellent spectra with clear molecular ions, in spite of the fact that the spectra were obtained at an ionization energy of 70 eV, rather than at 25 eV as recommended by Harvey (4). With almost every component of the samples that was adequately resolved, the chain length and the number of double bonds could be recognized, and diagnostic ions were present in the spectra that permitted location of the double bonds, even in polyenoic fatty acids. Methyl branches could be located in alkyl chains. All picolinyl esters gave distinctive ions at m/z 92 (usually the base ion), m/z 108, m/z 151 (or 150) and m/z 164. Some of the important ions in the spectra of the picolinyl ester derivatives of those fatty acids present in the two natural samples are listed in Table 1. With the exceptions described below, the identifications were consistent with the published compositions of the lipid extracts.

Saturated fatty acid derivatives gave clear spectra with a series of ions 14 amu apart, representing cleavage at each methylene group (4). When a methyl branch was present in the aliphatic chain, as in one component of the cod liver oil, a gap in this series was seen at the branch point, as reported elsewhere (4).

Picolinyl esters of monoenoic fatty acids could sometimes be identified by searching for a gap of 26 amu in the spectra, representing cleavage at either side of the double bond, although this was not always easy to find with minor components or with some of the C_{22} monoenes. The double bond location could, however, be determined unequivocally with great ease from a pair of ions 14 amu apart, which were often 30–40% of the base peak and stood out prominently from the other ions. It has been suggested that these ions could be rationalized in terms of initial hydrogen abstraction of the allylic hydrogens to form conjugated dienes with one or two carbon atoms more than the fragment with the single double bond (4). Thus, a double bond in position 9 was characterized by prominent ions at m/z 274 and m/z 288, as in 14:1ω5, 16:1ω7, 18:1ω9 and 20:1ω11 fatty acids. A double bond in position 11 was characterized by a doublet of prominent ions at m/z 302 and m/z 316, as in 16:1ω5, 18:1ω7, 20:1ω9 and 22:1ω11 fatty acids. Similarly, a double bond in position 7 characteristically gave a doublet of ions at m/z 246 and m/z 260, and a double bond in position 13 gave a doublet at m/z 330 and m/z 344. A heptadecenoic acid derivative, which was only 0.3% of the cod liver oil esters, was identified as 8-heptadecenoic acid from a distinctive doublet of peaks at 260 and 274, although this component had elsewhere been considered as having a double bond in position 9 (15). A further heptadecenoic acid component may have contained a methyl branch and will receive additional investigation. The picolinyl ester derivatives of oleic and elaidic acids had identical mass spectra.

Polyunsaturated fatty acids in the form of the picolinyl esters could also be identified from their mass spectra in the manner described by Harvey (6). Good molecular ions were generally obtained and a series of ions was present, 14 amu apart, representing cleavage at each of the methylene groups starting at the terminal end of the molecule. When a double bond was reached, a gap of 26 amu could be discerned, followed by a gap of 14 amu, a further gap of 26 amu, and so on. Those double bonds nearest the carboxyl group in a polyunsaturated component could not always be found in this way because of the complexity of ions in the intermediate m/z range, so it may on occasion be necessary to make the assumption that the progression of methylene-interrupted unsaturation is continued. As an example, the mass spectrum of the picolinyl ester of a 20:3ω9 fatty acid, which was a minor component of the pig testis lipids, is shown in Figure 3. A series of significant ions 14 amu apart, starting with the molecular ion, was seen until m/z 284 was reached, followed by a gap of 26 amu to m/z 258, which located the terminal double bond, a gap of 14 amu to m/z 244, and a gap of 26 amu to 218, which located the second double bond. Ions diagnostic of the proximal double bond were not apparent, although it is possible that a better spectrum, if the component had been more abundant in the sample, might have permitted location of this unequivocally. The mass spectrum of the picolinyl ester derivative of a 20:3ω6 derivative is shown in Figure 4 for comparison purposes. Again, there was a prominent

TABLE 1

Picolinyl Derivatives of Fatty Acids of Pig Testis Lipids and Cod Liver Oil

Component	Retention time (min)	Mol. ion (%)	Base ion	Other useful or diagnostic ions in the higher mass range						Wt %
Pig testis lipids										
14:0	11.39	319 (15)	108	290 (19)	276 (20)	262 (22)	248 (17)	234 (13)	220 (16)	1.4
16:1ω9	15.15	345 (40)	93	288 (6)	274 (7)	260 (26)	246 (31)	232 (7)	206 (3)	0.8
16:1ω7	15.38	345 (42)	92	316 (8)	302 (14)	288 (37)	274 (41)	260 (10)	234 (6)	0.9
16:0	16.06	347 (15)	108	318 (14)	304 (14)	290 (16)	276 (16)	262 (16)	248 (13)	28.5
17:0	18.97	361 (11)	92	332 (13)	318 (15)	304 (17)	290 (14)	276 (16)	262 (16)	0.7
18:2ω6	21.18	371 (50)	92	328 (13)	314 (16)	300 (8)	274 (13)	260 (17)	234 (4)	6.6
18:1ω9	21.52	373 (37)	92	316 (9)	302 (8)	288 (35)	274 (30)	260 (10)	234 (4)	10.7
18:1ω7	21.78	373 (34)	92	344 (6)	330 (9)	316 (29)	302 (38)	288 (14)	262 (3)	4.8
18:0	22.62	375 (14)	108	346 (9)	332 (11)	318 (12)	304 (13)	290 (13)	276 (14)	12.0
20:4ω6	26.88	395 (20)	92	338 (7)	324 (4)	298 (4)	284 (17)	258 (5)	244 (5)	11.6
20:3ω9	27.16	397 (21)	92	312 (3)	298 (7)	284 (8)	258 (9)	244 (7)	218 (5)	1.2
20:3ω6	28.15	397 (31)	92	354 (6)	340 (10)	326 (6)	300 (8)	286 (21)	260 (5)	6.0
20:2ω9	28.41	399 (33)	92	314 (8)	300 (13)	286 (8)	260 (12)	246 (9)	220 (6)	0.9
20:2ω6	29.17	399 (25)	92	356 (7)	342 (12)	328 (4)	302 (9)	288 (15)		0.7
20:1ω9	29.48	401 (29)	92	344 (8)	330 (9)	316 (26)	302 (35)	288 (11)	262 (7)	0.5
22:5ω6	34.84	421 (9)	92	350 (3)	324 (2)	310 (8)	284 (3)	270 (7)	244 (6)	5.9
22:6ω3	35.14	419 (7)	92	390 (3)	364 (2)	350 (5)	324 (2)	310 (6)	284 (2)	2.2
22:4ω6	36.01	423 (17)	92	352 (7)	326 (3)	312 (12)	286 (6)	272 (6)	246 (4)	2.1
22:3ω9[a]	36.29	425 (8)	92	326 (4)	312 (9)	286 (10)	272 (13)	246 (6)		0.7
Cod liver oil[b]										
14:1ω5	11.08	317 (29)	92	288 (24)	274 (22)	260 (7)	220 (9)			0.4
15:0 (isobr)	12.60	333 (16)	92	318 (18)	290 (44)	262 (21)	248 (10)	234 (11)	220 (11)	0.3
15:0	13.38	333 (18)	92	304 (13)	290 (11)	276 (21)	262 (8)	248 (14)	234 (12)	0.4
16:1ω5[a]	15.65	345 (36)	92	316 (17)	302 (22)	288 (19)	274 (11)	234 (6)		0.4
17:1ω9	17.30	359 (44)	92	274 (14)	260 (33)					0.3
18:4ω3	20.49	367 (13)	92	338 (5)	312 (4)	298 (17)	272 (7)	258 (7)	232 (7)	1.3
18:1ω5	22.16	373 (34)	92	344 (23)	330 (32)	316 (13)				0.6
20:5ω3	27.05	393 (8)	92	364 (2)	338 (2)	324 (7)	298 (2)	284 (7)	258 (5)	5.6
20:1ω11	29.27	401 (28)	92	302 (8)	288 (26)	274 (22)	260 (8)	234 (5)	220 (9)	1.6
20:1ω7	29.96	401 (33)	92	372 (7)	358 (9)	344 (24)	330 (29)	316 (14)		0.9
22:5ω3	36.32	421 (8)	92	392 (4)	366 (2)	352 (7)	325 (2)	312 (9)	286 (3)	1.8
22:1ω11	38.77	429 (35)	92	330 (10)	316 (31)	302 (40)	288 (12)	262 (4)	248 (8)	5.5
22:1ω9	39.10	429 (31)	92	358 (8)	344 (25)	330 (30)	316 (15)			0.8

[a]This peak may in fact represent more than one component.

[b]No data is given for the following components, which were also present in the pig testis lipids: 14:0 (5.0%), 16:1ω7 (11.6%), 16:0 (11.9%), 17:0 (0.6%), 18:1ω9 (24.8%), 18:1ω7 (5.6%), 18:0 (2.4%), 20:1ω9 (10.7%) and 22:6ω3 (6.2%).

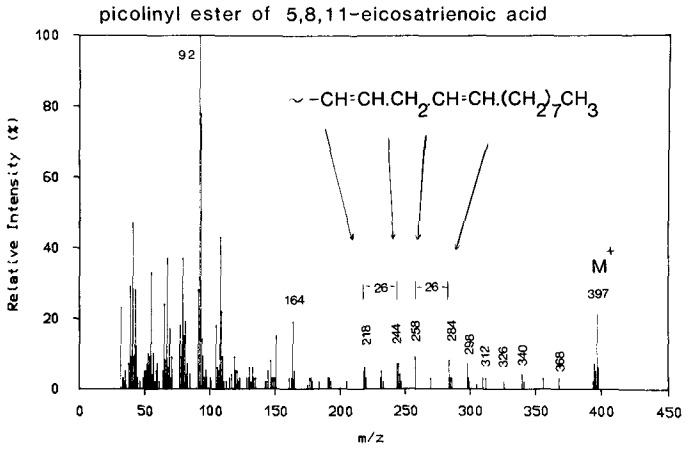

FIG. 3. The mass spectrum of the picolinyl ester derivative of 5,8,11-eicosatrienoic acid from pig testis lipids.

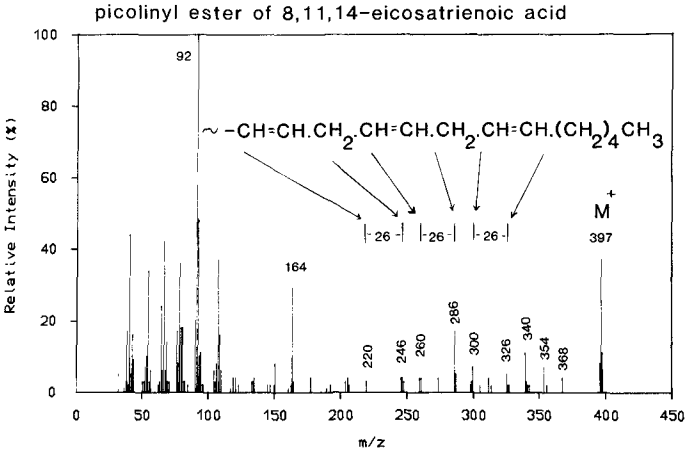

FIG. 4. The mass spectrum of the picolinyl ester derivative of 8,11,14-eicosatrienoic acid from pig testis lipids.

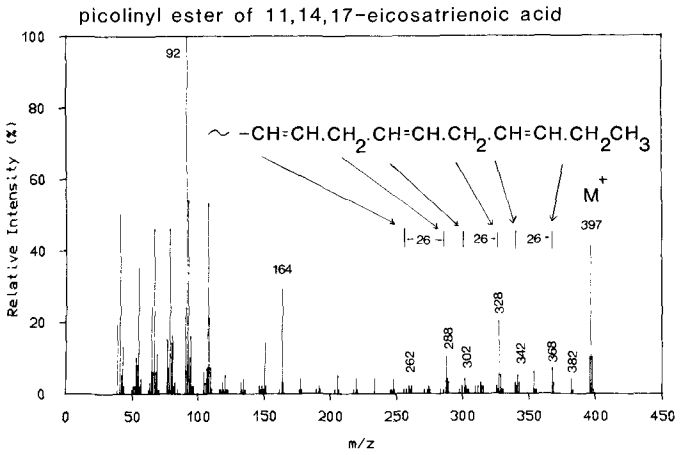

FIG. 5. The mass spectrum of the picolinyl ester derivative of 11,14,17-eicosatrienoic acid.

molecular ion and a series of ions 14 amu apart, representing cleavage at the methylene groups in the terminal region of the molecule. Gaps of 26 amu between m/z 326 and m/z 300, m/z 286 and m/z 260, and m/z 246 and m/z 220 were apparent and effectively located each of the double bonds. A spectrum of an authentic sample of the derivative of this acid was identical to that obtained from the pig testis fatty acid esters. If a 20:3ω3 fatty acid was present in either of the samples, it was masked by other components. Therefore a spectrum was obtained from an authentic derivative from a commercial source; this is illustrated in Figure 5. In this instance, gaps of 26 amu between m/z 368 and m/z 342, m/z 328 and 302, and m/z 288 and m/z 262 were diagnostic for the positions of the double bonds. All the polyunsaturated fatty acid picolinyl ester derivatives, which were adequately resolved and in sufficient abundance in the two natural samples to give good mass spectra, were identifiable in a similar manner.

DISCUSSION

The results of this study have shown that fatty acids in natural samples can be adequately resolved in the form of the pyrrolidides and picolinyl esters on fused silica capillary columns, especially when these are coated with stationary phases of low polarity. Although pyrrolidide derivatives from natural samples had been subjected to GC-MS analysis previously (7-11), the nature of the separation was depicted in only one paper (8). Positional isomers of the monoenoic components were not resolved, for example, as was the case here. Direct sample introduction into the ion source, as opposed to using a helium separator, preserved the quality of the separation. The picolinyl esters were found to be of greatest value, as every component in the two lipid samples used in this study (32 different fatty acids in total) from which a good mass spectrum was obtained could be identified by this means. With a little practice, workers could identify the diagnostic ions with little difficulty. Such an approach to mass spectral identification of fatty acids is certainly much simpler than methods involving derivatization at the double bond, and it is probably capable of wider applicability. On the other hand, the latter procedure may give more definitive results when a pure fatty acid is available. The simplest and most elegant method of double bond derivatization for mass spectral identification yet published would appear to consist in deuteration of the pyrrolidide derivatives of unsaturated fatty acids (2,16,17). From the results of this study, it could be argued that deuterated picolinyl esters might now be preferred if this approach were to be followed. The ideal derivative would also permit identification of double bond configuration, and it is probable that a combination of both mass spectral and chromatographic data would be essential to elucidate this information.

Picolinyl esters contain an aromatic moiety that would potentially facilitate spectrophotometric detection after separation by high performance liquid chromatography. Such a procedure might prove of value for the isolation of minor components in natural samples to obtain simpler fractions for GC-MS analysis or to obtain better mass spectra. This approach is currently being investigated.

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New Synthesis of *cis*-3,*cis*-5- and *trans*-3,*cis*-5-Tetradecadienoic Acids, Pheromone Constituents of *Attagenus elongatulus* and *A. megatoma*

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A new method for the synthesis of *cis*-3,*cis*-5- and *trans*-3,*cis*-5-tetradecadienoic acids, **pheromone constituents of the dermestid beetles *Attagenus elongatulus* and *A. megatoma*, was developed. The syntheses are based upon the formation of *trans*-2-tetradecen-5-ynoic acid by reaction of 4-bromo-2-butenoic acid with 1-decynylmagnesium bromide. The enynoic acid undergoes alkali-induced isomerization to yield a mixture of acids from which *cis*-3- and *trans*-3-tetradecen-5-ynoic acids were separated in 31% and 34% yields, respectively. Methyl *trans*-2-tetradecen-5-ynoate was similarly prepared and isomerized to furnish methyl *cis*-3-tetradecen-5-ynoate in 8% yield. Reduction of the tetradecenynoic acids with dicyclohexylborane gave *cis*-3,*cis*-5- and *trans*-3,*cis*-5-tetradecadienoic acids in 4% and 39% yields, respectively. A better yield (49%) in the reduction of *cis*-3-tetradecen-5-ynoic acid to *cis*-3,*cis*-5-tetradecadienoic acid was obtained by hydrogenation over Lindlar's catalyst. Similarly, reduction of methyl *cis*-3-tetradecen-5-ynoate with disiamylborane gave 22% methyl *cis*-3,*cis*-5-tetradecadienoate.**
Lipids 21, 662–665 (1986).

Heslinga et al. (1) reported the synthesis of *trans*-2-alken-5-ynoic acids by coupling 4-bromo-*trans*-2-butenoic acid (4-bromocrotonic acid) and 1-alkyn-1-ylmagnesium bromides. The report noted that when the coupling reaction was heated mixtures of *cis*- and *trans*-3-alken-5-ynoic acids were formed. This last reaction could constitute a convenient synthesis of *cis*- and *trans*-3-tetradecen-5-ynoic acids (c3,a5-14:2 and t3,a5-14:2, respectively), provided that their separation could be effected easily. Reduction of the triple bonds of these enynoic acids would yield *cis*-3,*cis*-5- and *trans*-3,*cis*-5-tetradecadienoic acids (c3,c5-14:2 and t3,c5-14:2, respectively), which are pheromone constituents of the dermestid beetles *Attagenus elongatulus* (Casey) and *A. megatoma* (Fabricius), respectively.

The pheromones of these beetles were shown to contain c3,c5- and t3,c5-14:2 as principal if not sole constituents. The beetles are destructive pests of stored foodstuffs, and investigation has been made of t3,c5-14:2 as bait for monitoring studies (2). Rather complicated syntheses of these acids have been developed, which depend upon gas chromatographic (GC) separation of isomers (3–5). An extraordinarily simple synthesis was accidently discovered (6). Recently, a new synthesis of t3,c5-14:2 with many steps and low yield was reported (7).

We report here syntheses of c3,c5- and t3,c5-14:2 by a short reaction sequence that start with the reactions described by Heslinga et al. (1) and can provide gram quantities of these pheromones.

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EXPERIMENTAL PROCEDURES

Methods. GC analyses were carried out with a Packard Model 428 instrument fitted with a 0.3 × 300-cm glass column packed with 3% EGSS-X or a 50-m 007-CPS-2 (J & S Scientific Inc., Crystal Lake, Illinois) glass capillary column. Also used were a 25-m OV 275 (Chrompack, Bridgewater, New Jersey) and a 100-m SP 2560 (Supelco Inc., Bellefonte, Pennsylvania) glass capillary column.

Liquid chromatographic separations were carried out on 35 × 3.7-cm or 45 × 4.7-cm glass columns (Michel-Miller type, Ace Glass Co., Vineland, New Jersey) packed with ca. 200 or 400 g, respectively, of 30 μm silica or $AgNO_3$ on silica (20:100, w/w). Columns of this type, packed by ordinary tap-fill procedure, tested ca. 2000 plates. When particularly crude samples were separated, a small column (13 × 1 cm, Michel-Miller) was connected as precolumn in series with the larger columns. A 35 × 3.7-cm column of the same type but packed with air-elutriated silver ion-saturated XN1010 exchange resin (8) was also used. A Schoeffel model GM 770 UV detector was employed. The columns were pumped with a Metering Pumps Ltd. type SII/100 unit with two variable stroke no. 3 heads 180 degrees out of phase to minimize flow fluctuations. Samples were applied with a Chromatronix slider valve with a 2-ml loop.

Proton and ^{13}C nuclear magnetic resonance (^1H-NMR and ^{13}C-NMR) spectra were determined on a Bruker spectrometer. Tetramethylsilane was used as internal standard with samples in $DCCl_3$ solution.

Mass spectral data were obtained with a Finnigan GC/MS spectrometer with butane as ionizing gas for chemical ionization (CI) technique.

Infrared (IR) determinations were made with a Perkin-Elmer model 621.

Preparations. 4-Bromocrotonic acid was prepared in 42% yield as described by Heslinga (1). Methyl 4-bromocrotonate was prepared as described by Vogel (9).

Trans-2-tetradecen-5-ynoic acid (t2,a5-14:2) was prepared by Heslinga's method (1) in 22% yield as off-white crystals (98% pure by GC). The structure was confirmed by NMR: ^1H-NMR δ .88 (t, 3H, CH_3CH_2—), δ 1.3–1.5 (m, 12H, —CH_2—), δ 2.17 (m, 2H, —CH_2—C≡C), δ 3.11 (m, 2H, C≡C—CH_2—C=C), δ 6.15 (fine-split d, 1H, J = 15 Hz, = C—*H* trans), δ 7.1 (m, 1H, =C—*H*). ^{13}C-NMR (δ-carbon no.) 14.1-C14; 18.8-C7; 22.2-C4; 22.7-C13; 31.9-C12; 29.0-29.2-C8-11; 74.2 and 84.7-C5,6; 121.9 and 146.3-C2,3; 170.7-C1. The mixture from which t2,a5-14:2 was isolated also contained small amounts of c3,a5- and t3,a5-14:2. The compound was crystallized from pentane/

hexane (PE) in 22% yield. Because the total tetradecenynoic acids is ca. 75% in these mixtures, the whole crude mixture was subjected to isomerization.

The preparation of methyl ester of t2,a5-14:2 from methyl 4-bromocrotonate was carried out by reaction with a molar equivalent of 1-decynylmagnesium bromide per the preparation of the acid just described. By GC analysis, the crude product contained 66% of the expected isomer together with 5% of c3,a5-14:2 and 3% of t3,a5-14:2 (as methyl esters). A peak with a longer retention amounting to ca. 23% was identified as 9,11-eicosadiyne. The crude mixture was separated by vacuum distillation through a 15-cm indented column to yield 13.5 g (bp 25–40 C/0.5 mm), 12.7 g (bp 100–140 C/0.6 mm) and 1.6 g (bp 140–170 C/0.7 mm). The last fraction, which contained 93% of 9,11-eicosadiyne, was recrystallized from acetone to give a pure GC sample of 9,11-eicosadiyne: MS-CI m/e (rel int) 276 (m, 100); ^{13}C-NMR: 14:0-C1 and 20; 22.7-C2 and 19; 31.9-C3 and 18; 29.2, 29.1, 28.9-C4-6 and 17-15; 28.4-C7 and 14; 19.2-C8 and 13; 77.4-C9 and 12; 65.4-C10 and 11.

Tetradecenynoic acid mixtures and their methyl esters were isomerized by various methods to attain high conversions to c3,a5- and t3,a5-14:2. Method 1: the second distillate fraction above (12.7 g of methyl esters of t2,a5- [71%], c3,a5- [13%] and t3,a5-14:2 [6%] as well as 5% 9,11-eicosadiyne) was treated with 70 mg sodium methoxide in 50 ml methanol at room temperature under N_2 for 24 hr. The reaction mixture was poured into 400 ml ice and saline (saturated NaCl solution), and the resultant mixture was extracted with three 100-ml portions (3 × 100 ml) PE. The combined extracts were washed with 2 × 400-ml portions of saline and 1 × 400 ml H_2O and dried over $MgSO_4$. Isolation gave 12.6 g yellow oil that by GC on EGSS-X was estimated to contain 76% of c3,a5- and 16% of t3,a5-14:2 (as methyl esters). About 3% of the t2-isomer remained, while the eicosadiyne was unaffected and served as internal standard. A sample of 0.5 ml of the isomerized mixture was separated on two coupled 35-cm silica columns by elution with tetrahydrofuran/hexane (25:1000, v/v) and monitoring in the UV at 260 nm. The first large peak was found to be pure Me c3,a5-14:2 (i.e., the methyl ester); ^{13}C-NMR: 14.0-C14; 22.7-C13; 31.9-C12; 28.3-29.1-4 peaks-C8-11; 19.6-C7; 76.5 and 96.6-C5 and 6; 112.9 and 132.5-C3 and 4; 35.2-C2; 171.6-C1; 51.8-methoxy. GC on EGSS-X showed one peak eluting at 6.8 min while GC on SP 2560 showed decomposition. Method 2: 4.77 g t2,a5-14:2 was dissolved in 160 ml H_2O/THF/methanol (50:100:10, v/v/v) containing 5 g KOH and allowed to stand in an N_2-atm. Samples were removed and converted to methyl esters with diazomethane to monitor the course of the isomerization by GC on EGSS-X.

A GC program of 160 C for 11 min followed by temperature rise to 190 C at 10 C per min was used to detect the formation of substances with long retention times. The GC results were (reaction time, GC percentages for c3,a5-14:2, 2,4,5-14:3 and t3,a5-14:2 [all as methyl esters; t2,a5-14 was not significant]) 1 hr, 71, 5, 24; 3 hr, 65, 7, 28; 22 hr, 46, 18, 36; and 43 hr, 51, 23, 34, respectively. After 43 hr, the solution was poured into 800 ml saline, acidified to pH 3 and extracted with 3 × 150 ml ether. The combined ether extracts were washed with 600 ml saline, dried over $MgSO_4$ and filtered. The filtrate freed of ether on the rotary evaporator yielded 4.2 g orange oil. The oil was applied in ca. 0.5-g portions to a 35 × 3.7-cm silica and eluted with 2-propanol/acetic acid/hexane (5:1:1000, v/v/v). The last eluted peak yielded 0.98 g t3,a5-14:2. The first two large peaks were collected together to yield 2.51 g of a mixture that consisted of c3,a5-14:2 (77%), 2,4,5-14:3 (19%) and t3,a5-14:2 (2%) by GC of methyl esters. To obtain a higher conversion to 3t,a5-14:2, the last collected fraction was isomerized in a solution of 2.8 g KOH in aqueous methanol (1:1, v/v). After 100 hr, a mixture having 53% c3,a5-14:2, 16% 2,4,5-14:3 and 31% t3,a5-14:2 was found. To this solution was added 20 ml dimethylsulfoxide (DMSO). After seven days, the mixture contained 48% c3,a5- and 37% t3,a5-14:2.

As a result of these isomerization reactions followed by separation on the silica columns, 2.61 g of c3,a5-14:2 and 2.89 g of t3,a5-14:2 (both 98% pure by GC) were obtained. Yields were 31% and 34%, respectively, based on bromocrotonic acid. A typical silica separation is shown in Figure 1.

Reduction of c3,a5-14:2 to c3,c5-14:2 was carried out by adapting the procedure of Svirskaya for the reduction of a conjugated diyne with dicyclohexylborane (10). Thus, 2.61 g (11.8 mmol) c3,a5-14:2 was treated with 35.4 mmol dicyclohexylborane in THF and subjected to acetolysis overnight at room temperature and then at 40–50 C for 1 hr. The crude product (9.0 g), which also contained boron compounds, was separated on a 35-cm silica column using 2-propanol/acetic acid/hexane (5:1:1000, v/v/v). Two large partially resolved peaks (UV detection at 260 nm) were collected. This material was analyzed by GC on the 007-CPS-2 capillary (decomposed on SP 2560): 88% c3,c5- and 6% c3,a5-14:2. Attempts were made to separate this material (1.6 g) on a 1 × 20-cm reversed phase column (Ultrasphere-ODS) using acetonitile (ACN)/H_2O (80:20, v/v) and 35-cm silica columns without success. By using the Ag resin 35-cm column with eluent methanol/ether (5:100, v/v), an excellent separation was achieved to yield 0.11 g (4%) of c3,c5-14:2 of 98% purity by GC.

Another portion (3.28 g) of c3,c5-14:2 was reduced over 1.2 g Lindlar's catalyst in 50 ml PE containing 0.25 g quinoline at 10 C and 760 mm. The H_2 uptake leveled out after 83% of the theoretical uptake. The mixture was filtered through a pad of filter aid. The filter aid was washed with 50 ml PE. The combined PE solutions were washed with 300 ml 2.5 N H_2SO_4 and 300 ml saline and dried over $MgSO_4$. Filtration and solvent evaporation gave 2.81 g of solid. The product was recrystallized at low temperature from PE to give 1.61 g (49%) c3,c5-14:2 (98% pure by GC).

Reduction of t3,a5-14:2 (2.89 g, 13 mmol) with dicyclohexylborane gave 5t3,c5-14:2 (1.16 g, 39%) of 98% purity by GC following separation on the Ag resin column using eluent THF/hexane (1:1.2, v/v).

Reduction of Me c3,a5-14:2 (2.97 g, 12.6 mmol) with 2 mol disiamylborane in the same manner as the above reductions with dicyclohexylborane gave a crude product of 8.58 g. This material was separated by passage through a 45-cm column packed with $AgNO_3$/silica (20:100, w/w) in portions of 0.5 to 1.5 ml using eluent ACN/hexane (4:1000, v/v). A typical separation is shown in Figure 2. There were obtained 0.66 g (22%) of Me c3,c5-14:2, 94% pure by GC on 007-CPS-2.

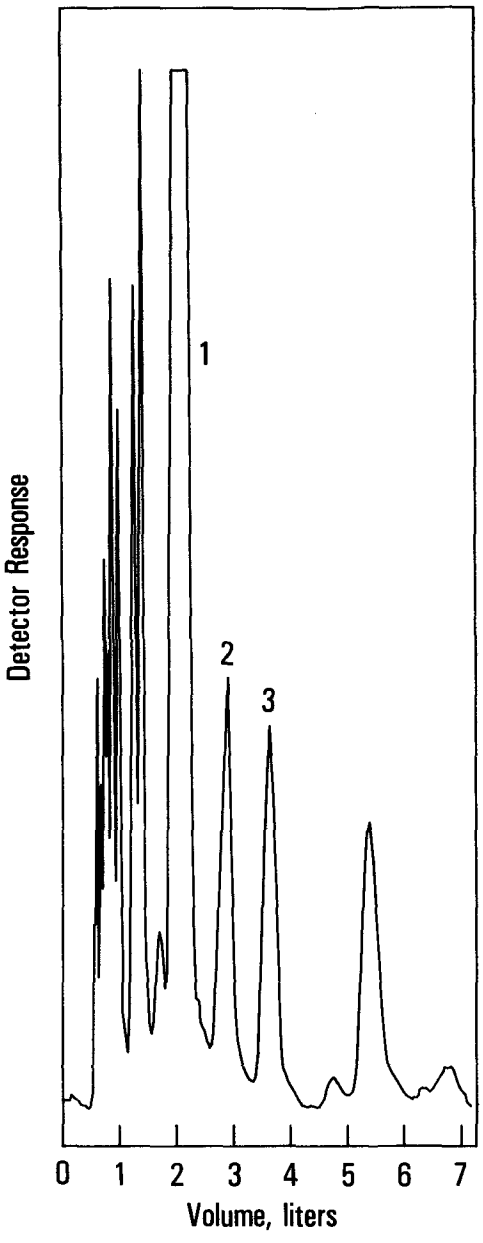

FIG. 1. Separation of *cis* and *trans*-3-tetradecen-5-ynoic acids (c3,a5- and t3,a5-14:2) from alkali-isomerization reaction. Eluent: acetic acid/2-propanol/hexane (1:5:1000, v/v/v). Column: 4.7 × 45–cm silica. Detector: UV at 257 nm. Peaks: (1) 2,4,5-14:3; (2) c3,a5-14:2; (3) t3,a5-14:2.

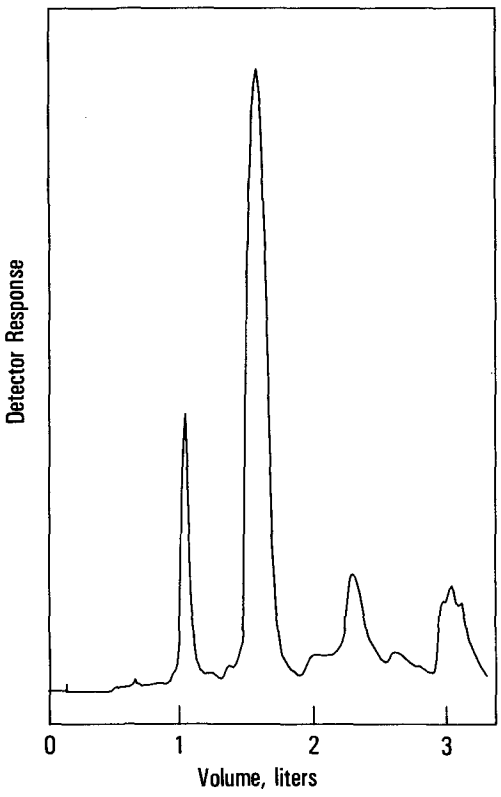

FIG. 2. Purification of methyl ester of c3,c5-14:2 on 4.7 × 45–cm $AgNO_3/SiO_2$ (20:100, w/w). Eluent: acetonitrile/hexane (4:1000, v/v). UV detector at 261 nm. Large peak is methyl ester of c3,c5-14:2.

RESULTS AND DISCUSSION

Preparation of t2,a5-14:2 by Heslinga's method for the dodecenynoic acid analog and subsequent alkali-catalyzed isomerization of the crude reaction product gave a mixture of c3,a5-14:2 (40%), t3,a5-14:2 (32%) and 2,4,5-14:3 (22%) as principal constituents. This mixture could be separated on silica columns using 2-propanol/acetic acid/hexane (5:1:1000, v/v/v). The columns adequately separated c3,a5- and t3,a5-14:2 (98% purity) when a sample size of ca. 0.5 g was injected (Fig. 1).

The yield of t3,a5-14:2 formed by alkaline isomerization was never greater than 37%, even when the sample was isomerized for seven days at room temperature. Although alkali isomerization was not studied extensively, some observations of the effect of varying conditions were made. When KOH-methanol solutions of the tetradecenynoic acids were refluxed for 4 hr, side products with long GC retention times were observed. Treatment of tetradecenynoic acids with KOH-ethylene glycol at 100 C also formed products with long GC retention times. GC/MS-CI tentatively identified these long retention peaks in the latter case as substances having molecular weights consistent with addition of ethylene glycol to methyl tetradecenynoates.

GC analyses of c3,a5- and t3,a5-14:2 were possible on EGSS-X packed columns or 007-CPS-2 capillary columns but not on the OV 275 or SP 2560 capillary columns because of decomposition. We also observed isomerization activity when we attempted to separate mixtures of c3,a5- and t3,a5-14:2 on a silver ion–loaded exchange resin liquid column. A very unusual aspect of the GC and liquid chromatographic behavior of c3,a5- and t3,a5-14:2 is the elution of the methyl ester or acid of the former first (see Fig. 1). This occurred on EGSS-X and 007-CPS-2 packings in GC and on both silica and argentation liquid chromatography. Ordinarily, *cis* isomers are more polar than *trans* isomers in GC or absorption and usually more strongly complex with Ag ion than do *trans* isomers, so the opposite elution order is normally observed.

Reduction of c3,a5- and t3,a5-14:2 was carried out with an excess of dicyclohexylborane. The yields of c3,c5- and t3,c5-14:2 in these reductions were 4% and 39%, respectively, of 98% purity. In the former case, the low yield

TABLE 1

^{13}C-NMR Signals Adjacent to Unsaturation Sites (w values-ppm)

Substance	Carbon position 2	4	7
1. *cis*-3-Tetradecen-5-ynoic acid	35.24		19.58
2. *trans*-3-Tetradecen-5-ynoic acid	37.81		19.41
3. *cis*-3,*cis*-5-Tetradecadienoic acid	32.85		27.69
4. *trans*-3,*cis*-5-Tetradecadienoic acid	37.86		27.78
5. *cis*-3-Tetradecen-5-yn-1-ol[a]	33.61		19.56
6. *trans*-3-Tetradecen-5-yn-1-ol[a]	36.36		19.31
7. Tetrahydropyran derivative of 5[a]	36.06		19.55
8. Tetrahydropyran derivative of 6[a]	38.84		19.37
9. *trans*-2-Tetradecen-5-ynoic acid		22.4	18.84

[a]Unreported syntheses this laboratory.

was partly due to the extensive experimentation in finding a proper separation method. Catalytic reduction of the remaining portion of c3,a5-14:2 gave a 49% yield of c3,c5-14:2. Reduction of the Me c3,a5-14:2 with disiamylborane gave a 22% yield of Me c3,c5-14:2. These reductions were largely stereospecific. For example, in reduction of c3,a5- to c3,c5-14:2, only about 3% of a possible isomer peak was seen on capillary GC together with about 6% of starting material.

Argentation column chromatography was used to raise the purity of c3,c5- and t3,c5-14:2. The acids c3,c5- and t3,c5-14:2 were obtained in 98% purity by chromatography on Ag resin, while Me c3,c5-14:2 was obtained in 94% purity by chromatography on $AgNO_3$/silica (Fig. 2). The ^{13}C-NMR signals of the carbons adjacent to the double bonds (allylic) are of importance in the assignment of the geometrical configuration of the double bonds. Table 1 shows these signals for compounds prepared during this work as well as others of related structure prepared in our laboratory. Bus et al. (11) reported that for conjugated dienes, where the allylic carbons are eight or more carbons from a carboxyl, the signal for an allylic carbon to the *trans* double bond was about 5.2 ppm higher than the signal for an allylic carbon to the *cis* double bond. A similar difference is seen in rows 3 and 4 of Table 1. This difference is not seen with the conjugated enyne systems in Table 1 (compare rows 1 and 2, 5 and 6, and 7 and 8). In these cases, the difference between *trans* and *cis* allylic carbon signals is about one-half the difference noted above, being 2.57, 2.75 and 2.78 for rows 1 and 2, 5 and 6, and 7 and 8 in Table 1, respectively. The most important factor is apparently the effect of the conjugated triple bond.

The synthesis of c3,c5- and t3,c5-14:2 by this method is quite simple chemically, with only three steps: coupling, isomerization and reduction. The method's feasibility for the preparation of gram quantities of the pheromones has been demonstrated using only ordinary liquid chromatographic equipment.

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COMMUNICATIONS

Pyrophosphatidic Acid in Mushrooms

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The distribution of pyrophosphatidic acid in mushrooms was investigated. Total lipids of mushrooms were extracted from their fruit body, and the pyrophosphatidic acid fraction was isolated by DEAE-silica gel column and silicic acid column chromatography. The presence of pyrophosphatidic acid was tested by thin layer chromatography. Of the 43 species of mushrooms examined, the acid was found in 30. The pyrophosphatidic acid was identified by chromatography, IR spectroscopy and quantitative degradation with aqueous pyridine to produce phosphatidic acid. Chemically synthesized pyrophosphatidic acid was used as standard.

Lipids 21, 666-668 (1986).

Pyrophosphatidic acid [*p,p'-bis*-(1,2-diacyl-*sn*-glycero-3-)-pyrophosphate] was first isolated from the yeast *Cryptococcus neoformans* CBS-132. Its fatty acid composition was found to be similar to that of phosphatidic acid isolated from the same source (1) with a positional distribution resembling that of other glycerophosphatides (2). In vivo incorporation of ^{32}P-labeled orthophosphate into the pyrophosphatidic acid of *Cr. neoformans* during cell growth showed pyrophosphatidic acid to be metabolically active (3). The results suggested that the pyrophosphatidic acid, through phosphatidic acid, plays an important role in glycerophospholipid metabolism.

Pyrophosphatidic acid was found exclusively in a limited number of yeast species, but not in other organisms (animals, plants and microorganisms) that have been investigated so far (4). All yeast species containing a detectable amount of pyrophosphatidic acid belong to the asporogenous and ballistosporogenous yeasts.

The present report describes the occurrence of pyrophosphatidic acid in basidiomyceteous mushrooms phyletically akin to yeast.

MATERIALS AND METHODS

Mushroom strains. Forty-three strains of mushrooms (listed in Table 1) were investigated. *Lentinus edodes* and *Flammulina velutipus* were obtained from a commercial source and others from various mountain areas in Japan. The mushrooms were preserved in a freezer at −20 C until use.

Lipid extraction. The fruit bodies (100 g) of the mushrooms were homogenized in a Waring blender in 200 ml of hot isopropanol according to Kates (5). The homogenate was filtered on a Buchner funnel. The residue was suspended in 200 ml of chloroform/methanol (2:1, v/v), stirred with a magnetic stirrer for 30 min at room temperature under an atmosphere of nitrogen and then filtered. Stirring and filtering were repeated three times. The combined filtrates were concentrated in vacuo, the residual lipids were taken up in 100 ml of chloroform/methanol (2:1, v/v), and the solution was washed several times with 20-ml portions of 0.9% sodium chloride according to Folch (6). The lipid extract was kept in a freezer at −20 C.

Fractionation of acidic lipids from total lipid extracts. The extracted lipids (about 100 mg) in 1 ml of chloroform/methanol/water (3:6:1, v/v/v) were applied onto a column (6 × 80 mm) of DEAE-silica gel (DEAE-Iatrobeads 6RS-2421A, Iatron Co., Tokyo, Japan). Following elution with 10 ml of chloroform/methanol/water (3:6:1, v/v/v) to remove phosphatidylcholine, phosphatidylethanolamine and other components, the acidic lipids were eluted with chloroform/methanol/0.8 M sodium acetate (3:6:1, v/v/v).

Isolation of the pyrophosphatidic acid/cardiolipin mixture. The acidic lipids (about 10 mg) in chloroform were chromatographed on a column (6 × 80 mm) of silica gel (Iatrobeads 6RS-80100, Iatron) using chloroform/methanol/conc. ammonium hydroxide (65:35:5, v/v/v) as solvent, and 2-ml fractions were collected. In the second and third fractions, the pyrophosphatidic acid/cardiolipin mixture was eluted. Phosphatidylinositol and phosphatidylserine were eluted in the fourth fraction (containing no pyrophosphatidic acid) and phosphatidic acid in the fifth.

Thin layer chromatography (TLC). The separation of phospholipids was performed by two-dimensional TLC on silica gel 60 plates (Art. 5626, 100 × 100 mm, Merck, Darmstadt, Federal Republic of Germany). Chloroform/methanol/conc. ammonium hydroxide (65:35:5, v/v/v) was used to develop the plates in the first direction and chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v) was used in the second direction. Phospholipids were detected after spraying the chromatogram with Dittmer reagent (7). To detect the total polar lipids, the chromatogram was charred at 140 C for 15 min.

Chemical synthesis of pyrophosphatidic acid. Pyrophosphatidic acid was synthesized from egg phosphatidylcholine as described previously (1).

Measurement of IR spectra. The IR spectra of a thin film of the acid placed on an AgCl plate were measured using a grating infrared spectrophotometer (JASCO IR-A-1) (1).

Hydrolysis of the pyrophosphate bond of pyrophosphatidic acid. The pyrophosphatidic acid isolated from *L. edodes* was hydrolyzed with 1% H_2O-pyridine at 100 C overnight (1).

Chemicals. Chloroform and methanol were of reagent grade and were freshly distilled before use. Other organic and inorganic reagents, of the highest analytical grade commercially available, were used without further purification.

RESULTS AND DISCUSSION

A typical thin layer chromatogram of the polar lipids of *L. edodes* is shown in Figure 1. Pyrophosphatidic acid can

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TABLE 1

Presence of Pyrophosphatidic Acid in Various Strains of Mushrooms

Mushroom strains	Pyrophosphatidic acid
Basidiomycota	
Polyporales	
Corticiaceae	
Stereum fasciatum	+
Thelephoraceae	
Sarcodon imbricatus	+
Hydnaceae	
Hydnum repandum	+
Creolophus spathulates	+
Polyporaceae	
Laetiporus sulphureus	+
L. versisporus	+
Ischnoderma resinosum	−
Fomitopsis cytisina	−
Polyporellus picipes	+
Favolus arcularius	−
Grifola frondosa	−
Canoderma lucidum	+
Agaricales	
Clavariaceae	
Ramaria formosa	+
Cantharellaceae	
Cantharellus cibarius	+
C. minor	+
C. cinnabarinus	+
Neurophyllum fujisanensis	+
Boletaceae	
Suillus bovinus	+
Beletus pulverulentus	+
Hygrophoraceae	
Hygrophorus erubescens	+
Tricholomataceae	
Laccaria laccata var. *proxima*	+
Clitocybe clavipes	+
Tricholoma matsutake	+
Pleurotus ostreatus	−
Lentinus lepideus	+
L. edodes	+
Flammulina velutipes	−
Mycena haematopus	+
M. crocata	+
Agaricaceae	
Macrolepiota procera	+
Agaricus arvensis var. *fulvus*	+
Coprinaceae	
Psathyrella velutina	−
P. candolleana	−
Strophariaceae	
Pholiota nameko	+
Amanitaceae	
Amanita vaginata	−
A. virosa	+
Sclerodermatales	
Sclerodermataceae	
Pisolithus tinctorius	+
Melanogastrales	
Rhizopogonaceae	
Rhizopogon rubescens	−
Lycoperdales	
Lycoperdaceae	
Calvatia craniformis	−
Lycoperdon pyriforme	−
Phallales	
Phallaceae	
Phallus impudicus	+
Ascomycota	
Pezizales	
Pezizaceae	
Peziza vesiculosa	−
Helvellaceae	
Morchella esculenta	+

−, Absence of pyrophosphatidic acid (below 1 μg/100 mg total lipid); +, presence of pyrophosphatidic acid.

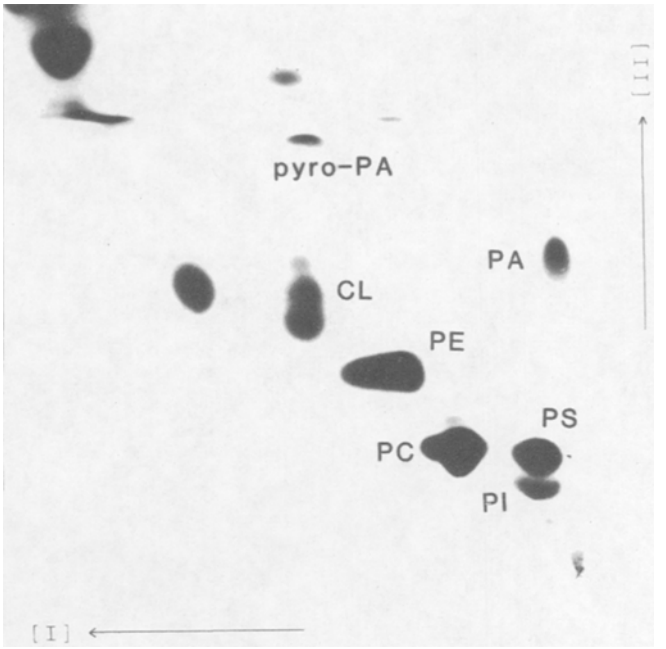

FIG. 1. Thin layer chromatogram of the total lipid extract of *L. edodes*. Chromatographic conditions are described in Materials and Methods. PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; PA, phosphatidic acid; pyro-PA, pyrophosphatidic acid.

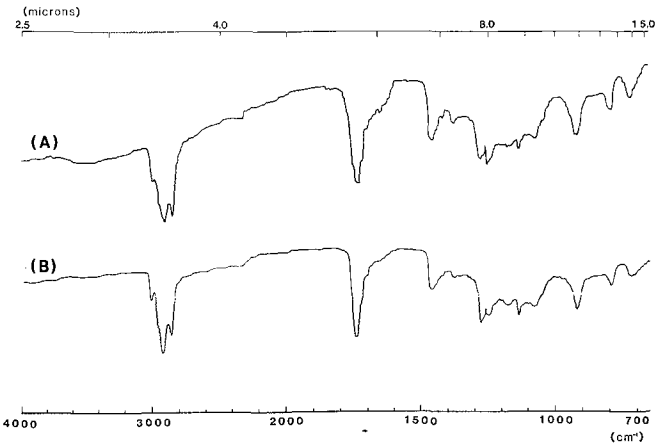

FIG. 2. IR spectra of pyrophosphatidic acid as a thin film placed on an AgCl plate. A, pyrophosphatidic acid (synthetic), B, pyrophosphatidic acid *(L. edodes)*.

be clearly distinguished from other lipids such as cardiolipin and phosphatidic acid. Pyrophosphatidic acid showed purple-blue staining different from that of the common phospholipids, which are stained blue with the Dittmer reagent. As little as 0.3 μg of pyrophosphatidic acid could be detected with this reagent.

The IR spectrum of pyrophosphatidic acid of *L. edodes* is shown in Figure 2. The pyrophosphatidic acid of *L. edodes* was isolated by TLC. The spectrum of this acid was completely identical to that of synthetic pyrophosphatidic acid; the absorption at 930 cm^{-1} indicated the presence of the pyrophosphate bond.

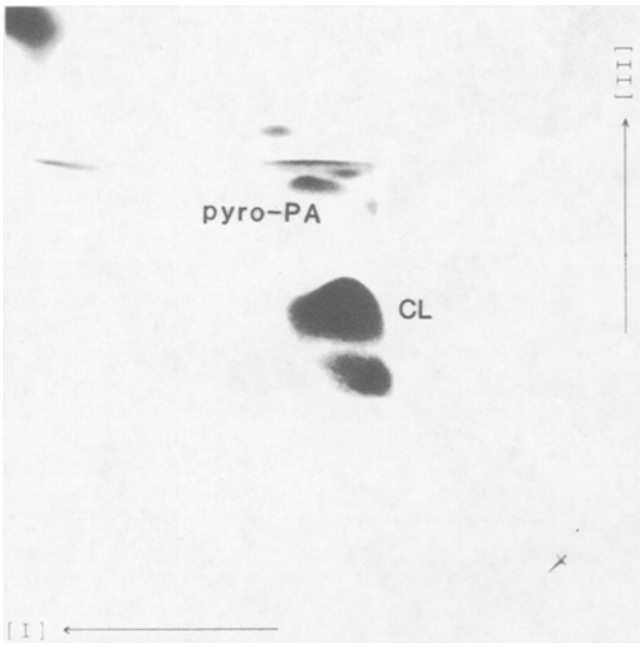

FIG. 3. Thin layer chromatogram of the fraction containing the pyrophosphatidic acid and cardiolipin of *R. formosa*. Chromatographic conditions and abbreviations are the same as in Fig. 1.

The pyrophosphate bond can be hydrolyzed with aqueous pyridine (8). The pyrophosphatidic acid of *L. edodes* was treated in 1% H_2O-pyridine at 100 C overnight. This acid was completely degraded to phosphatidic acid and no other reaction products could be detected. Using this method, the pyrophosphatidic acid of mushrooms was identified. A detectable amount of pyrophosphatidic acid was found only in *L. edodes*. For further identification of pyrophosphatidic acid, each total lipid extract was fractionated into acidic lipids on a column of DEAE-Iatrobeads.

The fractionation of the total lipid extract into acidic lipid resulted in the removal of phosphatidylethanolamine, phosphatidylcholine and other common lipids. The acidic lipid fraction in which the amount of pyrophosphatidic acid was below the limit of detection was fractionated into a mixture of pyrophosphatidic acid and cardiolipin by silicic acid column chromatography.

A two-dimensional thin layer chromatogram of the fraction of *Ramaria formosa*, which was obtained by silica gel column chromatography, is shown in Figure 3. In the case of *R. formosa*, the pyrophosphatidic acid was not detected at the level of the total lipid or even in the acidic lipid fraction, but it was detected in the silicic acid column fraction. Using ion exchange column and silicic acid column fractionations, pyrophosphatidic acid of mushrooms could be detected, provided it comprised at least 10^{-3}% of the total lipid content (i.e., about 1 μg in 100 mg of total lipid). The distribution of pyrophosphatidic acid in various species of mushrooms is shown in Table 1. Pyrophosphate was found in 30 species of mushroom strains examined.

A phylogenetic correlation could not be clearly established as pyrophosphatidic acid was distributed widely in Basidiomycota. Even in the same family, such as Polyporaceae and Tricholomataceae (Table 1), there were mushroom species both with and without pyrophosphatidic acid. It was reported previously that yeast strains in which the pyrophosphatidic acid was detected belonged to Basidiomycota, with the exception of *Kloechera apiculata* KK-3 which belonged to Ascomycota (4). In this study on mushrooms, we examined only two strains belonging to Ascomycota and found the presence of pyrophosphatidic acid in *Morchella esculenta*. The presence of pyrophosphatidic acid should be investigated in a large variety of Ascomycota mushroom species. It is assumed that pyrophosphatidic acid is widely distributed in Ascomycota.

Using the same procedure, involving column and thin layer chromatography, on lipid extracts of spinach, *chlorella*, rabbit kidney, dog liver, cow brain, hen yolk and commercially available packed baker's yeast, we found no detectable amount of pyrophosphatidic acid. Though pyrophosphatidic acid was found in yeast and mushrooms, its presence could not be confirmed in other organisms.

Pullarkat et al. (9) reported that pyrophosphatidic acid may be an immediate precursor of phosphatidylserine in rat brain. Thus, we cannot entirely eliminate the possibility that pyrophosphatidic acid occurs in animals and plants at very low levels which would not be detected by our methods.

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Fatty Acid Distribution in the Phospholipids of *Francisella tularensis*

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***Francisella tularensis,* LVS (live vaccine strain) grown in a chemically defined medium was found to have a lipid content of 21% by dry weight. The two major phospholipids were identified as phosphatidylethanolamine (PE; 76%) and phosphatidylglycerol (PG; 24%) by thin layer chromatographic analysis, staining characteristics and quantitative chemical analyses of fatty acid, phosphate and glycerol constituents. PE contained a high proportion of 24:0 fatty acid, with lesser amounts of 24:1, 22:0 and 10:0. The major fatty acids of PG were 18:1 and 22:0. Hydroxy fatty acids, which are prominent components of *F. tularensis,* were conspicuously lacking in these phospholipids; it is therefore concluded that hydroxy fatty acids are constituents of other structures of the organism.**
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The gram-negative bacterium *Francisella tularensis* is the causative agent of tularemia in humans and animals. Morphologically, the organism is bounded by a double-membraned, lipid-containing cell wall structure (1) in which lipopolysaccharide (2) is present. Often found encasing the bacterium is a labile capsule (1,3) that may contribute to virulence (1). Both the cell wall and capsule are rich in lipid (1). Cellular antigens of importance to the host immune response are to a large extent embedded in lipid, as suggested by studies on material extractable from *F. tularensis* by a mixture of ether and water (4-6).

Owing perhaps to its high pathogenicity and difficulties in culturing, *F. tularensis* has not been extensively investigated with respect to its structural constituents. Perhaps best characterized are the fatty acids of the organism, which comprise nonbranched saturated, monounsaturated and hydroxy species (1,7-9). Despite indications that cellular components are rich in lipid (1), information is lacking on the types of lipids present or their fatty acid compositions. We have undertaken the present study to shed light on these points.

MATERIALS AND METHODS

F. tularensis, LVS (live vaccine strain) was cultivated in a chemically defined medium (10) sterilized by filtration through a 0.45 μm Millipore filter. Flasks (6-l capacity) containing 2 l of medium were seeded with a 1% inoculum from an overnight grown culture. Growth was carried out for 24 hr at 37 C in a gyratory shaker (Psychrotherm, New Brunswick Scientific, New Brunswick, New Jersey) set at 150 rpm in an atmosphere of 5% CO_2/95% air. Cells were pelleted using a Beckman model J-21C centrifuge (20 min at 10,000 × g); cell pellets were stored frozen at −80 C until lipid extraction.

Frozen cell pellets were extracted according to the method of Bligh and Dyer (11). Resultant lipid extracts were concentrated under a nitrogen stream and analyzed by two-dimensional thin layer chromatography (TLC) on Silica Gel H plates developed in chloroform/methanol/28% ammonia (65:35:5, v/v/v) in the first dimension and chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v) in the second dimension (12). Lipids were detected using spray reagents consisting of one of the following: 50% sulfuric acid followed by heat charring, phosphorus detection reagent (13) or ninhydrin (14). Preparative isolation of phospholipids was performed on Silica Gel H plates run in chloroform/methanol/28% ammonia (65:35:5, v/v/v). Lipids were visualized as white bands without the use of detection reagents; elution of lipids was performed using a mixture of chloroform/methanol (1:1, v/v). Analytical determinations of fatty acid, phosphorus and glycerol were performed according to the procedures of Chen (15), Rouser et al. (12) and Renkonen (16), respectively. Fatty acid methyl esters were prepared from isolated phospholipids by hydrolysis with 5% methanolic HCl for 3 hr at 80 C in sealed tubes; trifluoroacetylation (to esterify hydroxy fatty acid methyl esters) was performed according to Jantzen et al. (17). Gas chromatography was performed on a Hewlett-Packard 5890 instrument equipped with a 25-m cross-linked 5% phenylmethyl silicone capillary column run on a temperature gradient from 180 C to 280 C. Verification of the identities of long chain and trifluoroacetylated hydroxy fatty acids was done using combined gas chromatography/mass spectrometry (7).

NMR spectra of samples dissolved in deuterochloroform were recorded at 22 C on a Varian XL-200 NMR spectrometer. Chemical shifts are given as ppm (δ) relative to tetramethylsilane as internal standard.

RESULTS AND DISCUSSION

Cells of *F. tularensis* were found to contain 21% lipid relative to total dry cell weight. This is a high value for bacteria in general and supports lipid-rich analyses previously reported for isolated cell wall and capsule of *F. tularensis* (1). As shown in Figure 1, two major lipids were detected, both of which stained positively for phosphate (13). The lipids were identified as phosphatidylglycerol (PG; 24%) and phosphatidylethanolamine (PE; 76%) by cochromatography with authentic phospholipids isolated from *Escherichia coli.* PE stained positively with ninhydrin (14). Following preparative isolation, both phospholipids were found to have the expected molar ratios of fatty acid/glycerol/phosphorus, as determined using the respective procedures of Chen (15), Renkonen (16) and Rouser et al. (12). No cardiolipin or phosphatidylserine was found. Depending on the rapidity of lipid extraction, varying amounts of minor phospholipids,

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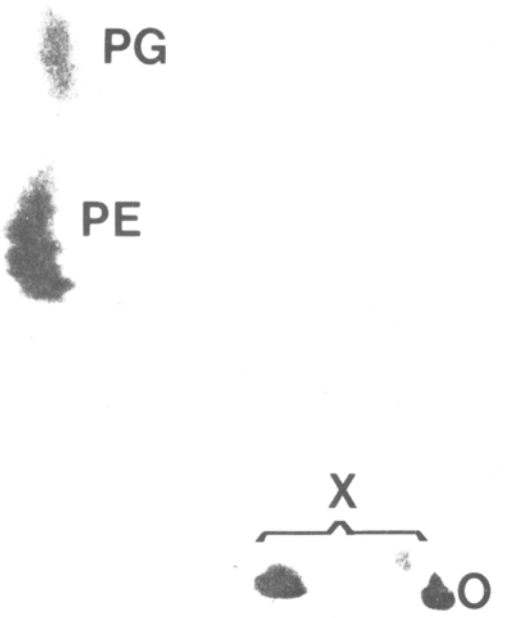

FIG. 1. Two-dimensional thin layer chromatogram of lipids from *F. tularensis*. Total lipid extract, applied at the origin (O), was chromatographed in the first (vertical) dimension in chloroform/methanol/28% ammonia (65:35:5, v/v/v) and in the second (horizontal) dimension in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v). Lipids were visualized by sulfuric acid spray and charring. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; X, unknown, probably nonlipid components.

tentatively identified as lysoPE and lysoPG, were observed. The occurrence of these minor phospholipids could be minimized by limiting the lipid extraction procedure to 6 hr or less.

The material labeled X in Figure 1 was found to vary in amount from extract to extract. By preparative isolation and elution from silica gel with chloroform/methanol/water (1:1:1, v/v/v), its proportion of total Bligh and Dyer-extractable material was never found to be more than 5% by weight. Its nature is uncertain.

It was found that authentic reference samples phosphatidylmonomethylethanolamine (PME) and PE (both obtained from Sigma Chemical Co., St. Louis, Missouri) migrated with identical Rf values in our two-dimensional TLC system. To exclude the possibility that PME may be a component of *F. tularensis*, the ninhydrin-positive phospholipid from *F. tularensis*, preparatively isolated as described above, was subjected to nuclear magnetic resonance (NMR) spectroscopy. While the NMR spectrum of authentic PME showed a clear three-proton singlet at 2.65 ppm ($N\text{-}CH_3$), no such signal was evident in the NMR spectrum of the ninhydrin-positive phospholipid from *F. tularensis*. Rather, this latter NMR spectrum was virtually identical to that obtained from authentic PE.

Fatty acid compositions, given in Table 1, differed strikingly between the two phospholipids. PE contained large amounts of 24:0, with lesser amounts of 24:1, 22:0 and 10:0, while the major fatty acid constituents of PG were 18:1 and 22:0.

Of significant interest is the observation that neither PE nor PG contained high proportions of hydroxy fatty acids. Nor did the percentage of hydroxy fatty acids exceed 2% of the fatty acids present in the total Bligh and Dyer extract. Hydroxy fatty acids, particularly the OH-18:0 species, have been shown to be major constituents of *F. tularensis* (7,8) and were found in the present study to be present in considerable amounts in whole cells (Table 1). Our finding that hydroxy fatty acids are lacking among the major phospholipid species of *F. tularensis* suggests that they are components of other structures, possibly the lipopolysaccharide, within the organism.

To date, *F. tularensis* and the animal pathogen *F. novicida* comprise the recognizable members of the genus *Francisella*. Despite indications of slight serological relatedness among *Francisella*, *Brucella* and *Yersinia* (18,19), there is little similarity in percentage GC base compositions (20). Moreover, DNA homology studies

TABLE 1

Fatty Acid Composition of *F. tularensis* and Its Major Phospholipids[a]

Fatty acid	Total cells	Phosphatidylethanolamine	Phosphatidylglycerol
10:0	7.5 ± 5.8	8.2 ± 6.4	1.0 ± 0.5
h-10:0	0.4 ± 0.3	0.7 ± 0.4	0.1 ± 0.1
14:0	2.9 ± 0.4	3.5 ± 0.9	4.5 ± 0.6
16:0	9.4 ± 0.5	2.9 ± 1.2	7.9 ± 0.6
h-16:0	2.9 ± 0.3	0.9 ± 0.8	1.2 ± 0.8
18:1	12.2 ± 1.1	2.8 ± 1.5	26.6 ± 0.5
18:0	3.8 ± 0.8	3.5 ± 0.8	7.5 ± 0.5
h-18:0	10.4 ± 2.3	0.7 ± 0.6	1.9 ± 0.2
20:1	1.0 ± 0.2	0.4 ± 0.2	3.8 ± 0.1
20:0	1.9 ± 0.2	1.2 ± 0.4	6.9 ± 0.3
22:1	1.8 ± 0.2	0.6 ± 0.4	7.0 ± 0.6
22:0	9.0 ± 0.7	10.2 ± 1.5	15.7 ± 0.9
24:1	11.7 ± 1.6	15.4 ± 3.4	9.2 ± 0.8
24:0	22.2 ± 1.8	43.0 ± 7.3	5.9 ± 0.2
26:1	0.7 ± 0.1	1.2 ± 0.5	0.2 ± 0.1
26:0	2.2 ± 0.2	4.8 ± 1.1	0.6 ± 0.2

[a]Results given as percentage composition ± standard deviation.

suggest little relatedness between *Francisella* and the genera *Pasteurella, Yersinia* or *Escherichia* (21). The apparent richness in cell wall lipid (1) and distinctive fatty acid composition (7) further serve to differentiate *Francisella* from other small, gram-negative, nonsporulating genera.

The phospholipids PE and PG are common constituents of a variety of gram-negative bacteria (22,23). In consideration of studies suggesting minor serological relationships between *Francisella, Yersinia* (18) and *Brucella* (19), it is of interest but probably not of phylogenetic significance that the phospholipid composition of *F. tularensis* shows some similarity to that of *Y. enterolytica* (24), in that PE and PG are major components. As a point of contrast, however, the phospholipid compositions of *F. tularensis* (present work) and at least one strain of *Brucella, B. melitensis* (25), show considerable differences.

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Growth Hormone and Liver Mitochondria: Effects on Phospholipid Composition and Fatty Acyl Distribution

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Effects of growth hormone on phospholipid composition and fatty acyl distribution were studied in liver mitochondria of hypophysectomized rats. After hypophysectomy, only cardiolipin showed a 25% decrease. Its fatty acyl distribution, which consisted mainly of linoleic acid (55–60%) and oleic acid (20%), was unchanged. In phosphatidylcholine and phosphatidylethanolamine fractions the contents of docosahexaenoic and arachidonic acids were decreased with a concomitant increase in linoleic acid content. These changes could be accounted for by small but significant decreases in the activities of Δ^9-desaturase (sucrose-induced), Δ^5-desaturase and mitochondrial elongation enzymes. The activities of Δ^6-desaturase, NADH cytochrome b_5 ferri-reductase, cytochrome b_5, NADH cytochrome c reductase and microsomal elongation enzymes remained virtually unchanged. Injection of bovine growth hormone daily for seven days restored cardiolipin and fatty acyl distribution and the enzyme activities. From these and other results, we conclude that growth hormone-dependent increase of respiratory activity of liver mitochondria may be partly mediated by the hormonal effects on membrane lipid distribution.
Lipids 21, 677-683 (1986).

Removal of the pituitary gland (hypophysectomy) in rats has been shown to modify a number of properties of liver mitochondria (1–4). These include decreases in the rate of state 3 respiration (2,3) and fatty acyl chain length and unsaturation (4). Also, the transition temperature in the Arrhenius plot of state 3 respiration was increased (4). Since transition temperature and membrane fluidity are inversely related, it was concluded that fluidity of the mitochondrial membrane was decreased after hypophysectomy. Among the hormones tested, injection of growth hormone daily for seven days to hypophysectomized rats was the most effective in restoring fatty acyl composition and transition temperature (4). Furthermore, time course studies after one injection of growth hormone showed that the hormone effects on fatty acyl composition precede its effects on respiration and that a strong association exists between the two parameters (5). Rates of respiration supported by polyunsaturated fatty acylcarnitines, in contrast to rates observed with palmitoylcarnitine or oleoylcarnitine, were slightly lower in hypophysectomized rats than in normal rats, but were partially restored by growth hormone. The effects were most pronounced with docosahexaenoylcarnitine, and growth hormone treatment of hypophysectomized rats caused a threefold increase in the activity of 2,4-dienoyl-CoA reductase in mitochondria (6).

In addition to fatty acyl composition, physical and biological properties of membranes depend on cholesterol content and composition of phospholipid head groups (7), but cholesterol content of the inner mitochondrial membrane is low (8). We have now extended the studies of the effects of hypophysectomy and subsequent growth hormone injection to include phospholipid composition and fatty acyl distribution of the three major phospholipids (8). The activities of Δ^9- (control and sucrose-induced), Δ^6- and Δ^5-desaturases as well as those of mitochondrial and microsomal elongation have also been measured.

METHODS AND MATERIALS

Animals. Male Sprague-Dawley rats (controls, 200–300 g; hypophysectomized, 100–150 g) were maintained on Purina Laboratory Chow as described (1–4). For sucrose induction of Δ^9-desaturase activity, animals were starved for 48 hr and refed with 20% (w/v) sucrose in 0.45% sodium chloride solution for 24 hr. Bovine growth hormone (NIH, BG-17) or an equivalent volume of saline was injected subcutaneously to hypophysectomized rats daily (0.1 IU/100 g body weight); rats were weighed at the same time of day.

Preparation of mitochondria, inner mitochondrial membranes and microsomes. Rats were killed, and livers were removed into ice-cold isolation medium containing 0.25 M sucrose, 1 mM EDTA, 30 mM Tris-HCl (pH 7.4) and 0.5% bovine serum albumin. Mitochondria were isolated from a 10% homogenate and washed as described (3,4). Inner mitochondrial membranes were prepared by digitonin and lubrol treatment as described (4). The microsomal fraction was prepared from a 10% homogenate of liver in 0.25 M sucrose, washed and stored as described (9).

Estimation of phospholipids and fatty acids. Isolation of phospholipids and fatty acids was carried out by established methods of this laboratory, as described previously (4,9–11). Freshly prepared mitochondria and microsomes were extracted twice with chloroform/methanol (2:1, v/v) (5 ml solvent/mg protein) under N_2 as described previously (4,9,10). The extract was filtered, and the pooled filtrate was washed with 0.2 vol of 0.9% NaCl according to the method of Folch et al. (12). The lower phase was collected and evaporated. The residue was dissolved in chloroform and fractionated chromatographically into neutral and polar phospholipids on a column of silicic acid (100 mesh, 1 g/30 mg total lipids). Neutral lipids were first eluted with 20 ml chloroform and phospholipids with 20 ml methanol. Phospholipids were separated by thin layer chromatography (TLC) on silica gel either by single migration using a solvent mixture of chloroform/methanol/water (72:25:4, v/v/v) or by two-dimensional migration using first chloroform/methanol/water/28% ammonia (130:70:8:0.5, v/v/v/v) followed by chloroform/acetone/methanol/acetic acid/water (100:40:20:20:10, v/v/v/v/v) according to Parson and Patton (13). The chromatographic plates were exposed to iodine vapor. Individual phospholipids were identified by using phospholipid standards. Spots were scraped off and eluted

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by washing with chloroform/methanol (2:1, v/v). Quantitative analysis of phospholipid fractions was carried out by phosphorous determination according to Bartlett's method (14). Fatty acids were analyzed by gas liquid chromatography (GLC) after conversion to methyl esters by refluxing for 2 hr with 6 ml of a mixture of 1 ml concentrated H_2SO_4, 61.6 ml methanol and 123 ml benzene. Identification of methyl esters was carried out on a Hewlett-Packard 5710A gas chromatograph as previously described (4,9,10). Cholesterol content of total lipids was measured after saponification and methanolysis by GLC on a 10% Silar 10C column at 170–200 C (flame ionization detector, 250 C). The unsaturation index of fatty acids was calculated as the sum of the mol% fraction multiplied by the number of double bonds in each acid.

Assay of fatty acid desaturation and elongation activities. Incubations for the assay of terminal desaturase activities (Δ^9-, Δ^5- and Δ^6-desaturases) were carried out at 30 C for 10 min in a total volume of 2 ml containing 10 μmol $MgCl_2$, 0.3 mmol KCl, 0.5 mmol sucrose, 3 μmol glutathione, 10 μmol ATP, 0.6 μmol CoA, 2.5 μmol NADH, 0.1 mmol potassium phosphate (pH 7.0), 80 μmol KF, 5 mg microsomal protein and 120 nmol [U-^{14}C]-palmitic acid (Δ^9-desaturase) or 90 nmol[1-^{14}C]linoleic acid (Δ^6-desaturase) or 100 nmol [1-^{14}C]eicosa-8,11,14-trienoic acid (Δ^5-desaturase). The reaction was terminated by adding alcoholic KOH (25% 10 M KOH and 7.5% ethanol). The reaction mixture was hydrolyzed by incubating at 60–70 C under N_2 for 1 hr and titrated to pH 4.0. Fatty acids were extracted with petroleum ether and methylated using boron trifluoride in methanol (15). The fatty acid methyl esters were separated by argentation TLC using the solvent hexane/benzene (50:50, v/v) for Δ^9-desaturase and toluene/acetone (95:5, v/v) for Δ^6- and Δ^5-desaturase activities (16). Radioactivity was measured in a Packard Model 3310 liquid scintillation spectrometer. Radioactivity recovery after completing the procedure was about 80%. Under the assay conditions, the formation of palmitoleic (Δ^9-), linolenic (Δ^6-) and arachidonic (Δ^5-) acids was proportional to time (up to 15 min) and protein concentration.

NADH cytochrome b_5-reductase was assayed spectrophotometrically by measuring the rate of oxidation of NADH at 25 C as described (16). The reaction mixture contained 60 nmol NADH, 140 nmol potassium ferricyanide and 20 μg microsomal protein in a final volume of 0.54 ml of 0.05 M Tris-acetate (pH 8.1) and 1 mM EDTA.

NADH cytochrome c-reductase was assayed spectrophotometrically at 550 nm by following the reduction of cytochrome c at 25 C (16). The reaction mixture contained 40 nmol cytochrome c, 60 nmol NADH and 20 μg microsomal protein in a final volume of 0.54 ml of 0.05 M Tris-acetate (pH 8.1) and 1 mM EDTA.

The content of cytochrome b_5 was calculated from the difference spectrum between NADH-reduced and oxidized samples. The mixture in a total volume of 3 ml contained 60 μmol phosphate buffer (pH 7.4) and 1.5 mg microsomal protein with and without (oxidized) 0.3 μmol NADH. An extinction coefficient of 185 cm^{-1} mM^{-1} was used for calculation.

The fatty acid elongation activity of microsomal fraction was measured by the incorporation of [1,3-^{14}C] malonyl CoA into microsomal fatty acids as described (17). The reaction mixture in a total volume of 1.25 ml contained 25 μmol [1,3-^{14}C]malonyl-CoA, 215 nmol rotenone, 0.625 μmol ATP, 1.25 μmol NADH or NADPH and 125 μmol Tris-HCl (pH 7.4). The mixture was preincubated for 5 min at 37 C and the reaction was started by adding 100 μg microsomal protein. At the end of three minutes, the reaction was stopped by adding 0.5 ml of 15% methanolic-KOH. Isolation, methylation and identification of fatty acids were as described above. Mitochondrial elongation activity was assayed by a similar procedure, except that incubation was carried out under N_2 with 100 μg mitochondrial protein, 56 nmol [1-^{14}C]acetyl CoA and 2.5 μmol NADPH instead of malonyl CoA and NADH.

The rate of reoxidation of cytochrome b_5 was measured as described by Keyes et al. (18). All spectrophotometric procedures were done in an Aminco DW-2 Recording Spectrophotometer. Protein was measured by the method of Lowry et al. (19) using bovine serum albumin as the standard.

Materials. All biochemicals were from Sigma Chemical Co. (St. Louis, Missouri). [U-^{14}C]palmitic acid (800 mCi/mmol), [1-^{14}C]linoleic acid (57 mCi/mmol), [1,3-^{14}C]malonyl CoA (15 mCi/mmol) and [1-^{14}C]acetyl CoA (45 mCi/mmol) were purchased from New England Nuclear (Boston, Massachusetts).

RESULTS

Effects of growth hormone on phospholipid composition and fatty acid distribution. Total phospholipid (mg/mg protein) or cholesterol (g/g fatty acyl methyl ester) did not significantly change after hypophysectomy or growth hormone administration daily for seven days (Table 1). Phospholipid composition of whole mitochondria and inner membrane fraction, as shown in Table 1, is similar to the reported composition (20). Among the phospholipids (Table 1), only the cardiolipin fraction showed a significant decrease ($p < 0.05$, 25% of control) in whole mitochondria and inner membrane fraction after hypophysectomy. The loss in cardiolipin fraction appears to be made up by a small increase in phosphatidylcholine content. Growth hormone administration increased the cardiolipin fraction to more than the control level.

Fatty acid composition of the three major phospholipids of whole mitochondria and inner membrane are shown in Tables 2–4. In general, the pattern of distribution (unsaturation and average chain length) in the three phospholipid fractions is similar to the reported distribution (21–23). In the phosphatidylcholine fraction, there appears to be an almost even distribution of saturated and unsaturated fatty acids, as well as of C_{18} and C_{20} fatty acids. After hypophysectomy, arachidonic (20:4) and docosahexaenoic (22:6) acids decreased significantly ($p < 0.001$ and $p < 0.002$, respectively) but the linoleic acid (18:2) content was increased ($p < 0.001$) (Table 2). As a result, unsaturation index and arachidonic acid/linoleic acid ratio were decreased. Administration of growth hormone restored them almost to the control level. Fatty acid composition of the phosphatidylethanolamine fraction (Table 3) showed some unusual features: linoleic acid (18:2) is lower but arachidonic acid is higher than in phosphatidylcholine fraction. Hypophysectomy decreased ($p < 0.05$) the arachidonic acid level (20:4), giving rise to lower

unsaturation index and arachidonic/linoleic acid ratio, which were restored after growth hormone treatment. The fatty acid composition of the cardiolipin fraction is quite different from that of other phospholipids (Table 4) in that linoleic (18:2) and oleic (18:1) acids constitute about 55–60% and 20% of the total fatty acids, respectively (21–23). After hypophysectomy, the linoleic acid level increased ($p < 0.01$), but the total unsaturation remained unchanged. From these and earlier results (4), it becomes clear that hypophysectomy generally decreases the levels of higher polyenoic fatty acids (20:4 and 22:6) but concomitantly increases linoleic acid in phosphatidylcholine

TABLE 1

Effects of Growth Hormone on Phospholipid Composition and Cholesterol Content of Rat Liver Mitochondria

	Control	Hypophysectomized + saline	Hypophysectomized + growth hormone
Cholesterol (g/g fatty acyl methyl ester)	0.065 ± 0.003	0.067 ± 0.005	0.066 ± 0.001
Total phospholipid mg/mg protein	0.380 ± 0.025	0.364 ± 0.025	0.363 ± 0.025
Phosphatidylcholine	40.0 ± 3.0 (37.5 ± 2.6)	44.6 ± 3.1 (42.2 ± 3.0)	39.2 ± 2.7 (34.8 ± 2.3)
Phosphatidylethanolamine + phosphatidylserine	32.8 ± 1.6 (39.0 ± 3.0)	33.4 ± 1.6 (40.2 ± 2.7)	30.6 ± 1.7 (38.5 ± 2.8)
Cardiolipin + phosphatidic acid	15.4 ± 1.3 (20.2 ± 2.2)	11.82 ± 1.2* (14.9 ± 1.4)	20.3 ± 1.4 (24.1 ± 2.0)
Phosphatidylinositol	6.8 ± 1.4 (1.7 ± 1.2)	6.6 ± 1.0 (1.5 ± 1.0)	6.3 ± 1.2 (1.7 ± 1.2)
Sphingomyelin	2.0 ± 1.0 (1.3 ± 0.2)	2.2 ± 0.9 (1.5 ± 0.3)	1.9 ± 1.2 (1.6 ± 1.0)
Lysophosphatidylcholine	1.3 ± 0.2	1.4 ± 0.3	1.3 ± 0.3

Results are shown as mean ± S.D. Individual phospholipid content is expressed as percentage of total lipid phosphorous. Numbers in parentheses represent the composition of isolated inner mitochondrial membrane. Hypophysectomized rats were injected daily with 100 μg of bovine growth hormone. Each group had 5–6 animals.

*These values are significantly lower than that of control, $p < 0.05$.

TABLE 2

Effects of Growth Hormone on Fatty Acid Composition of Phosphatidylcholine Fraction from Whole (WM) and Inner Membrane (IM) of Rat Liver Mitochondria

Fatty acid (% mole fraction)	Control		Hypophysectomized + saline		Hypophysectomized + growth hormone	
	WM	IM	WM	IM	WM	IM
16:0	27.4 ± 2.1	22.8 ± 2.3	27.1 ± 2.0	22.1 ± 2.2	26.8 ± 2.0	22.2 ± 2.4
16:1	2.8 ± 0.4	3.7 ± 0.5	2.0 ± 0.2	3.5 ± 0.4	2.5 ± 0.2	3.6 ± 0.5
18:0	22.0 ± 0.8	17.5 ± 0.5	21.5 ± 0.6	17.9 ± 0.4	22.4 ± 1.0	15.8 ± 0.8
18:1	12.2 ± 0.2	16.2 ± 0.4	13.5 ± 0.2	15.6 ± 0.2	11.9 ± 0.6	16.0 ± 0.9
18:2	12.1 ± 0.1	15.5 ± 0.6	17.0 ± 0.4	19.9 ± 0.4[a]	15.9 ± 0.3	17.3 ± 0.3
18:3	—	—	0.1 ± 0.0	0.2 ± 0.0	—	—
20:3	1.1 ± 0.1	1.2 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	0.7 ± 0.1
20:4	17.4 ± 0.2	18.9 ± 0.4	14.5 ± 0.4[a]	16.3 ± 0.2[a]	17.2 ± 0.6	18.5 ± 0.5
22:4	1.0 ± 0.2	0.1 ± 0.0	0.6 ± 0.2	0.4 ± 0.0	0.5 ± 0.0	0.1 ± 0.0
22:5	1.0 ± 0.2	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	0.1 ± 0.0
22:6	2.8 ± 0.3	4.0 ± 0.2	2.0 ± 0.2[b]	2.6 ± 0.2[b]	2.5 ± 0.3	4.7 ± 0.4
20:4/18:2	1.43	1.21	0.85	0.81	1.14	1.06
Unsaturation index[c]	137.8	156.0	128.2	143.9	135.4	171.4

Results are shown as mean ± S.D. Hypophysectomized rats were injected daily with 100 μg of bovine growth hormone. Each group had 5–6 animals.

[a]These values are different from control, $p < 0.001$.

[b]These values are lower than control, $p < 0.02$.

[c]Unsaturation index is computed as Σmol % of each fatty acid × number of double bonds in the acid.

and phosphatidylethanolamine fractions. These changes are reversed by growth hormone administration.

Effects of growth hormone on enzyme activities of desaturation and elongation. The important desaturases are those that introduce double bonds in the Δ^9, Δ^6 and Δ^5 positions of fatty acids. The Δ^9-desaturase activity in normally fed animals is low but could be induced by fasting and refeeding of sucrose. Therefore, basal and inducible activities of Δ^9-desaturase were measured. Activities of the terminal desaturase enzymes and the other segments of electron transport chain of microsomal desaturase system of rat liver under different growth hormone status are shown in Table 5. The activity of NADH-cytochrome c reductase, which is related to the nonphosphorylating microsomal electron transport but not to desaturation, was also measured. In control rats, feeding sucrose doubled the activity of Δ^9-desaturase. After hypophysectomy, basal activity of Δ^9-desaturase was slightly decreased (15%, $p < 0.01$), whereas sucrose-inducible activity was decreased (37%, $p < 0.001$) to a larger extent. Growth hormone injection increased only the sucrose-inducible activity (30%, $p < 0.001$). The activity of Δ^6-desaturase remained unchanged, whereas the activity of Δ^5-desaturase was decreased slightly but significantly (19%, $p < 0.001$) after hypophysectomy and was restored after growth hormone administration. The activity of NADH-cytochrome b_5 ferri-reductase, which may be common to the fatty acid desaturases, remained unchanged but the level of cytochrome b_5 was increased (21%, $p < 0.01$) after hypophysectomy. A similar increase

TABLE 3

Effects of Growth Hormone on Fatty Acid Composition of Phosphatidylethanolamine Fraction from Whole (WM) and Inner Membrane (IM) of Rat Liver Mitochondria

Fatty acid (% mole fraction)	Control		Hypophysectomized + saline		Hypophysectomized + growth hormone	
	WM	IM	WM	IM	WM	IM
16:0	26.9 ± 2.2	20.5 ± 2.1	29.5 ± 2.4	22.8 ± 2.2	28.1 ± 2.5	19.2 ± 1.9
16:1	3.0 ± 0.2	1.4 ± 0.3	3.1 ± 0.1	1.2 ± 0.2	3.0 ± 0.2	1.5 ± 0.1
18:0	27.5 ± 2.4	25.0 ± 2.2	27.8 ± 2.5	25.9 ± 2.6	25.8 ± 2.5	24.9 ± 2.7
18:2	5.2 ± 0.4	9.0 ± 0.5	5.3 ± 0.2	0.6 ± 0.3	5.5 ± 0.2	9.4 ± 0.5
18:3	—	—	0.4 ± 0.0	0.5 ± 0.1	—	—
20:3	—	1.7 ± 0.3	0.3 ± 0.1	1.5 ± 0.2	0.4 ± 0.0	1.5 ± 0.2
20:4	22.3 ± 1.7	22.3 ± 1.9	19.5 ± 1.6	19.8 ± 1.4[a]	23.0 ± 1.5	23.9 ± 1.9
22:6	3.4 ± 0.2	5.9 ± 0.5	3.2 ± 0.3	5.5 ± 0.6	3.5 ± 0.2	6.2 ± 0.3
20:4/18:2	4.28	2.47	3.67	2.06	4.18	2.54
Unsaturation index[b]	134.7	166.5	124.0	151.6	140.3	170.8

Results are shown as mean ± S.D. Hypophysectomized rats were injected daily with 100 μg of bovine growth hormone. Each group had 5-6 rats.

[a]These values are lower than control, $p < 0.05$.

[b]Unsaturation index is computed as Σmol % of each fatty acid × number of double bonds in the acid.

TABLE 4

Effects of Growth Hormone on Fatty Acid Composition of Cardiolipin Fraction from Whole (WM) and Inner Membrane (IM) of Rat Liver Mitochondria

Fatty acid (% mole fraction)	Control		Hypophysectomized + saline		Hypophysectomized + growth hormone	
	WM	IM	WM	IM	WM	IM
16:0	7.3 ± 0.4	7.0 ± 0.2	7.0 ± 0.4	7.3 ± 0.4	6.9 ± 0.3	7.2 ± 0.5
16:1	7.5 ± 0.2	5.5 ± 0.2	7.2 ± 0.4	4.9 ± 0.1	6.8 ± 0.4	5.9 ± 0.4
18:0	7.8 ± 0.5	4.4 ± 0.3	7.7 ± 0.5	4.8 ± 0.2	8.2 ± 0.6	4.8 ± 0.4
18:1	19.5 ± 1.1	20.2 ± 1.0	16.2 ± 0.9	15.3 ± 0.9	14.0 ± 1.2	20.6 ± 1.3
18:2	55.0 ± 1.6	59.7 ± 2.0	61.5 ± 2.2	67.4 ± 2.3[a]	55.2 ± 2.0	57.0 ± 1.8
20:3	1.2 ± 0.3	2.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	1.3 ± 0.0	2.6 ± 0.3
20:4	1.7 ± 0.2	0.9 ± 0.2	—	—	—	—
22:6	—	—	—	—	—	—
Unsaturation index[b]	147.4	155.3	147.0	156.6	151.7	155.5

Results are shown as mean ± S.D. Hypophysectomized rats were injected daily with 100 μg of bovine growth hormone.

[a]These values are higher than control, $p < 0.01$.

[b]Unsaturation index is computed as Σmol % of each fatty acid × number of double bonds in the acid.

in cytochrome b_5 has been noted in hypothyroid (24) and diabetic (25) rats. Neither the activity of cytochrome b_5 reductase nor the concentration of cytochrome b_5 is rate-limiting to desaturases. NADH-cytochrome c reductase activity remained unchanged, suggesting that the general microsomal electron transport system was not affected.

Elongation of fatty acids occurs both on the endoplasmic reticulum membrane and in mitochondria, but the cofactor and other requirements are different (17,18). Activities of microsomal elongation reactions (Table 6) were higher (16-17.8%, $p < 0.01$) after hypophysectomy and remained unchanged after growth hormone treatment for seven days. It has been shown that cytochrome b_5 also plays a role in the microsomal elongation of fatty acids (17). The rate of reoxidation of reduced cytochrome b_5 in the presence of malonyl-CoA has been considered related to elongation. The rates, measured as described (18) in the microsomal fraction, were 1.18 ± 0.15, 1.12 ± 0.28 and 1.09 ± 0.12 nmol cytochrome b_5/min/mg protein in control, hypophysectomized and hypophysectomized + growth hormone-injected rats, respectively. In contrast to that observed with the microsomal fraction, the rate of mitochondrial elongation decreased (Table 6) significantly (21%, $p < 0.002$) after hypophysectomy, and growth hormone injection showed a slight but significant increase (11%, $p < 0.01$).

DISCUSSION

Phospholipid composition and fatty acid distribution of mitochondrial inner membranes have been well established as to both the head group type and acyl chain length distribution (8,21-23). The significant features are lower cholesterol and higher cardiolipin contents than other membranes. The latter characteristic has a bearing on several functions of mitochondria. Also, there is a high

TABLE 5

Effects of Growth Hormone on Enzyme Activities of Fatty Acid Desaturation of Rat Liver Microsomes

Enzyme	Control	Hypophysectomized + saline	Hypophysectomized + growth hormone
Δ^9 Desaturase (nmol/mg protein/10 min)			
Normal feeding	3.97 ± 0.23	3.40 ± 0.28[a]	3.18 ± 0.08
Sucrose feeding	9.54 ± 0.33	6.01 ± 0.30[b]	7.92 ± 0.46
Δ^6 Desaturase (nmol/mg protein/10 min)	1.54 ± 0.20	1.45 ± 0.26	1.41 ± 0.18
Δ^5 Desaturase (nmol/mg protein/10 min)	0.300 ± 0.017	0.243 ± 0.022[b]	0.344 ± 0.041
NADH cytochrome b_5 ferri-reductase (μmol/mg protein/min)	2.58 ± 0.22	2.93 ± 0.73	2.60 ± 0.18
Cytochrome b_5 (nmol/mg protein)	0.38 ± 0.02	0.46 ± 0.02[c]	0.42 ± 0.05
NADH cytochrome c reductase (μmol/mg protein/min)	0.75 ± 0.07	0.87 ± 0.05	0.86 ± 0.07

Enzyme activities are shown as mean ± S.D. Each group had 5-6 animals. Hypophysectomized rats were injected daily with 100 μg of bovine growth hormone or an equivalent volume of saline for seven days.

[a]These values are lower than control, $p < 0.01$.

[b]These values are lower than control, $p < 0.001$.

[c]These values are higher than control, $p < 0.01$.

TABLE 6

Effects of Growth Hormone on Fatty Acid Elongation Activities[a] in Microsomal and Mitochondrial Fractions of Rat Liver

Endocrine state	Microsomal fraction		Mitochondrial fraction
	With NADH	With NADPH	
Control	1.55 ± 0.07	2.07 ± 0.05	0.82 ± 0.03
Hypophysectomized + saline	1.81 ± 0.06[b]	2.44 ± 0.08[b]	0.65 ± 0.02[c]
Hypophysectomized + growth hormone	1.80 ± 0.08	2.38 ± 0.10	0.75 ± 0.05

[a]The nmoles of product formed/min/mg protein are shown as mean of 5-6 determinations ± standard deviation. Hypophysectomized rats were injected daily with 100 μg bovine growth hormone or an equivalent volume of saline for seven days.

[b]These values are higher than control, $p < 0.01$.

[c]These values are lower than control, $p < 0.002$.

degree of unsaturation in the fatty acyl chains. Both chain length and unsaturation vary between wide limits in response to dietary and environmental factors. The present studies reinforce these observations and further demonstrate that growth hormone status can be another contributing factor.

The response of the anionic phospholipid, cardiolipin, but not its fatty acyl distribution, to growth hormone status is noteworthy. Cardiolipin, in contrast to other phospholipids, can be synthesized by mitochondrial enzymes (26). It is mostly located on the inside layer of the inner membrane bilayer (27,28). Turnover of its fatty acyl moiety is slower than that of other phospholipids, and the acyl composition is independent of growth stage and nature of the diet of rats (22). Because of the asymmetric distribution, the decreased content (25%) of cardiolipin after hypophysectomy should result in a lowering of net negative charge on the mitochondrial inner membrane toward the matrix side. This, indeed, appears to be the case, as we have shown that unenergized submitochondrial particles from hypophysectomized rat liver bind the negatively charged probe 1-anilino-8-naphthalene sulfonate with higher affinity than from control rat liver (3). Cardiolipin has been shown necessary for optimal functioning of ATPase (29), complex I (29) and complex III (30). This is reflected in the decreased activities of cytochrome oxidase, NADH dehydrogenase and succinate dehydrogenase after hypophysectomy (1). Furthermore, cytochrome c (31) and bivalent cations (32) have been found to induce the formation of nonbilayer structures in liposomes containing cardiolipin. Such transformations are thought to be important for ATP synthesis and ion transport. Liver mitochondria of hypophysectomized rats show a decreased rate of Ca^{++} efflux induced by acetoacetate (33) and t-butylhydroperoxide (unpublished results).

Arachidonic (20:4) and docasahexaenoic (22:6) acids were the major polyunsaturated fatty acids that were responsive to growth hormone status in unresolved (4) as well as in some individual (phosphatidylcholine and phosphatidylethanolamine) phospholipids. The level of linoleic acid (18:2) was also responsive to the hormone status, but the changes were in contrast to that of arachidonic. These changes could be accounted for by changes in the activity of Δ^5-desaturase, which is considered to be important for the synthesis of arachidonic and docosahexaenoic acids. For example, hypophysectomy decreased slightly but significantly the activities of Δ^5-desaturase and mitochondrial elongation as well as the content of the two polyenoic fatty acids. Growth hormone injection increased the Δ^5-desaturase activity and restored the distribution of the three fatty acids to control levels. It should be noted that microsomal but not mitochondrial elongation was found to depend on thyroid hormone (24). In addition to hormones, factors such as diet secondary to hormone changes and transport between adipose tissue and liver must be considered to influence fatty acyl distributions.

As discussed earlier (4), transition temperature in the Arrhenius plots of state 3 respiration depends on the growth hormone status. Interpretation of transitions in the Arrhenius plots is complex for many reasons, one of which is the paucity of information on the rate-limiting step(s) in state 3 respiration. It is the general view that transition temperature is inversely related to membrane fluidity. We have further found that the temperature of transition in the plots of fluorescence of 1-anilino-8-naphthalene sulfonate with vesicles of lipids extracted from liver mitochondria was increased after hypophysectomy and was restored by subsequent growth hormone administration (unpublished results). All these results suggest that hypophysectomy may decrease fluidity of liver mitochondrial membrane and that subsequent growth hormone injection restores it. We conclude that modulation of membrane fluidity by altering fatty acyl unsaturation and carbon chain length may be one of the mechanisms of regulation of cellular metabolism by growth hormone. It remains to be established whether growth hormone affects also the biosynthesis of prostaglandins and leukotrienes, possibly as a consequence of its effect on the biosynthesis and oxidation of polyunsaturated fatty acids.

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Animal Foods in Traditional Australian Aboriginal Diets: Polyunsaturated and Low in Fat

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Australian Aborigines develop high frequencies of diabetes and cardiovascular diseases when they make the transition to an urban lifestyle. The composition of the traditional diet, particularly its lipid components, is a most important aspect of the hunter-gatherer lifestyle that would bear on the risk of these diseases. We have examined the fat content and fatty acid composition of a variety of animal foods eaten traditionally by Aborigines from different regions of Australia. The muscle samples of the wild animals from all over Australia were uniformly low in fat (<2.6% wet weight) with a high proportion of polyunsaturated fatty acids (≥20% PUFA). Liver samples had a higher range of fat content (5–10% wet weight) but were also rich in PUFA (33–42%). Depot fat samples varied widely in their PUFA content (5–40%). In terms of their PUFA composition the foods tended to fall into three groups: (i) those rich in both n-3 and n-6 PUFA, which included land-based, coastal and freshwater animals; (ii) those rich in n-3 PUFA, i.e., marine species; (iii) those rich in n-6 PUFA, mainly land-based species. The results of these analyses suggest that even when the traditional Aboriginal diet contained a high proportion of animal foods it would have been low in fat with a high proportion of PUFA and thereby could have protected Aborigines against cardiovascular diseases and related conditions through a combination of factors: low energy density, low saturated fat and relatively high PUFA content.
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Although Australian Aborigines develop high frequencies of diabetes and diseases of the cardiovascular system when they are living in an urban environment (1–3), there is no evidence that they suffered from these conditions when living traditionally as hunter-gatherers. Indeed, we have shown that when urbanized Aborigines revert temporarily to their hunter-gatherer lifestyle, there are significant reductions in the risk factors for diabetes and heart disease in nondiabetic subjects (4,5) and marked improvement in the metabolic abnormalities associated with these diseases in diabetics (6).

Although the impact of other aspects of the lifestyle change should not be underestimated, the composition of the traditional diet, particularly its lipid components, is a most important factor of the hunter-gatherer lifestyle that would bear on the risk of these diseases. Most information available on the composition of bush foods relates to vegetable foods (7–10). The nutrient composition of animal foods has been less extensively covered (11), and there are few comprehensive data on the lipid composition (12–14).

Given the well-established relationship between dietary fat and the risk of cardiovascular disease, in the present study we have analyzed a selection of animal foods eaten traditionally by Aborigines from different regions of Australia (northwest Australia, coastal and desert; central Australia; southeast Australia) in terms of fat content and fatty acid composition, with particular attention to the longer chain polyunsaturated fatty acids.

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MATERIALS AND METHODS

In most cases photographs of the animals were taken upon capture for later identification of species. Samples of muscle and, from the larger animals, depot fat and liver were stored in liquid nitrogen until their analyses in Melbourne. Samples from southeastern Australia were stored below −20 C.

Some foods, cooked in the traditional manner (baked whole in a covered pit), were treated in the same way before analysis. No difference was observed among either the fatty acid profiles or the lipid contents of the raw and extremely lightly cooked meats.

Lipid analyses were carried out as described previously (15). Briefly, lipids were extracted into 20 vol of chloroform/methanol (2:1, v/v) containing 10 mg/l butylated hydroxytoluene. Fatty acid methyl esters were separated in Packard 427 and 437A gas chromatographs fitted with flame-ionization detectors and using a 45-m × 0.5-mm id SCOT glass capillary column coated with OV-275 on Gas Chrom R (Chromalytic Technology, Melbourne, Australia), a 35-m × 0.25-mm id WCOT glass capillary column coated with SP 2340 (Chromalytic Technology) and an 8-m × 0.22-mm id vitreous silica capillary column with BP-20 as the bonded phase (S.G.E., Melbourne, Australia). The glass columns were operated by temperature programming from 135–195 C at 2 C/min, and the silica column was programmed from 100–195 C at 10 C/min. The carrier gas was helium at a flow rate of 0.20 cm/sec. Response factors and retention times were determined by routinely chromatographing standard mixtures of methyl esters (Nu-Chek Prep, Elysian, Minnesota). Lipid contents were determined gravimetrically from the lipid extract.

RESULTS AND DISCUSSION

A wide range of animal foods frequently eaten traditionally by Aborigines in quite different geographic and climatic regions of Australia was analyzed. Locations where samples were collected are shown in Figure 1, and the species (with descriptions as necessary) are listed in Table 1. Results of the lipid analyses are reported in Tables 2–4 according to the geographical location of the animals: Table 2, northern Australia; Table 3, desert areas; and Table 4, southeastern Australia (Victoria). In terms of lipid composition, they exhibited many common characteristics unrelated to location. The muscle samples of wild animals from all over Australia were uniformly low in fat (≤2.6% wet weight) with a high proportion of polyunsaturated fatty acids (PUFA). All muscle samples

TABLE 1

Animal Species from Which Food Was Obtained for Analysis

Northwest Kimberley

Mammals
- Antelope kangaroo *(Macropus antilopinus)* [most common in this region]
- Dugong *(Dugong australis)*

Birds and reptiles
- Pacific black duck *(Anas superciliosa)*
- Freshwater crocodile *(Crocodylus johnstoni)*
- Northern snake-necked turtle *(Chelodina rugosa)* [small freshwater turtle]
- Flatback turtle *(Chelonia depresa)* [large sea turtle]
- Gould's goanna *(Varanus gouldii)* [0.25–0.4 m long]

Fish and crustaceans
- Mangrove ray *(Himantura granulata* [stingray]
- Black bream *(Hephaestus fuliginosus)*
- Bluebone *(Choerodon albigena)*
- Freshwater barramundi *(Lateo calcarifer)*
- Yabbie *(Cherax sp.)* [freshwater king prawn]

Southeast Kimberley

Birds and reptiles
- Australian bustard *(Ardeotis australis)* ["wild turkey"]
- Gould's goanna *(Varanus gouldii)*

Central Australia

Mammals
- Black-footed rock wallaby *(Petrogale lateralis)*
- Antelope kangaroo *(Macropus antilopinus)* [young]

Birds and reptiles
- Emu *(Dromaius novaehollandiae)*
- Gould's goanna *(Varanus gouldii)*
- Perente *(Varanus giganteus)* [large goanna, 1–1.5 m long]

Insects
- Witchetty grub *(Xyleutes sp.)*

Northeast Arnhemland

Molluscs
- Land snail *(Xanthomelon durillii)*

Southeast Australia

Mammals
- Eastern grey kangaroo[a] *(Macropus giganteus)*
- Eastern wallaroo[a] *(Macropus robustus)*
- Red kangaroo[a] *(Megaleia rufa)*
- Black-tailed wallaby[a] *(Wallabia bicolor)*
- Long-nosed potoroo[a] *(Potorous tridactylus)*
- Common wombat *(Vombatus ursinus)*
- Koala *(Phascolarctos cinereus)*
- Brushtail possum *(Trichosurus vulpecula)*
- Ringtail possum *(Pseudocheirus peregrinus)*
- Platypus *(Ornithorhynchus anatinus)*

Reptiles
- Red-bellied black snake *(Pseudechis porphyriacus)*

Crustaceans and molluscs
- Yabbie *(Cherax sp.)* [freshwater king prawn]
- Mussel *(Mytilus sp.)*

[a]Marsupial kangaroo-like animal.

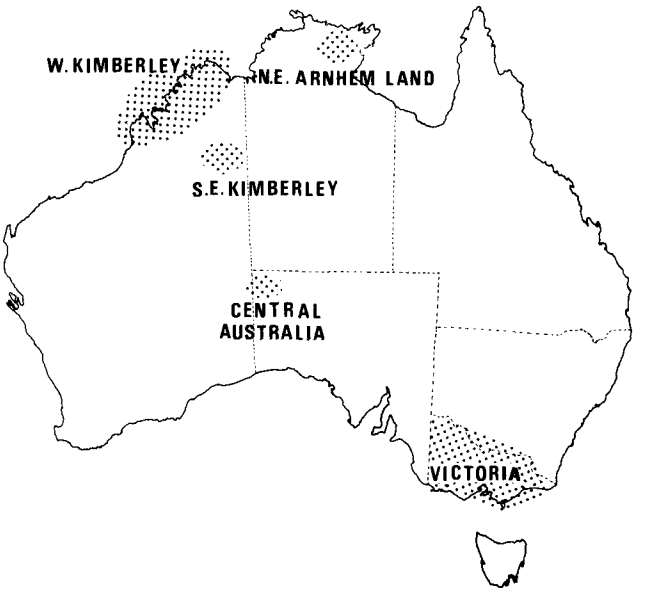

FIG. 1. Map of Australia showing locations where foods were collected.

analyzed contained more than 20% PUFA, most contained more than 30%, and a small number contained over 50% PUFA.

Liver samples had a higher range of lipid content (3.5–10% wet weight) than the muscle samples but were generally also rich in PUFA (28–58% of total fatty acids). The exception was mangrove ray liver, which was very high in lipid (59% wet weight) and contained a lower proportion of PUFA (15%). The PUFA in the liver samples were predominantly, although by no means exclusively, n-6 PUFA. Platypus liver was extremely rich in arachidonic acid, while koala and brushtail possum livers were rich in linoleic acid. The remainder were relatively good sources of both arachidonic and linoleic acids (antelope kangaroo, Australian bustard, Gould's goanna and perente).

Depot fat samples varied widely in their PUFA content, ranging from around 5% of total fatty acids in antelope kangaroo and Gould's goanna to 30% in the perente and 40% in Pacific black duck. The type of PUFA in the fat samples was almost exclusively shorter chain (linoleic and linolenic acids) with very little longer chain PUFA of either the n-3 or n-6 series. Witchetty grubs were the only food analyzed that contained essentially no PUFA. They were, however, very rich in the monounsaturated fatty acid, oleic acid, with an overall composition similar to olive oil.

In terms of their PUFA composition, the foods could be grouped into three main categories: (i) those rich in n-3 PUFA, which were sea or estaurine in origin (mussels, freshwater barramundi muscle and mangrove ray muscle and liver); (ii) those rich in n-6 PUFA, which were land-based (muscle from eastern wallaroo, black-tailed wallaby, long-nosed potoroo, antelope kangaroo, perente, long-necked turtle, emu, Gould's goanna and Pacific black duck; liver from Gould's goanna, Australian bustard and koala; and depot fat from Pacific black duck); and (iii) those rich in both n-3 and n-6 PUFA, which included

TABLE 2

Fatty Acid Composition and Lipid Content of a Range of Animal Foods in the Northwest Kimberley (Coastal) Region

	Fatty acids (as percentage of total fatty acids)[a]																		
								Polyunsaturates											
	Saturates				Monounsaturates			n-6 Series						n-3 Series					Lipid content (%)
	14	16	18	Total	16	18	Total	18:2	20:3	20:4	22:4	22:5	Total	18:3	20:5	22:5	22:6	Total	
Mammals																			
Antelope kangaroo																			
Muscle (n = 5)	—[b]	17.6	12.4	30.0	3.4	22.9	26.9[c]	22.1	1.7	7.7	—	—	31.5	6.1	2.0	2.7	0.8	11.6	1.2 (n = 5)
Liver (n = 4)	0.9	17.6	14.2	32.7	2.3	31.3	34.2[c]	15.0	1.0	7.3	0.4	—	24.3[d]	3.6	0.8	1.8	1.3	8.8[j]	4.3 (n = 4)
Fat (n = 1)	3.3	23.1	15.0	50.9[e]	3.2	40.9	45.0[f]	2.7	—	—	—	—	2.7	1.4	—	—	—	1.4	86 (n = 1)
Dugong																			
Muscle (n = 1)	6.4	23.0	7.2	36.6	4.6	38.2	43.2[c]	7.4	0.2	3.6	—	—	11.9[d]	5.1	1.7	1.5		8.3	1.2 (n = 1)
Birds and reptiles																			
Pacific black duck																			
Muscle (n = 1)	—	15.5	15.5	31.0	1.2	19.9	21.1	20.5	0.7	14.9	0.4	—	36.9[d]	6.2	0.5	1.2	3.1	11.0	1.9 (n = 1)
Fat (n = 1)	0.6	22.1	5.4	28.1	1.8	30.9	33.0[c]	24.8	1.3	2.8	0.3	—	29.5[d]	8.7	0.5	0.1	0.1	9.4	91 (n = 1)
Freshwater crocodile																			
Muscle (n = 3)	0.3	18.3	12.2	30.8	0.6	12.3	13.0[c]	10.5	0.5	24.4	1.2	1.2	37.9[d]	0.7	1.7	1.8	14.1	18.3	0.8 (n = 3)
Northern snake-necked turtle																			
Muscle (n = 4)	0.7	21.7	10.8	33.2	3.6	14.3	18.0[c]	11.1	1.2	20.2	1.4	0.9	34.8	1.3	1.3	2.0	9.4	14.0	0.6 (n = 4)
Fat (n = 4)	1.9	25.7	9.6	37.2	8.7	26.3	35.6[c]	10.4	0.6	3.8	0.8	0.5	16.5[d]	5.8	1.2	1.1	2.6	10.7	81 (n = 4)
Flatback turtle																			
Whole egg (n = 3)	6.8	17.9	5.5	35.7[g]	10.0	39.3	49.9[c]	0.8	0.5	6.1	2.0	0.5	10.3[d]	tr	0.6	1.1	2.4	4.1	7.7 (n = 3)
Gould's goanna																			
Muscle (n = 1)	0.2	16.1	12.8	29.1	0.3	27.8	28.4[c]	15.3	0.6	15.9	2.5	0.8	35.4[d]	0.3	0.6	1.4	4.8	7.1	1.3 (n = 1)
Fat (n = 1)	0.5	30.2	6.6	37.3	1.1	42.8	44.2[c]	13.9	0.3	0.7	0.4	0.2	15.8[d]	1.7		0.3	0.7	2.7	92 (n = 1)
Fish[h] and crustaceans																			
Mangrove ray																			
Muscle (n = 3)	0.9	16.2	17.1	34.2	10.8	26.8	38.2[c]	1.8	0.3	7.1	1.8	0.9	11.9	0.3	3.7	3.1	8.6	15.7	1.1 (n = 3)
Liver (n = 2)	2.8	34.6	6.0	43.4	21.2	20.1	41.6[c]	1.5	0.1	1.6	0.3	0.3	3.9[d]	0.4	5.0	1.4	4.2	11.1[j]	59 (n = 2)
Black bream																			
Muscle (n = 7)	2.2	28.4	9.6	40.2	5.6	24.3	30.4[c]	7.7	1.4	5.9	1.7	0.8	17.8[d]	3.7	1.8	1.6	4.2	11.6[j]	2.4 (n = 2)
Fat (n = 2)	2.7	26.8	9.5	39.0	5.9	36.5	43.5[c]	8.7	0.8	1.5	0.9	0.4	12.8[d]	2.8	0.5	0.5	0.9	4.7	78 (n = 2)
Bluebone																			
Muscle (n = 1)	—	19.2	9.7	28.9	0.2	13.0	13.2	0.3	0.1	24.0	1.9	1.8	28.2[d]	—	4.0	2.1	23.6	29.7	0.6 (n = 1)
Freshwater barramundi																			
Muscle (n = 1)	4.4	24.7	8.0	37.9[i]	12.8	14.4	28.1[c]	3.7	0.4	6.0	0.8	1.4	12.7[d]	1.5	6.4	3.6	9.8	21.3	1.0 (n = 1)
Yabbie																			
Muscle (n = 1)	1.5	20.4	10.3	32.2	6.7	24.0	31.3[c]	12.1	0.3	13.1	—	0.6	27.1[d]	1.4	5.4	0.2	1.6	9.4[j]	1.4 (n = 1)

[a]17:0 and 20:0 found in small amounts in all samples; 14:1 and 22:0 occasionally present in trace amounts.
[b]Not detected.
[c]Includes 20:0.
[d]Includes 20:2 n-6.
[e]Includes 15:0, 17:0, 20:0 and branched-chain fatty acids.
[f]Includes 14:1.
[g]Includes 12:0.
[h]Lipid and fatty acid composition of a further 10 species of marine fish caught in this area are reported in a previous publication.
[i]Includes 20:0.
[j]Includes 18:4 (n-3).

TABLE 3

Fatty Acid Composition and Lipid Content of a Range of Animal Foods in the Desert Regions (Southeast Kimberley, Central Australia and Northeast Arnhemland)

	Fatty acids (as percentage of total fatty acids)[a]																		
								Polyunsaturates											
	Saturates				Monounsaturates			n-6 Series						n-3 Series					Lipid content (%)
	14	16	18	Total	16	18	Total	18:2	20:3	20:4	22:4	22:5	Total	18:3	20:5	22:5	22:6	Total	
Southeast Kimberley																			
Birds and reptiles																			
Australian bustard																			
Liver (n = 1)	—[b]	18.6	24.8	43.4	1.2	16.5	17.7	21.8	0.2	13.8	0.2	—	36.2[d]	0.8	0.8	0.8	0.3	2.7	4.7 (n = 1)
Fat (n = 1)	0.2	29.7	4.8	34.7	3.4	48.8	52.4[c]	7.3	—	—	—	—	7.3	5.6	—	—	—	5.6	83 (n = 1)
Gould's goanna																			
Muscle (n = 2)	0.1	15.9	12.7	28.7	0.5	47.8	48.5[c]	11.6	0.1	9.2	0.3	0.1	21.3	0.3	0.2	0.1	0.9	1.5	2.1 (n = 2)
Liver (n = 1)	—	18.8	9.7	28.5	0.5	42.9	43.6[c]	16.7	0.3	8.2	0.5	—	25.7	0.9	—	0.4	0.9	2.2	10 (n = 1)
Fat (n = 2)	0.2	23.8	7.6	31.6	0.8	61.8	62.8[c]	5.1	—	0.2	—	—	5.3	0.3	—	—	—	0.3	84 (n = 2)
Central Australia																			
Mammals																			
Black-footed rock wallaby																			
Muscle (n = 1)	0.2	17.0	14.6	31.8	0.4	37.1	37.7[c]	18.0	0.4	5.5	—	—	24.1[d]	3.5	0.9	1.5	0.3	6.4[f]	2.0 (n = 1)
Antelope kangaroo (young)																			
Muscle (n = 1)	—	22.7	12.1	34.8	0.6	34.5	35.1	13.2	1.1	5.3	—	—	19.6[d]	1.9	2.7	3.4	1.9	10.5[f]	2.5 (n = 1)
Birds and reptiles																			
Emu																			
Muscle (n = 1)	0.5	13.3	13.8	27.6	1.4	30.1	31.5	26.0	0.2	9.7	—	—	36.0[d]	3.1	1.0	0.6	0.2	4.9	2.6 (n = 1)
Fat (n = 1)	—	22.	7.5	29.7	1.7	48.7	50.7[c]	11.1	—	—	—	—	11.1	8.5	—	—	—	8.5	ND[e]
Gould's goanna																			
Muscle (n = 7)	0.4	11.0	15.8	27.2	0.4	34.0	34.6[c]	17.2	0.2	14.3	0.6	0.2	32.6[d]	1.2	0.7	0.9	2.7	5.6[f]	1.4 (n = 7)
Liver (n = 2)	0.2	17.0	13.6	30.8	0.4	29.9	30.6[c]	20.2	0.3	11.2	0.7	0.4	33.1[d]	1.9	0.7	0.8	1.9	5.5[f]	4.5 (n = 2)
Fat (n = 3)	0.6	22.8	7.5	30.9	1.0	55.8	56.9[c]	10.3	0.1	0.4	trace	—	10.9[d]	1.1	0.1	—	0.1	1.3	74 (n = 3)
Perente																			
Muscle (n = 2)	0.1	15.7	16.9	32.7	0.9	22.8	23.8[c]	19.2	0.1	11.1	0.6	—	31.1[d]	6.3	1.0	2.9	2.1	12.4[f]	1.5 (n = 2)
Liver (n = 2)	0.1	20.9	10.4	31.4	2.4	24.3	26.8[c]	14.1	0.3	7.0	0.5	—	22.2[d]	14.5	1.5	1.8	1.8	19.6	9.1 (n = 2)
Fat (n = 2)	1.2	27.5	9.8	38.5	1.3	29.2	30.7[c]	17.3	0.1	0.8	0.1	—	18.5[d]	11.6	—	0.6	—	12.3[f]	92 (n = 2)
Egg (n = 4)	0.3	28.7	7.6	36.6	1.3	25.6	27.0[c]	19.5	0.1	1.7	0.1	—	21.5[d]	13.5	0.5	0.5	0.4	14.9	14 (n = 4)
Insects																			
Witchetty grub (n = 11)	—	29.4	3.1	32.5	tr	67.1	67.1	0.4	—	—	—	—	0.4		—	—	—		38 (n = 11)
Northeast Arnhemland																			
Molluscs																			
Land snails (n = 6)		13.8	10.9	24.7	1.6	28.1	31.1[c]	34.8	0.5	3.6	0.8	—	43.3[d]	0.9	—	—	—	0.9	4.3 (n = 6)

[a-d]See Table 2.

[e]ND, not determined.

[f]Includes 18:4 n-3.

TABLE 4

Fatty Acid Composition and Lipid Content of a Range of Animal Foods in Southeast Australia (Various Habitats)

	Fatty acids (as percentage of total fatty acids)[a]																		
								Polyunsaturates											
	Saturates				Monounsaturates			n-6 Series						n-3 Series					Lipid content (%)
	14	16	18	Total	16	18	Total	18:2	20:3	20:4	22:4	22:5	Total	18:3	20:5	22:5	22:6	Total	
Mammals																			
Eastern grey kangaroo																			
Muscle																			
Adult (n = 5)	0.8	18.8	12.1	31.7	2.5	26.4	29.4[c]	18.3	1.1	7.5	0.4	—[c]	27.9[d]	4.8	2.0	2.4	0.8	11.0[i]	ND[e]
Pouch young (n = 1)	0.2	14.1	13.1	27.4	1.8	15.5	17.6[c]	12.4	3.9	18.9	1.3	0.7	38.0[d]	0.3	3.6	6.4	6.1	17.0[i]	ND
Eastern wallaroo																			
Muscle (n = 1)	0.7	18.0	10.1	28.8	1.6	21.5	23.5[c]	33.2	1.0	8.5		—	43.2[d]	1.6	0.4	1.1	0.6	4.5[i]	ND
Red kangaroo																			
Muscle (n = 2)	0.8	20.8	13.5	35.1	1.7	30.2	32.4[c]	19.1	0.6	5.5	0.3	—	25.8[d]	3.2	0.7	1.2	0.5	6.7[i]	ND
Black-tailed wallaby																			
Muscle (n = 2)	0.1	12.2	12.9	25.2	1.2	13.1	14.8[c]	31.6	1.5	19.3	1.1	—	53.9[d]	0.8	0.8	3.1	0.8	6.1[i]	ND
Long-nosed potoroo																			
Muscle (n = 1)	0.4	17.9	13.2	31.5	1.4	24.2	25.8[c]	27.5	0.7	7.9	0.5	0.9	37.9[d]	0.4	0.7	0.5	3.0	4.8[i]	ND
Common wombat																			
Muscle (n = 2)	0.5	20.3	16.0	36.8	1.5	11.3	12.9[c]	27.3	1.6	13.2	0.3	0.1	43.1[d]	1.1	1.6	2.8	1.7	7.2	ND
Koala																			
Muscle (n = 3)	0.5	19.7	10.8	31.0	1.0	10.6	11.9[c]	32.2	1.8	13.9	2.4	0.1	50.8[d]	1.2	1.1	2.8	1.1	6.3[i]	0.8 (n = 1)
Liver (n = 1)	0.3	12.9	12.9	26.1	1.1	16.5	18.1[c]	35.4	2.8	9.5	0.9	trace	49.7[d]	2.2	1.3	1.2	1.0	6.1[i]	5.0 (n = 1)
Brushtail possum																			
Muscle (n = 5)	0.9	23.5	11.4	35.8	0.8	14.4	15.3[c]	28.4	0.7	5.7	0.2	tr	35.3[d]	6.3	2.5	3.2	1.3	13.6[f]	1.1 (n = 2)
Liver (n = 2)	0.2	14.3	18.0	32.5	0.6	17.0	17.6	26.6	1.6	7.6		0.2	37.3[d]	5.0	1.7	2.3	3.0	12.6[f,i]	7.9 (n = 2)
Fat (n = 1)	3.3	36.7	4.7	44.7	2.6	29.7	32.3	11.3	—	—	—	—	11.3	11.7	—	—	—	11.7	72 (n = 1)
Ringtail possum																			
Muscle (n = 1)	0.1	23.4	7.1	30.6	5.5	29.0	34.7[c]	18.0	0.6	9.9	0.7	0.8	30.2[d]	0.7	0.1	1.5	2.2	4.5	
Platypus																			
Muscle (n = 1)	2.0	17.6	10.0	29.6	4.2	13.0	18.7[c]	7.8	0.7	15.0	2.1	0.7	26.9[d]	7.2	4.9	5.6	6.7	24.8[i]	1.8 (n = 1)
Liver (n = 1)	1.2	15.6	14.5	31.3	2.7	11.3	14.5[c]	4.5	1.4	26.0	2.0	0.6	34.9[d]	3.1	3.1	5.6	7.3	19.3[i]	10.1 (n = 1)
Reptiles																			
Red-bellied black snack																			
Muscle (n = 1)	0.4	15.1	13.9	29.4	1.1	14.0	15.5[c]	30.9	0.4	14.2	0.7	0.4	46.9[d]	0.5	0.8	1.0	5.9	8.2	1.0 (n = 1)
Fat (n = 1)	1.8	25.3	6.0	33.1	3.2	28.8	32.4[c]	29.9	0.2	1.4	0.3	—	32.1[d]	1.0	0.1	0.3	1.0	2.4	89 (n = 1)
Crustaceans and molluscs																			
Yabbie																			
Muscle (n = 2)	0.4	14.9	7.5	22.8	3.4	21.7	25.6[c]	6.8	0.1	17.0	—	0.2	25.0[d]	2.4	20.7	0.5	2.0	26.6[g]	0.7 (n = 2)
Mussel (n = 6)	4.0	18.5	4.1	26.6	9.8	4.9	19.4[c]	1.5	0.2	2.9	0.3	0.3	6.0[d]	1.3	21.8	1.4	20.1	48.0[h,i]	1.7 (n = 6)

[a-e]See Table 3.

[f]Includes 20:3 n-3.

[g]Includes 16:4 n-3 (0.7%) and 20:3 n-3 (0.3%).

[h]Includes 20:4 n-3.

[i]Includes 18:4 n-3.

land-based, coastal and freshwater animals (perente liver and egg; platypus muscle and liver; and muscle from Eastern grey kangaroo pouch young, bluebone fish, crocodile and yabbie). Despite its low fat content (1.2% wet weight), dugong muscle had relatively low levels of both n-3 and n-6 PUFA.

Before European settlement of Australia 200 years ago, Aborigines lived as hunter-gatherers all over the continent, from the tropical coastal regions of the north (latitude 11°S) through the vast arid regions of the center (latitude 20–30°S) to the cool-temperate regions of the south (latitude 30–43°S). The richer coastal areas, both north and south, could sustain larger populations than the arid inland or desert areas. Little information is available about the traditional diet and lifestyle of the Aborigines from southeastern Australia, as their land was the first taken over by European settlers for farming. It is clear, however, from recent studies of traditional vegetable foods (16) and the continuing presence of abundant animal foods that this area could have supported the greatest population density of Aborigines of any part of the continent. These people would have had access to a mixed and varied diet of fruits, vegetables, roots, seeds, wild animals and fish throughout the year. The desert regions of Australia supported many fewer people. Desert Aborigines traditionally covered vast distances as they moved from place to place in search of food. They too had a mixed animal and vegetable diet that was very dependent on the season. The most detailed diet information exists for the Aborigines of northwest Australia. This derives from actual weighed food intake measurements taken over a two-week period in which a group of Aborigines reverted temporarily to their traditional hunter-gatherer lifestyle as part of a study on the effect of lifestyle change on diabetes in Aborigines (6,17). The diet over this two-week period was derived from antelope kangaroo, freshwater bream, freshwater prawns (yabbies), small freshwater turtle, crocodile, birds (Australian bustard, Pacific black duck), yams (a form of wild potato), wild figs and honey. Energy intake over this period was only 1200 kcal/person/day, much lower than the usual urban intake (6). In terms of total dietary energy consumed over the two-week period, antelope kangaroo accounted for 36%, freshwater bream 19% and yams 28%. All other listed foods accounted for only 17% of total energy intake. Traditionally, everything on an animal carcass is eaten, including muscle, fat depots and internal organs (although usually not intestinal contents). Fat depots are highly prized, as are brain, liver and other organ meats. However, fat depots tend to be very small on wild animals throughout most of the year. The muscle of wild animals always has a low lipid content irrespective of season. As such, the muscle lipid content is primarily structural lipid (phospholipid and cholesterol) with little contribution from triglyceride. Muscle provides the largest contribution in volume and energy from an animal carcass. Despite the relatively large contribution of animal foods to this traditional diet (64% of total energy), it was low in fat (13% of total energy) with approximately equal contributions from saturated, monounsaturated and polyunsaturated fat (4%, 5% and 4% of energy, respectively). This particular diet (6) was high in protein (54% of energy) and relatively low in carbohydrate (33% of energy). It is possible that at other times of the year vegetable foods could make up a higher proportion of the diet; however, in this region animal foods (including fish) were always a significant component.

How do these data on fat composition of animal foods in traditional Aboriginal diets help clarify the mechanisms by which the change to a western lifestyle promotes the development of obesity, diabetes and cardiovascular disease in Australian Aborigines? A major point to be made in this context is an obvious one. The carcass composition of wild animals is quite different from that of animals domesticated for human food consumption in western societies (18,19). The wild animal carcass contains much less fat both in the meat itself (18) and on the carcass as discrete depots (19). Thus, the foods most readily available in the largest quantities for the hunter-gatherer (muscle from wild animals, fish and shellfish) were low in fat with a higher proportion of PUFA. In contrast, the high fat foods were either less readily available (witchetty grubs, perente eggs) or were present only in small quantities on the animal (mangrove ray liver, depot fat). Thus, even when the traditional Aboriginal diet contained a high proportion of animal foods, it was low in fat with a high proportion of PUFA. Furthermore, the types of PUFA in the western and traditional Aboriginal diets are quite different; in the western diet linoleic acid is overwhelmingly the major PUFA (20), whereas the hunter-gatherer diet contained a wide variety of 18, 20 and 22 carbon long chain PUFA of both the n-6 and n-3 types. The data in the present paper, along with our previous studies on traditional Aboriginal diets (16,17,21), bear this out. In addition to these differences in the amount and type of PUFA, there were major differences in type and quality of the carbohydrate in traditional aboriginal and western diets (7–10) that could also have an important bearing on the risk of the "diseases of affluence." Finally, in addition to the composition of the traditional diet, the energy expended in the process of obtaining it (hunting and gathering) was an important factor both in maintaining physical fitness and in limiting the amount of food consumed. It is possible that the traditional hunter-gatherer lifestyle protected Aborigines against cardiovascular disease and related conditions through a combination of factors: low energy density of the diet, low fat content, high PUFA content and high physical activity in obtaining the diet.

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Correlation of Side Chain Mobility with Cholesterol Retention by Phospholipid Vesicles

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Multilamellar vesicles were prepared from choline phospholipids with various fatty acyl chains, singly and in mixtures, with and without cholesterol. Mobility of acyl side chains for each type of vesicle was measured by fluorescence polarization with diphenylhexatriene, and the amounts of cholesterol and phospholipid retained by them after extraction with a nonpolar solvent were determined. The data suggest that structures of acyl chains determine the extractability of cholesterol. Phosphatidylcholines with unsaturated or short saturated side chains above transition temperature retain less cholesterol upon extraction with petroleum ether than phosphatidylcholines with saturated side chains below transition temperature. Correlation of cholesterol retention with side chain mobility showed that cholesterol is more easily removed from vesicles with mobile acyl side chains than from vesicles with rigid side chains. The presence of cholesterol also alters extractability of phospholipids from vesicles and suggests that sterol affects the polarity rather than spacing of headgroups on vesicle surfaces.
Lipids 21, 691–696 (1986).

Cholesterol and the related phytosterols—campesterol, stigmasterol and β-sitosterol—are polycyclic alcohols with a single hydroxyl group. They are readily soluble in nonpolar solvents such as petroleum ether and benzene and should be extractable from an aqueous lipid suspension with these solvents. We were therefore surprised to find that neither solvent would extract even a trace of sterol from a total lipid extract prepared from seedlings of *Digitalis purpurea* (1,2). In reviewing the methods used in the *Digitalis* studies for extraction of lipids, it appeared that we may have prepared a crude suspension of multilamellar vesicles (MLV) and that free sterols could have been incorporated into phospholipid bilayers. Since the polar surfaces of MLV are incompatible with nonpolar substances, the solvent would not be permitted to penetrate to the hydrocarbon components of the bilayer core. If this hypothesis is correct, it should be possible to demonstrate a similar effect using purified phospholipids and cholesterol as a model system. We report here experiments to show that phosphatidylcholines (PC) with saturated side chains below their transition temperature are effective in preventing extraction of cholesterol by a nonpolar solvent, but PC with one or two long unsaturated side chains or short saturated side chains above their transition temperature are much less effective in this regard. The observations are correlated with mobility of acyl side chains and suggest that interaction of unsaturated side chains with cholesterol may alter polarity of the MLV surface in such a way that a nonpolar solvent has easier access to the bilayer core. Results also help in understanding why cholesterol-phospholipid liposomes with unsaturated acyl side chains are better donors of cholesterol than liposomes of phospholipid with saturated acyl side chains (3–6).

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MATERIALS AND METHODS

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine (DOPC), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) and 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (OPPC) were purchased from Sigma Chemical Co. (St. Louis, Missouri). The purity of all phospholipids was checked by thin layer chromatography. Phospholipids were made up as solutions of 10 mg/ml in chloroform. Cholesterol was recrystallized twice from methanol and was dissolved in chloroform to contain 5.0 mg/ml. [4-^{14}C]Cholesterol (60 mCi/mM) was purchased from New England Nuclear (Boston, Massachusetts). It was diluted with benzene to contain approximately 20,000 dpm/20 μl. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Sigma and was made up to 0.03 mg/ml in chloroform. Petroleum ether (boiling range 60–90 C) was obtained from Skelly Oil Co. (Kansas City, Missouri).

Scintillation counting was performed in an Intertechnique liquid scintillation counter, and fluorescence polarization measurements were made on a Perkin-Elmer Model MFP-44 fluorescence spectrophotometer. Polarization values, which were used as an indicator of the mobility of paraffin side chains (7), were calculated according to Shinitzky et al. (8) and were corrected for light scattering.

MLV were prepared. Each sample consisted of 1.0 mg phospholipid with or without addition of 500 μg of cholesterol. If cholesterol extractability was to be measured, 70 ng of [4-^{14}C]cholesterol, containing 0.01 μCi, was added to the unlabeled sterol. For polarization measurements, 20 μl of DPH stock solution was added to the lipid mixture. The lipids had been dissolved in a mixture of chloroform/methanol (2:1, v/v) (C/M) so that a final volume of 0.2 ml was always obtained. The solvent was removed under a stream of N_2 at 55 C. All other operations were carried out at 22 C. The dried lipid films remaining after removal of the solvent were vortexed with a glass bead for 2 min in either 0.2 or 0.4 ml of an unbuffered 0.9% NaCl solution, depending upon the ease of dispersal of phospholipid, and were made up to a volume of 3.0 ml with the same NaCl solution.

Samples were extracted three times with 3.0 ml of petroleum ether each time. For each extraction, the phases were mixed for 20 sec with 60 strokes of a glass rod flattened to a disk on its end. For reproducible results, it was important not to mix air into the samples. The flattened glass disk was kept under the surface of the liquid during mixing of phases to avoid introduction of air bubbles. The petroleum ether extracts for each sample were pooled and taken to dryness. The aqueous phases were

then extracted three times with 3.0 ml of C/M each time. The C/M fractions were pooled and solvents evaporated under N_2. Ten ml of scintillation fluid (OCS, Amersham-Searle, Arlington Heights, Illinois) was added to the dried petroleum ether and C/M fractions. Samples were counted; the recovery of radioactivity, by the sum of counts from both extracts, was within 1% of the amount of [4-^{14}C] cholesterol added in each case. After extraction with C/M, no further radioactivity remained in the aqueous phase.

To determine the amounts of phospholipids in the aqueous phases, vesicle preparations were extracted with petroleum ether, and the phospholipid in the extracts, after drying, was assayed by the method of Bartlett (9). All determinations were done in triplicate. Identical conditions were maintained for all experiments. The validity of the method is demonstrated by the constancy of results. Each point on the curves is an average of three to eight separate experiments; the standard deviations are shown by error bars about each point.

The solubility in petroleum ether of the phospholipids used in these studies was determined by placing 1 mg of each phospholipid dissolved in chloroform into a test tube, evaporating the solvent, adding 3 ml of petroleum ether and assaying aliquots of the resultant solution for phosphate content by Bartlett's procedure (9). Three or more replicate assays were done. The residues after evaporation of the solvent contained the entire amount of phospholipid expected for complete solubility in petroleum ether with a standard deviation of 5% or less. Solubility of cholesterol was tested similarly by assay of [4-^{14}C] cholesterol. The sterol showed complete solubility at the 1-mg level.

Although we report the contents of lipid in the C/M extracts only, we also measured the lipid contents of the petroleum ether extracts. In all instances, the sum of the lipids extracted by both solvent systems was within 5% of the amounts of lipid added.

RESULTS

The phospholipids used in these experiments were fully soluble in petroleum ether when they were coated upon the inner surface of glass tubes. However, when the lipids were dispersed as MLV in an aqueous phase, those with choline head groups could not be extracted into petroleum ether, regardless of whether the acyl side chains were saturated or unsaturated, long or short (Figs. 1, 2 and 5A). The data imply that lipids organized into bilayer structures with the polar head groups facing the aqueous phase do not permit the petroleum ether access to the acyl side chains.

If cholesterol was incorporated into MLV of PC with unsaturated or short acyl side chains, the amount of phospholipid extracted from the aqueous phase increased, but there was only a minor effect on the extraction of phospholipid from MLV prepared from DPPC (Figs. 1, 2 and 5A). Studies on the amount of cholesterol extracted showed that more sterol is extracted from vesicles made from phospholipids below their transition temperatures than from those above their transition temperatures. The transition temperatures for phospholipids used in these experiments are 41 C for DPPC (10); 0 C for DLPC (11); −22 C for DOPC (12); and about −5 C for OPPC (13). The extractability of phospholipids does not follow the same pattern as the extraction of cholesterol. In some cases, phospholipid and cholesterol were extracted in an equimolar ratio (Figs. 1 and 2), but in others cholesterol was extracted to a greater extent than phospholipids (Fig. 5A). The results of these studies suggested that extraction of suspensions of MLV with a nonpolar solvent would provide a means of comparing accessibility of the solvent for cholesterol in phospholipids with varying acyl side chains.

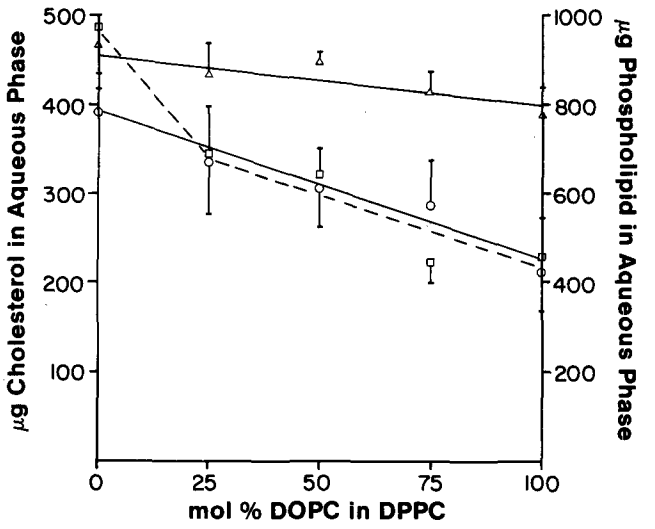

FIG. 1. Cholesterol and phospholipid retention in aqueous phase after petroleum ether extraction of vesicles prepared from DPPC and DOPC. Vesicles were prepared with and without cholesterol at a 1:1 molar ratio to phospholipid, as described in Materials and Methods. Sterol was determined by [4-^{14}C]cholesterol remaining in the aqueous phase after petroleum ether extraction. Phospholipid was determined similarly by assay of inorganic phosphate. Phospholipid in the aqueous phase was measured for vesicles prepared with and without cholesterol. Bars show s.d. Each point is the mean of 4 to 7 samples. ○, Cholesterol; □, phospholipid from vesicles with cholesterol; △, phospholipid from vesicles without cholesterol.

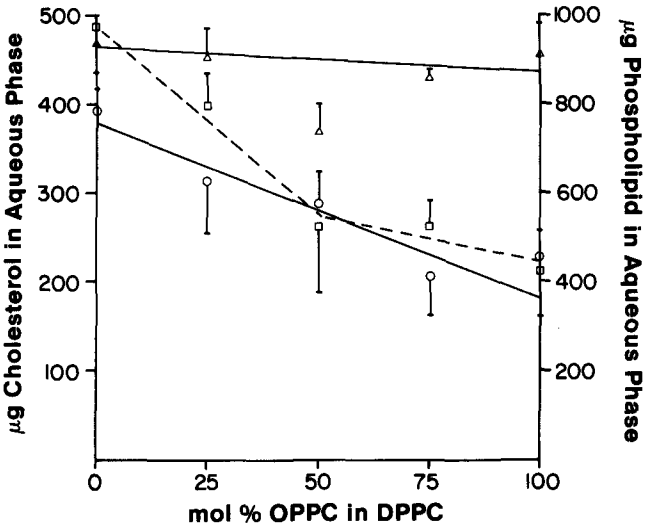

FIG. 2. Cholesterol and phospholipid retention in aqueous phase after petroleum ether extraction of vesicles prepared from DPPC and OPPC. See Fig. 1 for additional detail.

If phospholipid was not included with the cholesterol, 450 μg of cholesterol, or 90% of the total, was removed from the aqueous phase in the first extraction, followed by an additional 30 μg in the second extraction. Studies of the amount of cholesterol extracted from DPPC and DOPC vesicles showed that the amount of sterol removed in each of the three extractions was about 25 μg for DPPC vesicles. For DOPC vesicles 30 μg was removed by the first extraction, 60 μg by the second and 150 μg by the third. Comparison of amounts of phospholipid extracted from MLV with and without cholesterol showed that the sterol had no influence on extraction of DPPC; virtually all the phospholipid remained in the aqueous phase (Fig. 1). For MLV of DOPC, however, 20% of the phospholipid was extracted in the absence of cholesterol, but more than 50% was extracted in the presence of the sterol (Fig. 1). Apparently, the solvent interacts with the unsaturated vesicles to disrupt the bilayer structure.

To determine what factors affect the extractability of cholesterol from within phospholipid vesicles, MLV were prepared with phospholipids in which the head groups were held constant and the acyl side chains were varied. For phospholipid mixtures, the mol% of one phospholipid in the other was made up to 25, 50 and 75%. Every set was run with and without added cholesterol. Total phospholipids and cholesterol remaining in the aqueous phase after petroleum ether extraction were determined. The extracts were also assayed. The fluorescence polarization (p) of aqueous lipid suspensions, without petroleum ether extraction, was measured. The amount of lipid remaining in the aqueous phase was plotted against phospholipid composition of vesicles and against fluorescence polarization. Fluorescence polarization has been equated to microviscosity and side chain mobility in vesicles (7,8) and is used in this investigation as an index of the role of mobility in determining access to lipids by an extracting solvent.

The following pairs of phospholipids were assayed: DPPC and DOPC; DPPC and OPPC; and DPPC and DLPC. At 22 C, acyl side chains of DPPC are in the gel state, whereas side chains of OPPC and DOPC are in liquid state. Figures 1 and 2 show the influence of side chain unsaturation in phospholipids with choline head groups and two unsaturated side chains. In the absence of cholesterol, an increase in the proportion of unsaturated to saturated fatty acids had little effect on the amount of phospholipid retained by the aqueous phase after extraction with petroleum ether. However, when cholesterol was present, both phospholipid and cholesterol were extracted by petroleum ether in increasing amounts as the proportion of unsaturated side chains increased. Results showed only a small difference, whether one or two unsaturated side chains were present. The molar ratio of cholesterol to phospholipid remaining in the aqueous phase shifted from 0.8 to 1.0 as the proportion of unsaturated side chains increased. Cholesterol had a pronounced effect in modulating the mobility of acyl side chains within vesicle preparations (Fig. 3), although vesicles prepared from DOPC exhibited more side chain mobility than vesicles made from DPPC. Figures 4A and 4B show an inverse relationship between side chain mobility and amounts of cholesterol and phospholipid remaining in the aqueous phase; that is, the more mobile the side chains, the more lipid was extracted.

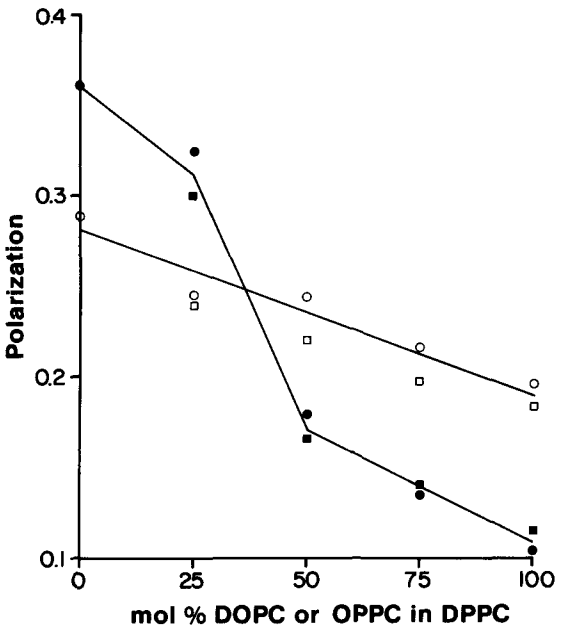

FIG. 3. Fluorescence polarization and composition of vesicles of DPPC and DOPC and of DPPC and OPPC, with and without cholesterol in a 1:1 molar ratio. ○, DPPC and DOPC with cholesterol; □, DPPC and OPPC with cholesterol; ●, DPPC and DOPC without cholesterol; ■, DPPC and OPPC without cholesterol.

To determine whether changing the state of the acyl side chains from gel to liquid-crystalline state would alter the cholesterol retention properties of DPPC, vesicles were incubated at 45 C prior to and during the extraction procedure. This temperature is above the transition temperature for DPPC. Less than 10% of the cholesterol was retained in the aqueous phase upon extraction with petroleum ether (data not shown). Thus, when the acyl side chains of DPPC are in liquid-crystalline state, cholesterol is much more accessible to the extracting solvent.

Figure 5A shows the effect of saturated side chains of different length and mobility on retention of PC lipids in the aqueous phase after solvent extraction. DLPC and DPPC were compared. At the temperature of these experiments (22 C), DPPC is below its transition temperature and DLPC is above its transition temperature (0 C). In the absence of cholesterol, almost the entire amount of phospholipid was retained. If cholesterol was present in mixtures of phospholipids with more than 50 mol% of DLPC, retention of phospholipid decreased, but sterol was extracted to a greater extent than phospholipid. One-half or more of the phospholipid was retained in the aqueous phase, but less than 20% of cholesterol was retained. As with phospholipid mixtures containing unsaturated acyl side chains, cholesterol modulated the mobility of phospholipid side chains (Fig. 5B). Although side chain mobility was similar for phospholipid-cholesterol mixtures containing mainly DPPC or DLPC, the amount of cholesterol retained by aqueous phases containing MLV prepared from the latter was much less (Fig. 5A). The molar ratio of cholesterol to phospholipid remaining in the aqueous phase was reduced from 0.8 for DPPC to 0.3 for DLPC. The amount of cholesterol extracted from vesicles was directly related to side chain mobility for vesicles containing predominantly shorter

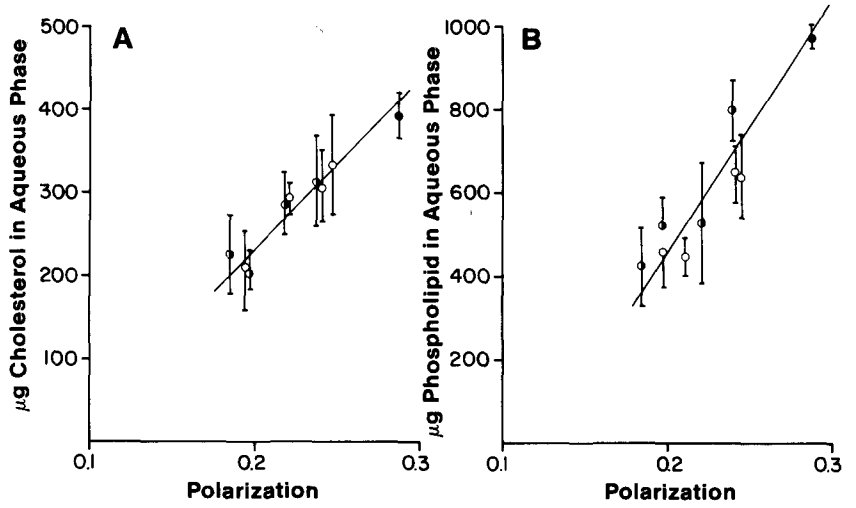

FIG. 4. (A) Cholesterol retention and polarization values of vesicles prepared from DPPC and DOPC and from DPPC and OPPC, with cholesterol. Polarization measurements were made on suspensions of vesicles of phospholipid and cholesterol in a 1:1 molar ratio with DPH fluorescent probe in 0.9% NaCl, as described in Materials and Methods. Polarization determinations were done in triplicate and deviated by no more than 0.01 units. Bars show s.d. for cholesterol retention, after petroleum ether extraction, for samples without DPH. ●, Vesicles of 100 mol% DPPC; ○, vesicles of DPPC and DOPC; ◑, vesicles of DPPC and OPPC. (B) Phospholipid retention and polarization values of vesicles prepared from DPPC and DOPC and of DPPC and OPPC, with cholesterol in a 1:1 ratio. Additional details as stated for (A).

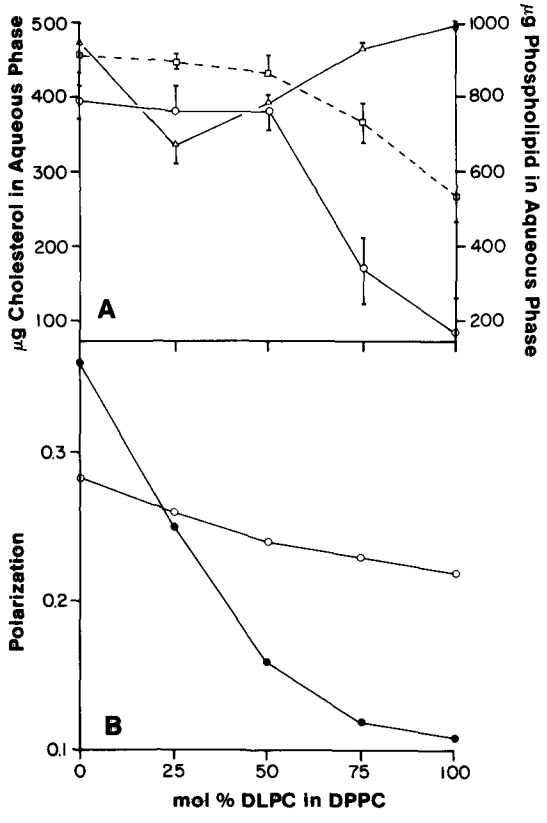

FIG. 5. (A) Cholesterol and phospholipid retention in aqueous phase after petroleum ether extraction of vesicles prepared from DPPC and DLPC, with and without cholesterol in a 1:1 molar ratio. See Fig. 1 for additional details. (B) Fluorescence polarization and composition of vesicles prepared from DPPC and DLPC with and without cholesterol. ○, Vesicles with cholesterol; ●, vesicles without cholesterol.

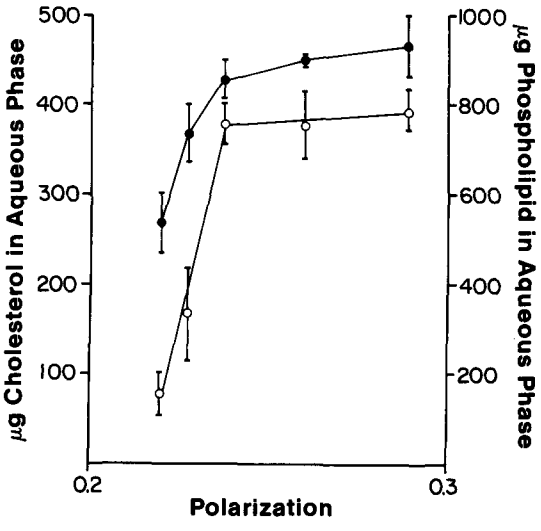

FIG. 6. Cholesterol and phospholipid retention and polarization values for vesicles prepared from DPPC and DLPC with cholesterol in a 1:1 molar ratio. See Fig. 4A for additional detail. ○, Cholesterol retention; ●, phospholipid retention.

acyl side chain preparations, but leveled off for vesicles with longer side chains (Fig. 6).

DISCUSSION

These studies show that behavior of phospholipid-cholesterol systems in aqueous suspension can explain, in part, the anomalous features of the procedures described before in which petroleum ether was incapable of extracting sterols from plant homogenates of *Digitalis* (1,2). In vesicles composed of PC and cholesterol, the present

studies show that extractability of sterol into a nonpolar solvent is directly related to mobility of acyl side chains; mobile side chains decrease and rigid side chains increase retention of cholesterol by vesicles. Naturally occurring phospholipids frequently contain one unsaturated acyl side chain, but unsaturated phospholipid vesicles did not retain sterol upon extraction with petroleum ether. The presence of other lipids, ions or proteins carried over into the aqueous homogenate prior to lipid extraction may influence the process in ways that have not been investigated. It will be necessary to ascertain the level of saturated and unsaturated phospholipids in *Digitalis*.

Several observations in these experiments warrant discussion. First, none of the phospholipids examined was extractable from an aqueous solution into petroleum ether to an extent exceeding 25% of its total quantity in absence of cholesterol. This implies that, despite their lipid composition, they are capable of forming vesicular structures with polar surfaces that hide their lipid components from the nonpolar solvent. The polar properties of the surfaces are sufficiently strong to repel nonpolar solvents. Second, incorporation of cholesterol into MLV increased extractability of phospholipids by the solvent in every case, but the loss of phospholipid from vesicles with long unsaturated acyl side chains was much greater than from vesicles with long saturated side chains (Figs. 1 and 2). In the case of DOPC, increasing amounts of sterol were removed with each successive extraction, and equimolar amounts of sterol and phospholipid were extracted at each step.

These results could be explained if cholesterol were to alter the spacing of polar head groups in the bilayer so as to allow solvent to enter the hydrocarbon core. The association of cholesterol with phospholipid acyl side chains is well established (14–18). It is generally believed, and our fluorescence data are in complete accord, that sterol increases the mobility of rigid side chains, while reducing the mobility of liquid ones (19–24). Cholesterol is known to reduce the area occupied by monomolecular films of phospholipids with liquid side chains (10,18,25), but it increases the area occupied by phospholipids with rigid side chains (14). Oldfield et al. (26) and Kawato et al. (27) reported an increase due to cholesterol of the cone angle between head groups of phospholipids with saturated side chains in bilayers. However, Plank et al. (28) reported no change in surface charge density of MLV containing cholesterol and concluded that cholesterol does not alter the spacing between head groups of phospholipids in bilayers, regardless of side chain composition. The former observations imply that cholesterol should increase the amount of phospholipid extracted from vesicles of DPPC, which has rigid side chains, and decrease the amount of phospholipid extracted from vesicles of DLPC, DOPC or OPPC, which have liquid-crystalline side chains, at the temperature of these experiments. The results of Plank et al. (28) suggest that extractability of phospholipid from MLV should be unaffected by the presence of cholesterol. Neither of these predictions was borne out; phospholipid extraction from DPPC vesicles was unaffected by cholesterol (Fig. 1), while extraction from vesicles of DOPC, OPPC or DLPC was greatly increased with cholesterol present (Figs. 1, 2 and 5A).

Cholesterol may exercise an influence upon the spacing of head groups, but if it does, the effect could not be the only factor that determines the extractability of lipids from a bilayer. Our data suggest that cholesterol may alter the polarity of vesicle surfaces, particularly in situations where the length, mobility or shape of acyl side chains limit the depth to which the sterol can enter the hydrocarbon core.

Cholesterol may intercalate itself into bilayers of phospholipids with liquid-crystalline or unsaturated acyl side chains. Upon extraction with petroleum ether, microdrops of solvent may enter the bilayer, causing disintegration of the liquid-crystalline structure. The main effect of cholesterol, supported by the present data, would be to interact noncovalently with the aliphatic side chains of ordered phospholipids in bilayers and thus change their surface properties.

At 22 C, DLPC is above its transition temperature of 0 C and the side chains are mobile, as indicated by the low polarization value (Fig. 5B). DPPC is below its transition temperature of 41 C and the side chains are rigid. Incorporation of cholesterol into vesicles of DLPC increases the polarization value from 0.11 to 0.25, indicating that sterol is reducing the mobility of side chains. Since the p value for DLPC + cholesterol is slightly lower than for DPPC + cholesterol, one might expect cholesterol retention by both types of vesicles to be similar, but this is not the case. DPPC vesicles retain 90% of cholesterol, while DLPC vesicles retain less than 20%. When the temperature of DPPC vesicles was raised above the transition, they did not retain sterol. The space required by a mobile side chain may limit the depth to which the sterol can penetrate into the hydrocarbon core of the bilayer, with the result of producing a less polar vesicular surface.

DOPC or OPPC vesicles without cholesterol lost 20–25% of their phospholipid upon extraction with petroleum ether and more than 50% of phospholipid when cholesterol was included (Figs. 1 and 2). Fluorescence polarization curves show side chains of both of these lipids became less mobile in the presence of cholesterol (Fig. 3). One might expect that reducing mobility would also reduce space requirements of the hydrocarbon core, allowing head groups greater proximity and increasing the polar properties of the surface, but this was clearly not the case. The solvent exhibited greater interaction with the hydrocarbon phase, extracting both sterol and phospholipid in equimolar amounts. This could happen only if polarity of the surface had been decreased. Presence of a double bond at C-9 of the acyl side chain may position a hydrocarbon portion of the sterol at or near the surface of the bilayer, making the surface less polar. One other possibility for the effect of cholesterol is that the sterol induces localized reverse or hexagonal phases in the bilayer, causing surfaces to be less polar (29).

Clejan and Bittman (6) proposed that mobility of acyl chains in the core of a membrane, bilayer or lipoprotein particle may control the rate of cholesterol exchange between intracellular membranes or between plasma membranes and plasma lipids. They found *Mycoplasma gallisepticum* membranes, adapted to a cholesterol content of less than 26 mol%, will exchange cholesterol faster with PC vesicles than will normal *M. gallisepticum* membranes, which have a cholesterol content of 48 mol%. The adapted membranes have been reported by Le Grimmellec et al. (30) to be more fluid than native membranes. Bloj and Zilversmit (3), Nakagawa et al. (4) and Lange

et al. (5) reported cholesterol to move from phospholipid vesicles with a preponderance of unsaturated side chains toward vesicles with saturated side chains. Very low density lipoproteins (VLDL) with a high proportion of unsaturated acyl side chains exchange cholesterol with red blood cells more rapidly than VLDL with saturated side chains (31). The present study suggests that increased side chain mobility will decrease polarity of vesicle surfaces, rendering vesicular sterol more accessible to exchange with another lipid particle or to extraction with a nonpolar solvent.

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Adam Alpers gave technical assistance and John Swaney provided helpful discussion. This project was supported in part by the U.S. Public Health Service, Grant AM 36122.

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Effects of Dietary Linolenate on the Fatty Acid Composition of Brain Lipids in Rats

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Weanling male rats were fed hydrogenated coconut oil to induce essential fatty acid (EFA) deficiency. After 15 weeks, the rats were divided into six groups. Five groups were fed graded amounts of purified linolenate (18:3ω3) with a constant amount of linoleate (18:2ω6) for six weeks. Fatty acid composition was determined in brain lipids. Increasing dietary 18:3ω3 resulted in a decrease in arachidonic acid (20:4ω6), docosatetraenoic acid (22:4ω6) and docosapentaenoic acid (22:5ω6), whereas 18:2ω6 and eicosatrienoic acid (20:3ω6) were increased both in total lipids and phospholipids. These results suggest that dietary 18:3ω3 exerts its inhibitory effect mainly on the desaturation of 20:3ω6 to 20:4ω6 in brain lipids. Linolenate was undetectable in brain lipids from any dietary treatments. The levels of eicosapentaenoic acid (20:5ω3) in groups receiving dietary 18:3ω3 were not different from that of the group receiving no 18:3ω3. These results indicate that, in the brain, 18:3ω3 is rapidly converted mainly to 22:6ω3 without being accumulated and imply that dietary 18:3ω3 can modulate the level of precursor of diene prostaglandins (PG) but not that of triene PG in the rat brain.
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Availability of direct precursor acid is an important factor regulating the biosynthesis of eicosanoids in animal tissues. Direct precursors of eicosanoids are free, unesterified fatty acids (1). Amounts and types of precursor acids released depend on the composition of fatty acids in tissue lipids, which in turn are affected by the composition of dietary fatty acids. It is well documented that there is competitive inhibition among oleic acid (18:1ω9), linoleic acid (18:2ω6) and linolenic acid (18:3ω3) for desaturases responsible for the synthesis of tissue polyunsaturated fatty acids (PUFA). Competitive inhibition between ω6 and ω3 fatty acids is dependent on the ratios of the fatty acids in diets (2). The inhibitory effect of 18:3ω3 on the conversion of 18:2ω6 to 20:4ω6 is much greater than that of 18:2ω6 on the conversion of 18:3ω3 to 20:5ω3 (3). Thus increasing dietary 18:3ω3 results in reduction of arachidonic acid (20:4ω6) in tissue lipids by inhibiting the conversion of 18:2ω6 to 20:4ω6 (4,5). Subsequently, it has been demonstrated that dietary 18:3ω3 suppressed the capacity of various tissues to synthesize prostaglandins (PG) and thromboxane (TX) derived from 20:4ω6 (6–8).

It has been suggested that the low incidence of thromboembolic disorders among native Greenland Eskimos, whose diets consist mainly of cold-water marine animals, might be attributed to an enhanced level of eicosapentaenoic acid (20:5ω3) in their diets (9–11). Ingestion of fish oil, which is rich in 20:5ω3, results in reduction of tissue 20:4ω6 with a concomitant increase in 20:5ω3. Accordingly, increasing dietary 20:5ω3 reduces the formation of eicosanoids derived from 20:4ω6 while enhancing those derived from 20:5ω3. The question then can be raised as to whether dietary 18:3ω3 has the same efficacy as 20:5ω3 in reducing tissue levels of 20:4ω6.

Brain lipids contain unusually high levels of docosahexaenoic acid (22:6ω3) in rats (12,13) and humans (14). The fatty acid composition in brain lipids is known to be resistant to dietary alteration as compared to other organs (15). In this report, we studied effects of various levels of dietary 18:3ω3 on the fatty acid composition of rat brain lipids, with emphasis on eicosanoid precursors and other long chain PUFA derived from 18:2ω6 and 18:3ω3.

MATERIALS AND METHODS

Sixty weanling male Sprague-Dawley rats were fed a diet containing hydrogenated coconut oil (HCO, 10% by weight of the diet) to induce essential fatty acid (EFA) deficiency. After 15 weeks, the rats were randomly divided into six groups. Five groups were fed graded amounts of linolenate. Safflower oil as a source of linoleic acid was held constant at 4%. The remaining group was kept on the EFA-deficient diet. The composition of dietary fat is shown in Table 1. The composition of the basal diet is described in Table 2.

After six weeks, the rats were sacrificed by anesthetizing with diethyl ether. After the blood and various organs were removed, the remaining carcasses, including the brains, were frozen and stored at −15 C until they were analyzed within three months. The fatty acid composition of liver and serum lipids and of serum levels of eicosanoids of the rats have been reported elsewhere (7).

The brain lipids were extracted with chloroform/methanol (2:1, v/v) as described by Folch et al. (16). Butylated hydroxytoluene (0.02%) was added to chloroform/methanol as an antioxidant.

An aliquot of the lipid extract was applied to thin layer chromatography (TLC) plates to separate total phospholipids using a solvent containing diethyl ether/petroleum ether/acetic acid (30:70:1, v/v/v). In this solvent

TABLE 1

Dietary Lipid

Group	Hydrogenated coconut oil (wt %)	Safflower oil (wt %)	Methyl linolenate (wt %)
A[a]	10.0	0.0	0.0
B	6.0	4.0	0.0
C	5.5	4.0	0.5
D	5.0	4.0	1.0
E	4.0	4.0	2.0
F	2.0	4.0	4.0

[a]Essential fatty acid-deficient.

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system, the phospholipid fraction remained at the origin. Another aliquot of the lipid extract was fractionated into individual phospholipid classes: phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine plus phosphatidylinositol (PS + PI). The solvent system used was originally devised by Vitiello and Zanetta (17). It contained ethyl acetate/chloroform/n-propanol/methanol/0.25% aqueous potassium chloride (25:25:25:10:9, v/v/v/v/v). The Rf values for the phospholipid classes were PE, 0.65; PC, 0.40; and PS + PI, 0.52. The identity of phospholipids was verified by using standard phospholipids (Sigma Chemical Co., St. Louis, Missouri). All TLC was carried out on precoated Silica Gel G plates (Kontes, Vineland, New Jersey). A small strip of each plate was broken off and visualized in an iodine vapor tank. The phospholipid bands were then matched to the remaining plate. The bands from both systems (total phospholipids and individual classes) were scraped off and removed, using a sample recovery tube attached to a vacuum pump. The lipids were eluted from the silica gel using 30 ml chloroform/methanol/acetic acid (2:1:0.1, v/v/v). Fatty acid composition of the phospholipid fraction was determined as described in a previous report (18). Regression analysis was used to determine the relationships between dietary linolenate levels and tissue fatty acid levels.

TABLE 2

Composition of the Basal Diet Fed to Experimental Rats

Ingredient	Weight (%)
Fat[a]	10.0
Casein (vitamin-free)	20.0
Salt mix[b]	4.0
Vitamin mix[c]	2.0
Cellulose	4.0
Choline chloride	0.2
Sucrose	to 100.0

[a]See Table 1.

[b]See Jones, J.H., and Foster, C.J. (1942) *Nutrition 24*, 245.

[c]ICN Pharmaceuticals Inc., Cleveland, Ohio, g/1 kg; vitamin A, 900,000 IU; vitamin D, 100,000 IU; all-α-tocopherol, 10 (additional 5 g were added to give 10 g); ascorbic acid, 45; inositol, 1.0; choline choride, 75.0; menadione, 2.25; paraminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine hydrochloride, 1.0; thiamine hydrochloride, 1.0; calcium pantothenate, 3.0; biotin, 0.02; folic acid, 0.09; vitamin B_{12}, 1.35 mg.

RESULTS AND DISCUSSION

The fatty acid composition of brain total lipids is shown in Table 3; those for total phospholipids, PE, PS + PI and PC are shown in Tables 4, 5, 6 and 7, respectively. The pattern of change in the fatty acid composition of total lipids was similar to that of phospholipids (Tables 3 and 4). As the amount of dietary 18:3ω3 increased, levels of 18:2ω6 and 20:3ω6 were also increased, whereas those of 20:4ω6, 22:4ω6 and 22:5ω6 were reduced both in the total lipids and the phospholipids. A similar trend was observed in liver lipids of the same rats (7). These results suggested that dietary 18:3ω3 exerted its inhibitory effect mainly on the desaturation of 20:3ω6 to 20:4ω6. It was shown that rats fed the diet deficient in 18:3ω3 for two generations accumulated greater amounts of 20:4ω6 and 22:5ω6 in various organ lipids than the control group (13).

TABLE 3

Fatty Acid Composition of Brain Total Lipids of Essential Fatty Acid (EFA)-Deficient Rats After Receiving Graded Amounts of Linolenate[a]

Fatty acid (wt %)	Percentage of 18:3ω3						Regression, $p < .01$
	0 (Group A)	0 (Group B)	0.5 (Group C)	1.0 (Group D)	2.0 (Group E)	4.0 (Group F)	
16:0	19.62 ± .19	20.27 ± .12	19.87 ± .23	19.99 ± .17	20.16 ± .47	19.78 ± .31	
16:1ω7	1.12 ± .03	1.07 ± .13	0.93 ± .04	0.92 ± .07	1.20 ± .29	0.99 ± .09	
18:0	19.25 ± .07	19.61 ± .19	19.38 ± 1.35	19.35 ± .10	18.98 ± .39	19.45 ± .13	
18:0DMA[b]	2.42 ± .36	3.04 ± .64	3.02 ± .77	2.58 ± .17	2.92 ± .59	1.98 ± .17	
18:1ω9	24.71 ± .22	23.40 ± .24	23.67 ± 1.05	23.32 ± .78	23.44 ± .50	23.63 ± .06	
18:1DMA[b]	0.99 ± .13	0.99 ± .06	0.98 ± .03	0.77 ± .00	0.97 ± .14	0.79 ± .20	
18:2ω6	0.30 ± .04	0.95 ± .03	1.09 ± .04	1.10 ± .04	1.31 ± .09	1.28 ± .02	Quadratic
20:1ω9	2.66 ± .05	2.86 ± .09	2.96 ± .07	2.83 ± .13	3.00 ± .06	2.88 ± .04	
20:3ω9	5.28 ± .14	1.31 ± .07	1.37 ± .06	1.24 ± .04	1.30 ± .07	1.48 ± .08	
20:3ω6	0.21 ± .01	0.25 ± .06	.30 ± .08	0.38 ± .06	0.42 ± .07	0.62 ± .05	Linear
20:4ω6	7.32 ± .08	10.90 ± .11	10.25 ± .13	9.92 ± .14	9.37 ± .14	9.16 ± .05	Linear
20:5ω3	1.69 ± .13	0.82 ± .08	0.78 ± .08	0.77 ± .02	0.91 ± .07	0.96 ± .02	
22:0	0.38 ± .01	0.21 ± .02	0.19 ± .02	0.21 ± .01	0.19 ± .02	0.23 ± .01	
22:4ω6	1.69 ± .09	3.11 ± .03	2.53 ± .08	2.51 ± .03	2.22 ± .87	2.12 ± .06	Quadratic
22:5ω6	4.64 ± .11	4.43 ± .88	1.75 ± .08	1.59 ± .04	1.37 ± .04	1.41 ± .06	Cubic
22:5ω3	0.11 ± .01	0.06 ± .01	0.16 ± .03	0.26 ± .02	0.35 ± .02	0.48 ± .02	Linear
22:6ω3	7.70 ± .28	6.59 ± .20	10.77 ± .29	12.26 ± .18	11.90 ± .21	12.70 ± .06	Cubic

Group A, EFA-deficient; group B, control.

[a]Values are mean ± SEM of six rats.

[b]Dimethyl acetals (DMA) were tentatively identified based on their retention times.

*Significantly different from control ($p < .05$).

TABLE 4

Fatty Acid Composition of Brain Phospholipids of Essential Fatty Acid (EFA)-Deficient Rats After Receiving Graded Amounts of Linolenate[a]

Fatty acid (wt %)	Percentage of 18:3ω3						Regression, $p < .01$
	0 (Group A)	0 (Group B)	0.5 (Group C)	1.0 (Group D)	2.0 (Group E)	4.0 (Group F)	
16:0	21.56 ± .37	22.99 ± 1.29	21.46 ± .93	21.47 ± .76	21.66 ± .59	22.40 ± .43	
16:1ω7	0.87 ± .03	0.77 ± .13	0.72 ± .05	0.70 ± .05	0.80 ± .11	0.76 ± .09	
18:0	20.19 ± .37	19.63 ± .83	20.18 ± .47	19.86 ± .68	19.83 ± .59	19.99 ± .48	
18:0DMA[b]	2.21 ± .52	2.25 ± .46	1.33 ± .12	2.09 ± .41	1.64 ± .12	1.98 ± .15	
18:1ω9	24.54 ± .18	23.38 ± .13	23.96 ± .13	23.80 ± .31	23.93 ± .17	23.73 ± .20	
18:1DMA	1.86 ± .64	2.16 ± .57	1.20 ± .16	1.77 ± .53	1.54 ± 1.9	1.99 ± .25	
18:2ω6	0.20 ± .08*	0.85 ± .08	0.94 ± .14	1.01 ± .05	1.02 ± .03	1.17 ± .05	Linear
20:1ω9	3.05 ± .08	3.45 ± .35	3.55 ± .04	3.16 ± .22	3.46 ± .25	3.18 ± .11	
20:3ω9	4.79 ± .21	1.18 ± .08	1.38 ± .10	1.13 ± .10	1.34 ± .04	1.31 ± .07	
20:3ω6	0.09 ± .01	0.06 ± .01	0.20 ± .04	0.19 ± .02	0.17 ± .01	0.21 ± .02	Cubic
20:4ω6	6.71 ± .11*	9.42 ± .45	8.74 ± .17	8.97 ± .31	8.12 ± .31	7.68 ± .51	Linear
20:5ω3	1.80 ± .13*	0.84 ± .09	1.01 ± .07	0.77 ± .12	0.94 ± .07	0.90 ± .09	
22:0	0.30 ± .05	0.15 ± .04	0.16 ± .04	0.18 ± .03	0.26 ± .06	0.13 ± .05	
22:4ω6	1.61 ± .03*	2.76 ± .25	2.80 ± .11	2.47 ± .07	2.34 ± .11	2.10 ± .10	Linear
22:5ω6	3.64 ± .18	3.94 ± .26	1.56 ± .07	1.29 ± .11	1.19 ± .08	0.98 ± .06	Linear
22:5ω3	0.05 ± .01	0.09 ± .02	0.05 ± .03	0.20 ± .03	0.20 ± .06	0.30 ± .07	Linear
22:6ω3	6.28 ± .52	6.12 ± .53	10.78 ± .31	10.97 ± 1.6	11.57 ± .51	10.86 ± 3.13	Cubic

Group A, EFA-deficient; group B, control.

[a]Values are mean ± SEM of six rats.

[b]DMA, dimethyl acetal.

*Significantly different from control ($p < .05$).

TABLE 5

Fatty Acid Composition of Brain Phosphatidylethanolamine of Essential Fatty Acid (EFA)-Deficient Rats After Receiving Graded Amounts of Linolenate[a]

Fatty acid (wt %)	Percentage of 18:3ω3						Regression, $p < .01$
	0 (Group A)	0 (Group B)	0.5 (Group C)	1.0 (Group D)	2.0 (Group E)	4.0 (Group F)	
16:0	6.98 ± .76	6.56 ± .64	7.45 ± 1.50	8.89 ± 1.27	9.85 ± .86	7.35 ± .48	
16:1ω7	0.78 ± .19	0.44 ± .13	0.37 ± .10	0.66 ± .27	0.35 ± .02	0.38 ± .08	
18:0	13.09 ± 1.77	15.12 ± 1.42	15.87 ± 2.17	17.69 ± 2.24	18.15 ± 1.19	16.93 ± 2.18	
18:0DMA[b]	8.42 ± 1.04	8.41 ± 1.07	8.28 ± 1.43	5.13 ± 1.9	6.67 ± 1.03	7.54 ± .49	
18:1ω9	20.86 ± 3.16	12.51 ± 1.58	13.18 ± 2.10	17.60 ± 3.14	17.19 ± 17.3	22.40 ± 2.04	
18:1DMA	12.98 ± 1.99	9.57 ± 2.13	8.06 ± 2.19	5.76 ± 2.4	5.63 ± 1.19	8.51 ± 1.22	
18:2ω6	0.16 ± .06	1.46 ± .11	0.47 ± .19	0.57 ± .12	0.52 ± .14	0.63 ± .16	
20:1ω9	2.30 ± .56	2.42 ± .44	2.12 ± .70	2.96 ± .96	2.82 ± .53	2.86 ± .81	
20:3ω9	6.00 ± .37	1.43 ± .17	1.82 ± .15	1.53 ± .19	1.83 ± .42	1.52 ± .14	
20:3ω6	0	0.31 ± .06	0.24 ± .03	0.24 ± .04	0.25 ± .04	0.34 ± .06	
20:4ω6	9.26 ± .97*	13.83 ± 1.36	12.82 ± .91	12.37 ± 1.66	10.81 ± .94	10.02 ± 1.32	Linear
20:5ω3	1.86 ± .26*	1.12 ± .21	1.39 ± .18	1.12 ± .19	1.21 ± .17	0.89 ± .05	
22:0	0.31 ± .09	0.31 ± .05	0.33 ± .06	0.32 ± .02	0.32 ± .07	0.30 ± .07	
22:4ω6	2.72 ± .42*	4.92 ± .58	4.51 ± .41	4.05 ± .74	3.95 ± .45	2.35 ± .18	Linear
22:5ω6	3.46 ± .51*	5.46 ± .66	2.23 ± .28	1.46 ± .33	1.42 ± .17	0.91 ± .13	Quadratic
22:5ω3	0.22 ± .01	0.13 ± .04	0.38 ± .11	0.21 ± .04	0.34 ± .06	0.47 ± .10	Linear
22:6ω3	8.40 ± .92	9.33 ± 1.00	15.45 ± .90	16.63 ± 1.83	16.08 ± 1.43	15.92 ± 1.65	

Group A, EFA-deficient; group B, control.

[a]Values are mean ± SEM of six rats.

[b]DMA, dimethyl acetal.

*Significantly different from control ($p < .05$).

TABLE 6

Fatty Acid Composition of Brain Phosphatidylserine and Phosphatidylinositol of Essential Fatty Acid (EFA)-Deficient Rats After Receiving Graded Amounts of Linolenate[a]

Fatty acid (wt %)	Percentage of 18:ω3						Regression, $p < .01$
	0 (Group A)	0 (Group B)	0.5 (Group C)	1.0 (Group D)	2.0 (Group E)	4.0 (Group F)	
16:0	6.18 ± .18	9.41 ± 1.60	8.05 ± .60	12.79 ± 1.84	10.08 ± 1.84	10.18 ± 2.10	
16:1ω7	0.82 ± .18	0.84 ± .17	1.15 ± .37	0.49 ± .05	0.95 ± .08	0.83 ± .18	
18:0	40.88 ± 6.88	43.64 ± 7.37	46.18 ± 5.06	46.85 ± 3.57	41.17 ± 5.76	39.42 ± .38	
18:1ω9	23.33 ± 7.27	12.27 ± 2.54	12.20 ± 2.59	10.90 ± 1.11	16.64 ± 2.57	27.31 ± 8.06	
18:2ω6	0.77 ± .19	1.06 ± .15	1.04 ± .16	1.03 ± .18	1.36 ± .33	0.62 ± .27	
20:1ω9	0.83 ± .19	0.87 ± .18	1.13 ± .19	1.16 ± .30	0.87 ± .25	Trace	
20:3ω9	6.61 ± .89	1.87 ± .35	2.23 ± .25	1.53 ± .24	1.82 ± .22	1.48 ± .21	
20:3ω6	0.32 ± .16	0.62 ± .19	0.34 ± .11	0.79 ± .23	0.43 ± .13	0.37 ± .20	
20:4ω6	6.31 ± 1.89	13.84 ± 3.22	10.30 ± 1.40	10.93 ± .25	12.45 ± 3.37	7.46 ± 1.85	
20:5ω3	1.61 ± .42*	0.81 ± .29	1.19 ± .27	0.97 ± .15	0.79 ± .11	0.40 ± .14	
22:0	0.17 ± .09	Trace	0.66 ± .17	Trace	0.21 ± .03	Trace	
22:4ω6	0.68 ± .23	1.30 ± .32	1.83 ± .67	1.04 ± .14	0.84 ± .04	0.45 .22	Linear
22:5ω6	5.35 ± .59	4.43 ± 76	2.19 ± .25	1.80 ± .15	1.25 ± .11	0.64 ± .11	Linear
22:5ω3	0.09 ± .01	0	0	0.31 ± .009	Trace	Trace	
22:6ω3	6.51 ± .70	6.51 ± 1.57	9.50 ± 1.84	9.90 ± .81	10.31 ± 1.37	9.39 ± 2.44	

Group A, EFA-deficient; group B, control.

[a]Values are mean ± SEM of six rats.

*Significantly different from control ($p < .05$).

TABLE 7

Fatty Acid Composition of Brain Phosphatidylcholine of Essential Fatty Acid (EFA)-Deficient Rats After Receiving Graded Amounts of Linolenate[a]

Fatty acid (wt %)	Percentage of 18:3ω3					
	0 (Group A)	0 (Group B)	0.5 (Group C)	1.0 (Group D)	2.0 (Group E)	4.0 (Group F)
16:0	44.77 ± 3.59	46.07 ± 2.12	43.47 ± 4.04	42.32 ± 3.42	42.21 ± 1.41	43.22 ± 3.25
16:1ω7	1.96 ± .42	1.70 ± .30	1.19 ± .39	3.02 ± 1.18	0.86 ± .44	1.87 ± .35
18:0	12.91 ± 2.44	14.83 ± 2.15	15.98 ± 3.33	15.40 ± 2.20	18.59 ± .22	11.64 ± 2.02
18:0	12.91 ± 2.44	14.83 ± 2.15	15.98 ± 3.33	15.40 ± 2.20	18.59 ± .22	11.64 ± 2.02
18:1ω9	34.70 ± 3.23	29.89 ± 1.05	31.20 ± .54	30.20 ± .63	31.91 ± 1.38	33.16 ± 4.37
18:2ω6	0.54 ± .06	2.17 ± .99	1.10 ± .04	1.44 ± .15	1.73 ± .23	1.92 ± .58
20:1ω9	1.34 ± .51	1.19 ± .35	1.34 ± .49	1.07 ± .29	1.80 ± .02	0.77 ± .28
20:3ω9	1.19 ± .29	1.07 ± .85	—	0.34 ± .25	0.05 ± .01	0.17 ± .95
20:3ω6	0.10 ± .06	0.27 ± .09	0.12 ± .05	0.11 ± .06	0.21 ± .07	0.15 ± .03
20:4ω6	1.24 ± .22	1.06 ± .84	2.92 ± .80	3.63 ± .59	1.60 ± .09	2.29 ± .58
20:5ω3	0.32 ± .10	0.28 ± .05	0.21 ± .12	0.40 ± .10	0.60 ± .16	0.07 ± .02
22:0	—	—	—	—	—	—
22:4ω6	0.11 ± .04	0.18 ± .04	0.27 ± .04	0.22 ± .06	0.02 ± 0.00	0.40 ± .005
22:5ω6	0.30 ± .05	0.32 ± .02	0.17 ± .01	0.12 ± .08	—	0.12 ± .03
22:5ω3	—	—	—	—	—	0.47 ± .03
22:6ω3	0.47 ± .11	0.56 ± .17	1.75 ± .61	1.51 ± .06	0.55 ± .06	1.12 ± .22

Group A, EFA-deficient; group B, control.

[a]Values are mean ± SEM of six rats.

The major PUFA derived from 18:3ω3 in total lipids and phospholipid fractions was 22:6ω3, and its level in groups receiving dietary 18:3ω3 was significantly ($p < 0.01$) increased compared with group B, receiving no 18:3ω3, as demonstrated by other investigators (13,15, 19). However, there was no difference in the level of 22:6ω3 among the groups receiving different amounts of 18:3ω3. These results were quite contrary to those of liver and serum lipids of the same rats. Levels of 18:3ω3, 20:5ω3 and 22:6ω3 in liver lipids were elevated as dietary 18:3ω3 increased in a dose-dependent fashion (7). Such a dose-dependent increase in 18:3ω3 and 20:5ω3 was also observed in serum lipids (7). Reduction of 20:4ω6 by dietary 18:3ω3 was much greater in liver and serum lipids (7) than in brain lipids.

The level of 20:5ω3 in brain lipids was greatest in the

EFA-deficient group. The level of 22:6ω3 in the EFA-deficient group was not significantly different from that of group B, receiving 18:2ω6 with no 18:3ω3. This may be due to the reduced inhibitory effect of oleate family fatty acids, compared to ω6 fatty acids, on the desaturation of ω3 fatty acids (3).

The change in fatty acid composition in total lipids as affected by dietary 18:3ω3 was similar to that of phospholipids. Among individual phospholipids, PE was the major phospholipid containing high levels of ω3 and ω6 PUFA (Table 5), as was demonstrated by Babcock et al. (13). Unusually high levels of dimethyl acetals in PE and their absence in other phospholipid classes indicated that substantial portions of PE in brain lipids may be plasmalogen. PC contained very little PUFA derived from both ω3 and ω6 fatty acids (Table 7).

No 18:3ω3 was detectable in brain lipids following any dietary treatment. The levels of 20:5ω3 in groups receiving dietary 18:3ω3 do not seem to be significantly different from those of group B, receiving no 18:3ω3. Brown et al. (19) showed that the level of 20:5ω3 in brain phospholipids of rats fed a linseed oil diet for two generations was not different from those of rats fed a corn oil or hydrogenated coconut oil diet. It was suggested by Aftergood and Alfin-Slater (20) that adequacy of dietary 18:3ω3 can be estimated by the triene/pentaene (20:3ω9/20:5ω3) ratio of tissue lipids. The ratio was 3.12 for total lipids and 2.66 for phospholipids of the brain from the EFA-deficient group in this study. However, supplementation of 18:3ω3 did not restore the ratio to the normal range of 0.4. This result and lack of 18:3ω3 in the brain lipids indicated that dietary 18:3ω3 taken up by the brain is rapidly desaturated and elongated to 22:5ω3, and particularly 22:6ω3, or that 18:3ω3 is desaturated and elongated to 22:6ω3 at the blood-brain barrier and then taken up by the brain. Consequently, little 18:3ω3 or 20:5ω3 remains in the tissue. It was demonstrated that the major radioactivity was incorporated into 22:6ω3 of brain lipids within 48 hr after administration of [1-^{14}C] linolenic acid (21). Therefore, in brain lipids, the triene/pentaene ratio does not seem to be a reliable criterion for determining the adequacy of 18:3ω3 in diets.

In summary, reduction of 20:4ω6 in brain lipids by dietary 18:3ω3 was less pronounced compared to other tissue lipids. Unlike in other tissues, the level of 20:5ω3 (the precursor of triene PG) in brain tissue was not affected by dietary 18:3ω3.

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Glucosylceramide and the Level of the Glucosidase-Stimulating Proteins

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The concentration of β-glucosidase-stimulating proteins (called cohydrolase here) was measured in mouse liver and brain by immunoassay. Factors that might influence the levels of cohydrolase were examined. Injecting mice with an inactivator of glucosidase (conduritol B epoxide) rapidly produced elevations in liver glucosylceramide (the enzyme's substrate) and in liver and brain cohydrolase. Injection of glucosylceramide emulsified with Myrj 52 produced the same two effects in liver but not in brain. The increases in cohydrolase level induced by the enzyme inhibitor persisted in both organs for at least seven days, reaching 61–70% above the normal level. Injection of emulsified galactocerebroside, sphingomyelin and mixed glucosphingolipids but not of ceramide also produced rises in cohydrolase level. An increase in cohydrolase level resulted from injection of phenylhydrazine, which produces hemolysis and consequently an increased workload for the glucosidase of liver. When the enzyme inhibitor and/or larger amounts of glucosylceramide emulsion were injected (750 mg/kg body weight), increases in liver weight of 13 to 37% appeared within one day. The increased weight was characterized by increases in the weights of protein, total lipid and DNA and a very high increase in glucosylceramide level. These procedures have produced a rapidly developing model version of Gaucher disease in mice. Injected glucocerebroside also induced an elevated level of glucosidase activity.
***Lipids 21,* 702–709 (1986).**

Gaucher disease is a heritable human disorder characterized by a deficiency in the activity of glucosylceramide (GlcCer) glucohydrolase (EC 3.2.1.45). A heat-stable glycoprotein preparation capable of stimulating this enzyme was discovered in the spleen of a patient with the disorder (1,2). The GlcCer glucosidase activator, variously called Factor P (1), heat-stable factor (3), sphingolipid activator protein 2 (4) and cohydrolase sphingolipid I (5), will be referred to here as cohydrolase (CH). The activator, a mixture of similar low molecular weight proteins, occurs in a variety of normal tissues and also activates two other sphingolipid hydrolases, galactosylceramide (GalCer) galactosidase and sphingomyelinase (4,6).

Considerable accumulations of both cohydrolase sphingolipid-I (CH) and GlcCer are found in the spleen of patients with Gaucher disease. In addition, there is a pathologically large increase in the size of the reticuloendothelial system, represented by a generalized increase in functioning tissue rather than the simple accumulation of CH and GlcCer. While accumulation of the enzyme's substrate is readily explained by the inadequate hydrolase activity, the mechanism causing CH accumulation and organ hypertrophy is still obscure.

The study reported here tests the hypothesis that the concentration of GlcCer in a reticuloendothelial tissue is an important controlling factor for the tissue level of cohydrolase and organ size. If this is correct, interventions that increase the level of GlcCer ought to increase the level of CH and the organ size. We report here on the results of three such approaches with mice.

MATERIALS AND METHODS

Materials. Most of the materials have been described (5,7). Conduritol B epoxide, which specifically inactivates glucocerebrosidase in mice (8), was prepared chemically from *myo*-inositol (9,10) and injected intraperitoneally in saline solution at a dose level of 0.1 mg/g body weight. Sphingomyelin and GalCer were isolated from bovine brain, ceramide (stearoyl sphingosine) was prepared chemically and GlcCer was isolated from a human Gaucher spleen. Calf thymus DNA, Type V, was from Sigma Chemical Co. (St. Louis, Missouri).

A mixture of glycosphingolipids was prepared from outdated human red cells by conventional procedures: extraction with hexane-isopropanol, alkaline methanolysis of the ester lipids and silica gel chromatography with chloroform/methanol. The mixture consisted primarily of di-, tri- and tetraglycosylceramides, with a small amount of an unidentified phospholipid migrating on TLC plates between lactosylceramide and hydroxy GalCer. We are calling these lipids "glucolipids" because they are catabolized to GlcCer.

Each sphingolipid was coevaporated from solution with half its weight of a low-toxicity nonionic detergent, Myrj 52 (polyoxyethylene 40 stearate, ICI America Inc., Wilmington, Delaware) and emulsified in 0.9% saline by slight warming in an ultrasonic bath for 15 min. The concentration of sphingolipid was 9 or 45 mg/ml. The slightly milky emulsion was injected intraperitoneally at a dosage of 0.15 or 0.75 mg/g.

Animals. Hsd CFl mice (Harlan Industries), nominally 16 days old, were matched by computer into groups of five, each group having a similar mean and distribution of weights; two groups were chosen at random from a computer-generated table for each control or treatment (11). Each nursing mother was assigned two groups of five. The brains or livers from each group of five were pooled and each homogenate was assayed in duplicate, so that the average values cited in this paper (and standard deviations, where shown) were obtained from four values derived from 10 mice. The purpose of choosing this unusual approach was to reduce the importance of animal variability as well as the analytical workload. The analytical data from the two five-mouse groups in each treatment group typically differed by 5% or less.

Assay methods. CH was measured by a peroxidase-linked immunoassay after extraction, heat treatment and purification with a size exclusion column (5). This method of measurement is much more sensitive than the original assay method (stimulation of glucosidase) and more accurate because it avoids interference by endogenous enzyme and activation inhibitors (12,13).

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Glucosidase was assayed with [^{3}H]GlcCer (14). This assay does not detect the cytosolic glucosidase of unknown function (15), nor is it dependent on the tissue content of CH since an exogenous activator (taurocholate) is included in the incubation medium. In some of the liver samples (see Results), a considerable accumulation of GlcCer was seen and the possibility existed that this lipid might interfere with the assay. However, comparisons with 1, 2 and 4 mg of liver per incubation tube yielded similar specific activities with control and experimental groups. Evidently the radioactive micellar substrate did not equilibrate with the suspension of GlcCer in the homogenate during the 1-hr incubation.

Protein was determined with the Folin-Ciocalteu reagent with bovine serum albumin as standard (16); DNA was determined by the diaminobenzoic acid method (17).

The liver homogenates were analyzed for lipids after extraction with hexane/isopropyl alcohol (18). Total lipids were determined by weighing the extract nonvolatile contents. TLC was performed with precoated plates of silica gel 60, 10 × 10 cm (EM Laboratories), which were heated with a charring reagent. Some plates were quantitated with a computer video camera (19). Chloroform/methanol/aqueous 0.02% KCl (60:35:8, v/v/v) was used for the polar lipids; chloroform/methanol/HOAc (90:2:8, v/v/v) was used for ceramide.

RESULTS

Inactivation of glucosidase with conduritol B epoxide (CBE). In experiment 1, mice (4.9–7.8 g) were injected at time zero with either saline (control mice) or a 0.9% solution of CBE in saline. Liver and brain homogenates were made in 0.25 M sucrose and compared with the membranous and cytosolic fractions (140,000 × g for 30 min). Virtually all of the glucosidase activity (>95%) in the control mice was found to be in membrane-bound form. As noted before (20), CBE injection produced almost a complete loss of glucosidase activity within 1 hr. There was no noticeable recovery of activity within 5 hr.

The CH concentrations in control brain and liver (Table 1) were similar to those found before (5). CBE injection produced increases in liver total CH levels, 28% above the control value by 3 hr and 45% by 5 hr. An increase of 14% was visible even within 1 hr. While this is a relatively small increase, it is likely to be significant in view of the monotonic nature of the concentration/time relationship.

Brain CH rose in response to CBE too, but only after a delay. By 5 hr after injection the concentration had risen 11% compared to zero-time animals and 24% compared to 5-hr controls.

The cytosol from brain contained no detectable CH, but that from liver contained about 10% of the total activator protein (Table 1). The changes in CH noted above were mirrored in the membrane-bound CH, but the amount of cytosolic CH in liver did not change after CBE injection.

Summing the values for the CH in the cytosolic and membranous fractions yielded recoveries that were 81–93% (87% average) of the values in the unfractionated homogenate. This suggests that CH was relatively stable to enzymatic degradation during the subcellular separation step. Only the total CH was measured in the subsequent experiments.

Longer-term effects of CBE. In experiment 2, the injection of saline or epoxide was repeated as above and the mice were killed after 1, 4 or 7 days. All animals, control and experimental, were killed at the same time of day to eliminate the possibility of diurnal changes. Homogenates were prepared in extraction buffer (5 mM phosphate pH 7, 1 M NaCl) (5) instead of sucrose solution.

Both organs showed some restoration of glucosidase activity within 1 day (left side of Table 2), and a substantial restoration several days after inactivation of the enzyme. Similar data were found elsewhere (8,14). Glucosidase activities in control mice peaked in the livers of the four-day group (at weaning time) but showed decreasing values in brain with age. A similar drop in rat brain glucosidase activity with the approach of weaning has been found (21), probably a reflection of a decreasing turnover rate for gangliosides.

In the CBE-treated mice, the CH concentrations in both organs (right side of Table 2) were markedly elevated over the control values at each time point. The degree of elevation was greater than that seen after 5 hr (Table 1), and it is evident that the effect of CBE continued well after all of the inhibitor had been excreted (22).

While the CH level in liver was quite constant with increasing age over the seven-day period studied, brain showed a gradual decrease. This may reflect the known slowdown in ganglioside turnover that occurs at this age.

A repetition of the first two experiments (experiment 3) again showed that CBE produced increases in liver total CH (19% after 1 hr, 54% after 4 days). As before, brain showed an increase only after an initial delay—65% above normal by 4 days.

Effects of glucosphingolipid injection. A second mode of manipulating GlcCer levels in tissues was tested. In experiment 4, mice (6.2–7.8 g) were injected with either detergent solution in saline (control mice) or with the emulsion of red cell ceramide oligosaccharides (dosage about 0.15 mg/g body weight). One set of 20 mice (10 control, 10 experimental) was killed after one day. A second set was injected three times—at time zero, after one day and after four days; they were then killed one day later, five days after starting.

TABLE 1

Changes in Cohydrolase Following Injection of Mice with Conduritol B Epoxide

	Cohydrolase concentration (ng/mg wet tissue) in liver in mice killed at				Cohydrolase concentration (ng/mg wet tissue) in brain of mice killed at			
	0 hr	1 hr	3 hr	5 hr	0 hr	1 hr	3 hr	5 hr
Controls								
Homogenate	3.34			3.26	1.67			1.50
Cytosol	0.30			0.31	—			—
Membranes	2.62			2.63	1.52			1.46
CBE-treated								
Homogenate		3.81	4.28	4.72		1.66	1.68	1.86
Cytosol		0.31	0.32	0.30		—	—	—
Membranes		2.90	3.43	3.81		1.48	1.62	1.78

TABLE 2

Longer-Term Changes in Glucosidase and Cohydrolase Levels in Liver and Brain after Injection of Glucosidase Inactivator, Conduritol B Epoxide

Group	Glucosidase activity (nmol/hr/mg)	% of control	Cohydrolase concentration (ng/mg)	% of control
Liver				
Controls, 1 day later	3.12 ± .15		3.17 ± .18	
+ CBE 1 day later	0.24 ± .11	8	5.10 ± .08	161
Controls, 4 days later	3.63 ± .07		3.05 ± .13	
+ CBE 4 days later	1.58 ± .06	44	4.90 ± .08	161
Controls, 7 days later	2.94 ± .03		3.10 ± .08	
+ CBE 7 days later	1.24 ± .03	42	4.58 ± .06	156
Brain				
Controls, 1 day later	1.23 ± .03		1.83 ± .13	
+ CBE 1 day later	0.14 ± .06	11	3.08 ± .09	168
Controls, 4 days later	1.07 ± .08		1.68 ± .06	
+ CBE 4 days later	0.27 ± .03	25	2.87 ± .05	171
Controls, 7 days later	1.06 ± .03		1.57 ± .05	
+ CBE 7 days later	0.48 ± .04	45	2.37 ± .05	151

Experimental design as in Table 1. Groups of five mice each were injected at time 0 with saline or CBE. Data, based on wet weight of tissue, are calculated from two groups killed at each time point.

The levels of brain CH and glucosidase did not change in either set. This may mean that entry of the lipid emulsion was blocked by the blood-brain barrier. However, both proteins responded positively in liver. The increase in liver glucosidase activity was 14% by one day and 23% by five days (Fig. 1). The CH concentration responded more vigorously to the glycolipid load: a 33% increase within one day and a 60% increase by five days (Fig. 2). Except for the glucosidase activities after one day, the observed increases are clearly significant. These results can be interpreted to mean that the injected glucosphingolipids had reached the liver and, after catabolic degradation to GlcCer, had induced an elevated level of CH and, to a smaller extent, of glucosidase. An elevation in liver glucosidase activity following injection of red cell sphingolipids has also been observed in rats (23).

The injection of erythrocyte glucosphingolipids was repeated (experiment 5) and again there was no change in the level of brain CH while the level of liver CH rose 34% in one day.

Effect of injecting simple sphingolipids. Since CH also activates GalCer and sphingomyelin hydrolases (4,6), we thought that elevations in the tissue levels of these lipids might act like the hypothesized elevation in GlcCer in experiments 4 and 5. A similar injection schedule and dosage (three injections) was followed in experiment 6 with individual sphingolipids—ceramide, GalCer and sphingomyelin—and the animals were analyzed only after five days. As expected from the previous experiment, there were no significant changes in brain, presumably because of the blood-brain barrier. In liver, only small changes were seen in the glucosidase activity, except for an 11% rise after sphingomyelin injection (left column of Table 3). Cohydrolase levels in liver (right column of Table 3) showed distinct increases after injection of sphingomyelin (25%) and GalCer (23%). Ceramide injection produced no clear effect.

The injection of the three sphingolipids was repeated (experiment 7), but with only one injection of lipid and animals being killed on the fifth day. Even with this lower lipid load, the glucosidase activity in liver was again slightly elevated (7%) by sphingomyelin injection. The CH concentration in liver again showed a distinct positive response to loading the animals with sphingomyelin and GalCer (+21% with both) but not to ceramide.

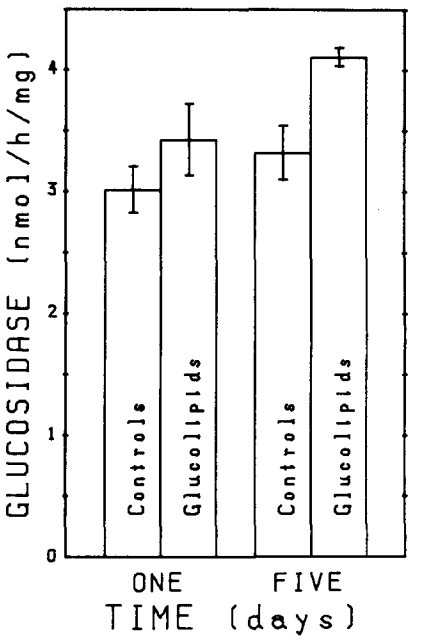

FIG. 1. Changes in liver glucosidase activity produced by injecting emulsified glucolipids from red cells (experiment 4). Liver glucocerebrosidase was assayed as described in the text. The left bar in each pair is from the control mice killed after 1 or 5 days. Error bars are calculated from the four analytical values obtained from the two five-mouse pools used for each bar (while the standard deviation formula used was used for the calculation, this is not strictly the same as the standard deviation obtained from single analyses of four different mice.)

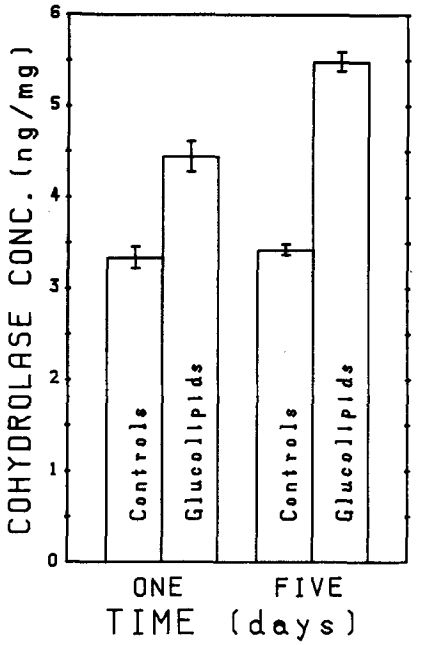

FIG. 2. Changes in liver cohydrolase concentration produced by injecting glucosphingolipids from red cells (experiment 4). Animals are the same ones indicated in Fig. 1.

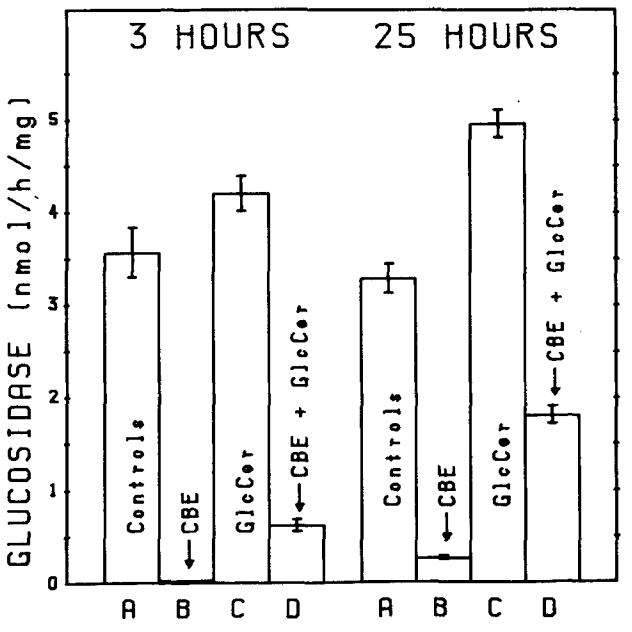

FIG. 3. Changes in glucosylceramide glucosidase of liver on injecting emulsified ceramide (A), emulsified ceramide with CBE (B), emulsified GlcCer (C) or emulsified GlcCer with CBE (D). Data are shown for mice killed 3 hr and 25 hr after injection (experiment 8).

TABLE 3

Effects on Liver of Injecting Sphingolipid Emulsions

	Glucosidase activity (nmol/hr/mg tissue)	Cohydrolase concentration (ng/mg tissue)
Myrj controls	2.78 ± .14	3.64 ± .15
Ceramide/Myrj	2.51 ± .22	3.46 ± .11
Galactosylceramide/Myrj	2.92 ± .11	4.46 ± .18
Sphingomyelin/Myrj	3.09 ± .25	4.54 ± .14

Each mouse was given three injections of detergent solution or lipid emulsion at time 0, after 1 day and after 4 days and then was killed after 5 days. Data reported are the averages of four analyses derived from two sets of five mice each.

The above loading experiments are similar to those of Kampine et al. (23), who showed that injecting rats with red cell sphingolipids (including sphingomyelin) induced a rise in liver sphingomyelinase. It appears likely that injected sphingomyelin and GalCer reach the liver.

In experiment 8, loading was carried out with emulsified GlcCer. The dosage of lipid in this experiment was fivefold higher, 0.75 mg/g body weight. Because of the difference in molecular weights between GlcCer and red cell glycolipids, the molar dosage of GlcCer was actually somewhat greater than fivefold higher. The control mice were injected with emulsified ceramide (at the same molar dosage as the cerebroside) instead of with detergent alone. In addition, since inactivation of tissue glucosidase should protect the GlcCer load against catabolic loss, half of the control and cerebroside-injected mice were also injected with CBE as in experiment 1. The animals were killed after 3 and 25 hr; only the livers were analyzed.

As seen before, the glucosidase levels in CBE-injected mice were virtually zero at the 3-hr time point, and some reappearance of active enzyme molecules was visible at the 25-hr point (Fig. 3, bars B). GlcCer injected into normal mice (bar C vs bar A) produced distinct increases in enzyme activity: 18% and 51% above normal after 3 and 25 hr, respectively. A similar augmentation in glucosidase activity was seen in the mice injected with both CBE and lipid (bar D vs B). The reappearance of enzyme activity was remarkably fast in the latter animals: 16% of normal at 3 hr and 55% of normal at 25 hr. It appears likely that the GlcCer absorbed by the liver induced more rapid synthesis of the glucosidase or protected it against normal catabolic loss.

Cohydrolase responded similarly (Fig. 4), with the rise in concentration being greatest 25 hr after injecting the combination of inhibitor and glucolipid (bar D is 70% higher than bar A). Even in a short interval (3 hr) after CBE or GlcCer injection, a small but distinct increase in CH was visible. As with the previous experiment with CBE (Table 1), CH synthesis or level seemed to respond quickly to an increased glucosidase workload. Calculation of the total CH in liver, taking into account the liver growth (see next section), shows that the amount of CH in the CBE/GlcCer animals was 96% above normal within 25 hr.

Liver enlargement. The liver weight (Fig. 5) was markedly increased by all three treatments of experiment 8, especially with the combination of CBE and GlcCer. The maximal effect was a 37% increase at 25 hr.

The control livers after 25 hr contained, on the average, 49.5 mg protein (18.1% of total weight), and the CBE/GlcCer livers each contained 77 mg protein (20.5% of total weight). Thus the gains in liver weight were accompanied by an even greater gain in total organ protein,

56%. This shows that the increase in liver size could not be the result of hydration or fat storage. This sudden enlargement was not a reflection of a generalized increase in body weight.

Since experiment 8 was done with controls injected with ceramide/Myrj, the possibility had to be considered that this emulsion had a toxic effect that made the livers smaller than those of the animals injected with GlcCer/Myrj. This question was tested in experiment 9, in which two five-membered groups of control mice were given Myrj alone (no ceramide) and two groups were given Myrj + GlcCer + CBE at the same dosages used in experiment 8. After 25 hr, the average body weights were 5.79 g and 5.50 g for the control and experimental mice (−5%). The average liver weights were 243 mg for control mice and 304 mg for experimentals (+25%). The effect of this treatment on CH concentration, as observed in the previous experiments, was to raise the level considerably (+77%). Injected GlcCer again speeded the restoration of glucosidase activities (to 49% of normal). Thus the effects on liver weight, CH level and glucosidase activity found in experiment 8 were repeatable and not artifacts due to the use of ceramide in the control animals.

In addition we tested the possibility that the Myrj in the control animals might have had some effect (experiment 10). Groups of 10 mice were injected with either *a*, saline; *b*, saline + CBE; *c*, saline + CBE + Myrj + ceramide; *d*, saline + CBE + Myrj; or *e*, saline + Myrj, and were killed 25 hr later. The individual liver weights were measured to permit use of analysis of variance with the conservative Bonferroni Multiple Comparison procedures (Table 4). The body weights were not significantly affected by the treatments. The two controls, *a* and *e*, also did not differ significantly with respect to liver weight (i.e., Myrj injection did not affect the liver weight). However, the two control groups differed from the three CBE groups with a high degree of significance. The largest effect, an increase in liver weight of 22% ($p < 0.0001$), was seen on comparing the CBE/ceramide/Myrj group with the Myrj control mice.

When the groups were compared on the basis of percentage of liver (Table 4, column 3), highly significant differences ($p < 0.0001$) were seen between the CBE groups and Myrj controls. The differences were significant with regard to the saline control mice also, but only when the ordinary t-test was used, evidently because of the greater statistical noise due to variability in body weights.

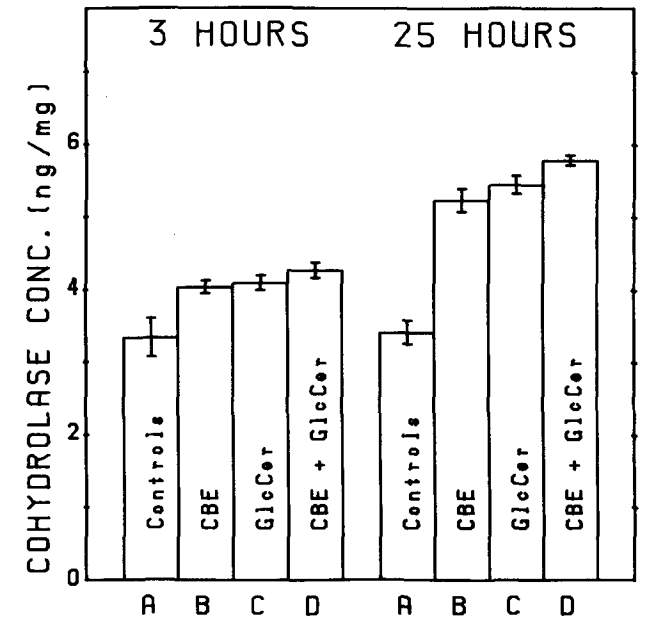

FIG. 4. Changes in cohydrolase concentration on injecting emulsified ceramide (A), emulsified ceramide with CBE (B), emulsified glucosylceramide (C) or glucosylceramide with CBE (D). See legend to Fig. 3 (experiment 8).

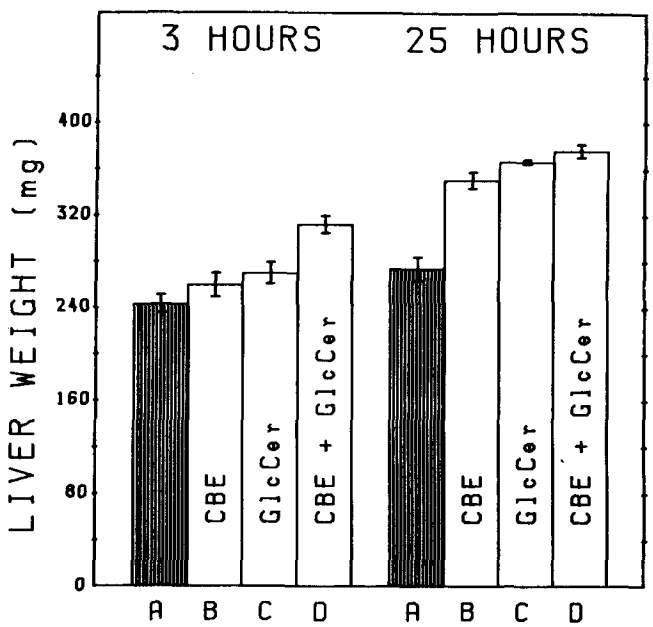

FIG. 5. Changes in liver weight on injecting ceramide (A), ceramide + CBE (B), glucosylceramide (C) or glucosylceramide + CBE (D) (experiment 8). Error bars show deviation from the mean of the two groups of mice (five mice/group).

TABLE 4

Effect of CBE Injection, With or Without Detergent and Ceramide, on Liver and Body Weight

Treatment	Body weight (g)	Liver weight (mg)	Liver/body (%)
a: Saline controls	6.43 ± .43	256 ± 16	4.00 ± .31
b: + CBE	6.51 ± .60	289 ± 17	4.47 ± .42
c: + CBE + Ceramide/Myrj	6.78 ± .59	304 ± 3	4.52 ± .41
d: + CBE + Myrj	6.31 ± .60	282 ± 9	4.52 ± .50
e: + Myrj	6.89 ± .51	249 ± 20	3.63 ± .43

Significant differences (p values): liver weight, 0.0001 (*a* vs *b* and *c*; *e* vs *b*, *c* and *d*); 0.002 (*a* vs *d*); 0.015 (*c* vs *d*); liver/body, 0.0001 (*e* vs *b*, *c* and *d*). Mice were injected with conduritol B epoxide (0.1 mg/g body weight) in saline, with added Myrj (0.27 mg/g) or with added stearoyl sphingosine (0.54 mg/g) in Myrj. They were killed 25 hr later and each animal's liver was weighed separately. The averages and S.D.s are listed. See experiment 10 for details.

The inclusion of ceramide with CBE and Myrj (*c* vs *d*) significantly enhanced the CBE stimulation of liver growth (Table 4). Thus it would appear that part of the growth effect seen in experiment 8, which included ceramide/Myrj in the CBE, was due to a synergistic interaction. This may arise from the fact that ceramide is the lipoidal precursor of GlcCer; the injected lipid may increase the rate of GlcCer synthesis and thus enhance the CBE effect on liver growth.

In none of the groups in this last experiment was there an effect on brain weight. We had previously reported a statistically significant increase of 13% in brain weight (and 9% in liver weight) of mice injected with eight daily doses of CBE, compared to saline control mice (20). Evidently a 25-hr period of glucosidase depletion is too short to produce this intriguing increase in brain weight.

Determination of the protein concentrations in the various livers from experiment 10 showed slightly elevated values in the mice with the heaviest livers: 11.3 and 11.6% in the two groups of saline control mice, *a* and *e*, and 12.3% in the CBE/Myrj/ceramide mice. This confirms the similar observation made in experiment 6.

In experiment 11, a comparison was made of mice injected with GalCer and GlcCer, both emulsified in Myrj and injected at the same dosage (0.75 mg/g). Twenty-five hours later the two groups did not differ significantly in body weight, but the GlcCer animals had livers that were 16% heavier ($p < 0.001$). Thus the weight enhancement, unlike the enhancement in CH concentration by both lipids (Table 3), was quite specific with respect to the sugar moiety of the cerebroside.

The DNA concentrations in the livers of experiment 10 were very similar in all groups (2.44 to 2.49 mg/g), and the weight of total DNA was 20% higher in the larger livers (*c* vs *e*). In experiment 11, the total DNA was 0.611 mg/liver in the animals injected with GalCer and 0.715 mg/liver in animals injected with GlcCer (+17%).

Changes in liver lipid composition. The concentrations of total lipids in the 25-hr livers in experiment 8 were 66, 77, 77 and 102 mg/g wet wt for controls, CBE mice, GlcCer mice and CBE/GlcCer mice, respectively. The maximum increase was $(102 - 66)/66 = 55\%$, a striking change. Even at the 3-hr time point there was a noticeable increase (12%) in liver lipids of the CBE/GlcCer group. The increases in the 25-hr mice were even more marked when compared on a total liver basis: the weights of lipid were 18, 27, 28 and 38 mg per liver for the same four groups (a maximal increase of 111%).

Examination of the total lipids from this experiment by semiquantitative thin layer chromatography (TLC) showed that the animals treated with CBE, GlcCer or the two together had accumulated a striking amount of GlcCer. At the sample level applied to the plate, equivalent to 0.13 mg of liver, the GlcCer spot from the control mice was barely visible while the experimental groups contained GlcCer at a level similar to those of the major lipids.

The GlcCer concentrations were 0.04, 6.5, 7.7 and 29 mg/g liver for the four groups. This lipid accounted for a substantial portion of the increases seen in the total lipids.

Ceramide typically shows a dual spot on TLC plates in which the upper one (very long chain acids) is heavier than the lower one (mainly stearoyl sphingosine) in non-neural organs. The mice injected with emulsified stearoyl sphingosine, compared with Myrj-injected mice, showed a small but definite increase in the intensity of the lower spot, suggesting that a small portion of the injected ceramide had reached the liver. The mice treated with GalCer and GlcCer emulsions showed a slight elevation in the concentrations of both ceramide spots, especially the ones with shorter fatty acids. This kind of fatty acid distribution is characteristic of the GlcCer that accumulates in Gaucher spleen (24) and illustrates how closely our Gaucher mice mimic the natural form.

Quantitative analysis (25) of the liver extracts from experiment 10 revealed ceramide concentrations of about 110 μg/g in the control mice and about double this in the mice given CBE (with or without ceramide).

Responses to internal hemolysis. We next tested a third method of raising the GlcCer concentration in liver. Injection of phenylhydrazine induces hemolysis and an increased requirement on the part of the reticuloendothelial system to destroy the damaged red cells. This was found to induce elevated activities of glucocerebrosidase, galactocerebrosidase and sphingomyelinase in rat spleen (23) and glucosidase in mouse spleen and liver (14). In experiment 12, 5.1–6.8 g mice were given a single injection of phenylhydrazine.HCl (0.04 mg/g body weight) in saline. As noted before (14), the glucosidase activity in liver rose in response (Table 5). Analysis after 1, 2 or 4 days showed activities that were 9, 15 and 54% above each pair of corresponding control groups, respectively. The CH concentrations also rose (Table 5), but with a longer lag period: 0, 11 and 30%, respectively.

DISCUSSION

Our data show that mouse liver, and brain in some cases, can respond to various treatments by developing an elevated concentration of cohydrolase. Three types of treatments produced this effect: inactivation of β-glucosidase by an inhibitor (experiments 1, 2, 3 and 8), injection of certain sphingolipids (experiments 4, 5, 6, 7 and 8) and induction of hemolysis (experiment 12). All three

TABLE 5

Effects of Phenylhydrazine Injection on Glucosidase and Cohydrolase Levels in Liver

	Glucosylceramide hydrolase (nmol/hr/mg tissue)	Cohydrolase (ng/mg tissue)
Controls		
0 time	3.36 ± .05	3.04 ± .05
1 day	3.39 ± .16	3.01 ± .08
2 days	3.17 ± .10	3.00 ± .07
4 days	2.42 ± .04	3.04 ± .05
Phenylhydrazine		
1 day	3.70 ± .17	2.98 ± .05
2 days	3.64 ± .29	3.34 ± .05
4 days	3.72 ± .22	3.96 ± .11

Groups of five mice each received a single injection of phenylhydrazine.HCl or saline and were sacrificed after 1, 2 or 4 days. Details in experiment 12. The data are calculated from two groups sacrificed at each time point.

treatments have in common the effect of increasing the tissue concentration of substrate for one or more of the three enzymes known to respond to cohydrolase (4,6). In the case of the first approach, increased levels of GlcCer are derived from the normal catabolic degradation of tissue glucolipids (gangliosides and ceramide hexosides); with liver there is the additional load of hydrolyzing blood elements as their life spans come to a conclusion. In the case of the hemolytic approach, the increased substrate levels are derived from the hydrolytic breakdown of red cells. Direct demonstration of an increase in GlcCer was shown by TLC in mice injected with CBE and with GlcCer (experiment 8). The same effect has been reported for CBE by Kanfer et al. (26).

Our observed accumulation of CH corresponds to that seen in a human genetic disorder, Gaucher disease. Our findings show that the increase in CH appears rapidly and cannot be attributed to some secondary, late-developing phenomenon that might occur in the natural disorder.

It is apparent from our TLC results that the tissue concentration of GlcCer was many-fold higher than normal in some animals, yet the increase in CH (and glucosidase) was much smaller. It may be that only a portion of the accumulated GlcCer was localized in a region that could influence the formation of CH. Another explanation is that the mechanism by which the CH accumulation was produced has a limited velocity, and longer-term experiments would show increases comparable to those seen in the human disorder. The latter develop over a much longer time.

Cohydrolase was not the only substance found to accumulate in liver as the result of injecting GlcCer. Total and specific activity of glucosidase also increased (Fig. 3) and, in the case of mice that were also given CBE (experiments 8 and 9), the restoration toward normal activity of the enzyme was hastened by the GlcCer. Injection of GlcCer also increased total protein and protein concentration (experiments 8 and 10) and total DNA weight per liver (experiments 6, 7, and 8). These findings may mean that GlcCer causes an actual increase in the concentration of tissue glucosidase or the proliferation of certain cell types that are rich in content of the enzyme. We have recently shown that GlcCer is absorbed by neuroblastoma cells and increases their utilization of [^{3}H]thymidine for the synthesis of DNA (Datta, S.C., Snider, R.M., and Radin, N.S., unpublished data).

The unexpectedly rapid liver growth produced in our mice by accumulating GlcCer (experiments 8, 9, 10 and 11) is consistent with the great enlargements in spleen, liver and bone marrow that develop over time in Gaucher disease. Because of the link sometimes observed between Gaucher disease and proliferation of B-cells, the proposal has been made that GlcCer could stimulate their formation (27). GlcCer stimulates the formation of fruiting bodies in a fungus and wheat (28). The possibility should be considered that this lipid plays a specific role in determining the size of certain organs or in controlling the rate of proliferation of certain cells.

Our finding that GlcCer, but not GalCer or ceramide, reached and entered the liver from the peritoneum rather efficiently finds a parallel in an in vitro uptake study with cultured macrophage (29).

While speculation over a mechanism for the effects of GlcCer may be inappropriate here, it should be mentioned that GlcCer is the lipoidal precursor of the gangliosides. That cells loaded with GlcCer send some of their GlcCer "upstream" (by addition of sugars) has been demonstrated for cultured Gaucher fibroblasts (30). Gangliosides have been reported to stimulate nerve growth in cultures (cf. 31). Ganglioside GM_1 is involved in thymocyte proliferation (32).

Other researchers have reported large, rapid increases in liver and DNA weight as the result of single injections: insulin in diabetic rats (33), trioiodothyronine in rats (34) and lead nitrate in rats (35). Further studies (to be published separately) in this laboratory of the growth phenomenon have confirmed the effect described in this paper.

Our unconventional research design has been used before (36) and has recently been described in more detail (11). It was found in preliminary studies that the primary source of variability in results arose in individual mice, rather than in the analytical procedures. The use of a "multimouse"—a pool of five mice—was adopted to reduce this source of experimental noise. Every experiment was run twice since two multimice were used for each treatment group. Each multimouse was independently assayed in duplicate, so that four values were averaged to yield many of the values reported here. If one mouse in a multimouse group exhibited a highly aberrant value, the effect on the pool value should be greatly reduced by the other four mice, and the chance of the second multimouse in the same experimental group having a similar aberrant value in the same direction would be very small. Since the duplicate enzyme, CH, and protein assays typically agreed within a few percent; since the time studies generally showed monotonic progression; since the two multimice at each point yielded similar values; and since all the experiments involving the same treatment, such as CBE injection, showed identical kinds of responses (smaller or larger increases above the control values), our observed relationships can be considered secure. The values for CH in the various multiple control groups were similar to each other within each experiment (see, for instance, the four control groups—8×5 mice—listed in Table 5).

In the case of CH, when 46 control multimice were compared with 56 multimice treated with CBE, sphingolipids or phenylhydrazine, an increased concentration was seen in the livers of every experimental multimouse. A total of 230 individual control mice and 280 experimental mice were used in this study. In one experiment, individual livers were weighed and all weight increases proved highly significant by a standard statistical test.

The idea of selecting animals to produce matched groups, with similar distributions around the mean of a selected variable, has been recommended by several biostatisticians (37–39).

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METHODS

An Improved Method for the Separation of Molecular Species of Cerebrosides

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A method employing reversed-phase high performance liquid chromatography (HPLC) has been developed for the analysis of molecular species of underivatized cerebrosides. By using a high carbon (20%) C18-silica bead column, monohexosylceramides isolated from bovine brain and Japanese quail intestine could each be separated into 42 and 56 peaks, respectively, and could subsequently be recovered. Under our analytical conditions, underivatized cerebrosides with 24:1 and 22:0 fatty acids were completely separated by single step HPLC. Each of the molecular species was then analyzed by high performance thin layer chromatography and gas chromatography-mass spectrometry (or fast atom bombardment mass spectrometry). This analytical method permits the complete identification of molecular species of glycosphingolipids.
Lipids 21, 710-714 (1986).

High performance liquid chromatography (HPLC) has been used for the separation of molecular species of glycosphingolipids with (1-3) or without (4-5) derivatization. The latter procedure is very useful since the chemical structure of glycosphingolipids can directly be examined. However, "critical pairs" of underivatized glycosphingolipids were not fully resolved by the previous procedures (6-8). Complex lipids having monounsaturated fatty acid side chains were eluted from a reversed-phase column together with those with saturated fatty acid side chains having two carbons less. For example, underivatized cerebrosides with 24:1 and 22:0 fatty acids were eluted together. Smith et al. (8) reported that by combined use of argentation and reversed-phase HPLC these molecular species could be almost completely resolved. However, use of analytical systems involving two subsequent chromatographic steps is time-consuming.

Here, we show that critical pairs of glycosphingolipids can be separated by single step HPLC using a high carbon C18-silica bead column. Using this procedure, the molecular species of monohexosylceramides in avian intestine have carefully been reexamined (9).

MATERIALS AND METHODS

Materials. Cerebrosides were prepared from bovine brain. Monohexosylceramide from Japanese quail intestine was purified as described previously (9). The composition of the intestinal monohexosylceramide used in the present study is shown in Table 1. Standard fatty acids were purchased from Applied Science (State College, Pennsylvania. HPLC solvents were from Wako Pure Chemicals (Tokyo, Japan).

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TABLE 1

Chemical Composition of Japanese Quail Intestinal Monohexosylceramide Used[a]

		Fatty acid composition		
			Nonhydroxy acids (%)	Hydroxy acids (%)
Ratios of carbohydrate (%)		16:0	5.18	6.03
		16:1	—	—
Galactose	80.65	18:0	3.14	0.41
Glucose	19.35	18:1	—	—
		20:0	1.79	4.36
Long chain base composition (%)		21:0	0.33	2.44
		22:0	3.76	42.83
t18:0	43.4	22:1	—	—
d18:1	27.0	23:0	1.31	13.15
d18:0	8.9	24:0	2.85	11.61
t20:0	5.8	24:1	—	—
d20:1	11.4			
d20:0	3.0	Subtotal	18.36	80.33

[a]Data were obtained by procedures described previously (11-13).

Instrumentation. HPLC analyses were done with a Waters Associates (Milford, Massachusetts) model M-45 solvent delivery system and model U-6K injector. Reversed-phase HPLC was performed using a water-jacketed ERC-ODS-1272 column (20 cm × 6 mm id, Erma Optical Works, Tokyo, Japan). A variable wave length spectromonitor model S-310A (Soma Kogaku, Tokyo, Japan) was used at 210 nm for the nonspecific detection of underivatized glycosphingolipids. Methanol was used as eluent at a flow rate of 1 ml/min. The column was kept at a given temperature by circulating water through the jacket. Using N-acetyl derivatives of galactosylceramides, apparent ε_{210}, $M^{-1} \times cm^{-1}$ values were determined to be 8.84×10^2, 5.71×10^2 and 5.54×10^2 for those containing *trans*-D-erythro-2-aminooctadecene-1,3-diol (sphingosine; d18:1), *trans*-D-erythro-2-amino-octadecene-1,3-diol (sphinganine; d18:0) and D-ribo-2-aminooctadecene-1,3,4-triol (4-D-hydroxysphingosine; t18:0) long chain bases, respectively. Under our assay condition, about 1.6 μg or 1.0 μg of each cerebroside which contains sphingosine or phytosphingosine, respectively, could be detected.

High performance thin layer chromatography (HPTLC). For analytical purposes, borate-impregnated HPTLC and borate-free HPTLC plates were used. Borate-impregnated HPTLC plates were prepared by soaking the plates in 2.5% sodium tetraborate in 50% methanol (10) prior to activation at 120 C for at least 90 min. Lipids were applied to the plates and the plates were developed using solvent system I (chloroform/methanol/water, 65:25:4, v/v/v) for borate-impregnated HPTLC or solvent system II (chloroform/methanol/water, 80:20:0.5, v/v/v) for borate-free HPTLC. Spots were detected by spraying with α-naphthol/H_2SO_4 reagent.

Other analytical methods. Samples were analyzed for carbohydrate, fatty acid and long chain base contents by gas liquid chromatography (GLC) and GC/mass spectrometry (MS) as described previously (11,12). Long chain bases were also analyzed by reversed-phase HPLC (13). Fast atom bombardment (FAB)/MS analysis was performed as described previously (9).

RESULTS AND DISCUSSION

It is well known that the separation of glycosphingolipids by reversed-phase HPLC is mostly according to fatty acid chain length in ceramide moiety. In this study the factors that affect the separation of molecular species were carefully examined using bovine brain cerebroside. Each isolated fraction was further subjected to carbohydrate, fatty acid and long chain base analyses.

We examined the effect of temperature on the separation of critical pairs of cerebrosides. Increasing temperature resulted in reduced retention times and back pressure. When chromatography was performed at a temperature higher than 40 C, critical pairs of cerebrosides could not be separated (Figs. 1 and 2A). Column temperatures of 20–26 C yielded the best results, with the ERC-ODS-1272 column giving as many as 42 peaks, as shown in Figure 2B. Critical pairs of cerebrosides could be clearly separated under this condition (see also Figure 3).

Columns containing 10% carbon used in the present study (Table 2) gave little separation of critical pairs of cerebrosides at any temperature examined. On the other hand, columns containing 20% carbon produced better cerebroside resolution (not shown). Thus, it can be concluded that the high carbon columns are best suited for the separation of molecular species of glycosphingolipids.

From these results, it is apparent that both the column temperature and the carbon content of the columns are important factors in determining the separation of molecular species of glycosphingolipids. The reasons for

TABLE 2

Columns Examined at 26 C of Column Temperature

Column	Carbon content (%)	Theoretical plate number
Nucleosil 7C-18 (Chemco)	10	13000
ERC-ODS-1161 (Erma)	10	11000
ERC-ODS-1262 (Erma)	20	10000
ERC-ODS-1272 (Erma)	20	17000

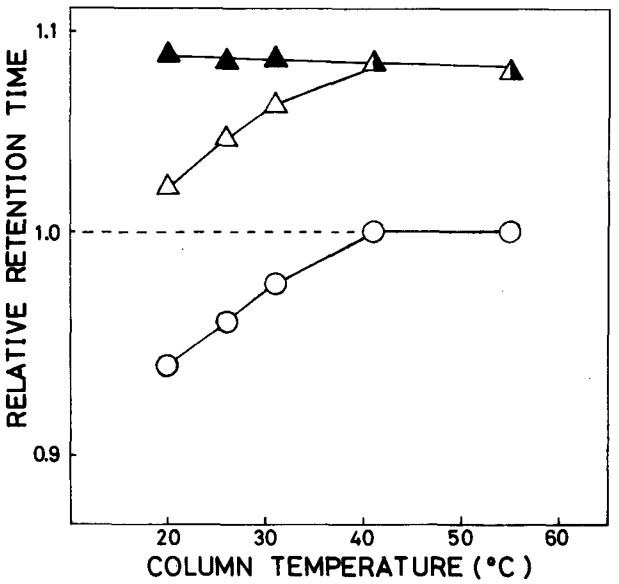

FIG. 1. Effect of column temperature on relative retention time. Chromatographic conditions are described in the text. The relative retention time of each galactosylceramide was calculated on the basis of sphingosine (d18:1)/h22:0 as 1.0. d18:1/22:0 (▲); d18:1/24:1 (△); d18:1/h24:1 (○).

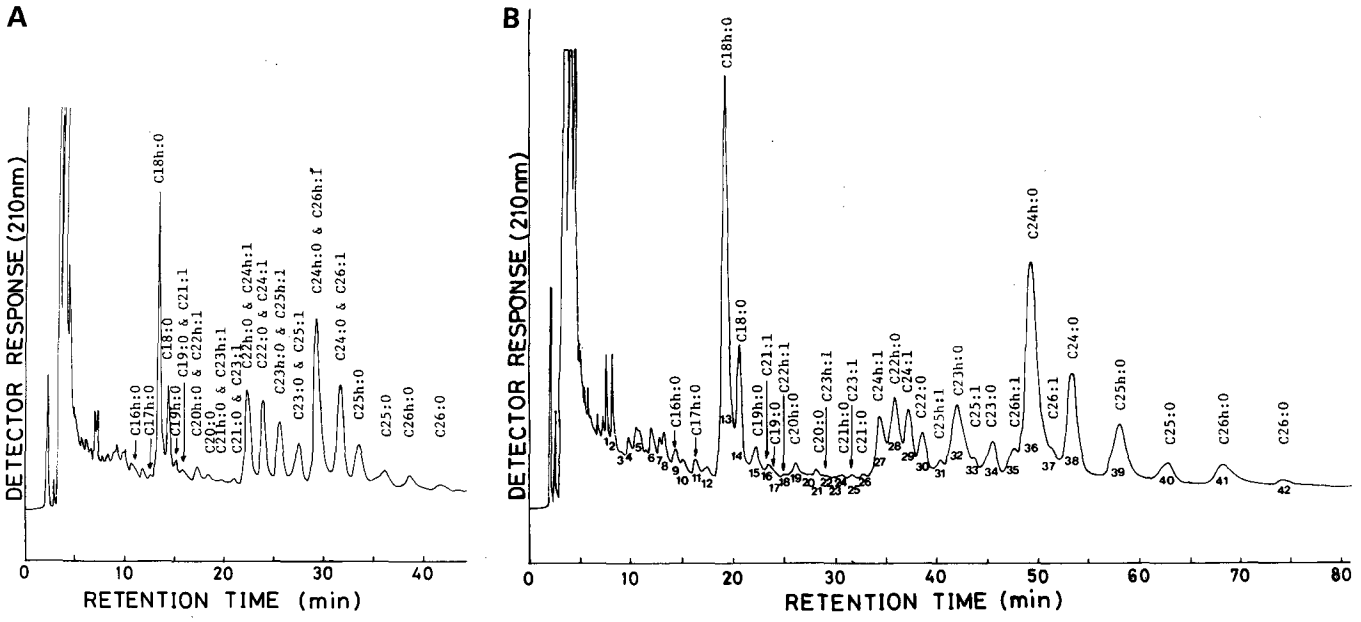

FIG. 2. HPLC chromatogram of bovine brain cerebroside. The ERC-ODS-1272 column was used. The column temperature was set at 41 C (A) or 26 C (B). About 500 μg of the glycolipid was injected. The detector range was set at the level of 0.05.

this are not well understood. Nevertheless, critical pairs are clearly separated by single step HPLC under these optimized conditions.

Chromatographic behavior of bovine cerebrosides in reversed-phase HPLC. Cerebrosides and their chromatographic behavior on reversed-phase columns can be subdivided into four groups according to the types of constituent fatty acids in the ceramide moiety: galactosylceramide with normal/saturated, normal/unsaturated, hydroxy/saturated, and hydroxy/unsaturated fatty acids attached to sphingosine (Fig. 4). The relative retention times of the groups increase logarithmically with increasing fatty acid carbon number.

Detection of the peaks of underivatized glycosphingolipids on HPLC by absorbance at 210 nm is not specific. Therefore, it was desirable to further characterize the glycosphingolipids by HPTLC. Most peaks were shown to consist of one type of molecular species of cerebroside (Fig. 3). The Rf value of each of the molecular species gradually increased with increasing fatty acid carbon number. HPTLC analysis showed that some overlap between the molecular species in the different peaks had occurred, as an attempt was made to collect the entire fractions (valley to valley). Pure molecular species could be prepared by repeated HPLC.

Analysis of monoglycosylceramide from Japanese quail intestine. Previously we have shown that monoglycosylceramides from Japanese quail intestine consist of several molecular species and that the major glycolipid was galactosylceramide containing considerable amounts of unsaturated hydroxy fatty acids and 4D-hydroxysphingosine (phytosphingosine) (9,13; see also Table 1). In the present experiment, the monohexosylceramides were analyzed under the optimum conditions worked out for the separation of bovine brain galactosylceramide. As shown in Figure 5, the glycosylceramides from Japanese quail intestine could be separated into 56 peaks. Semilogarithmic plotting of the peaks (Fig. 6) gave six parallel lines: A, B, C, C′, D and D′. Lines A and B corresponded to the series of galactosylceramides having C18-sphingosine with normal and hydroxy fatty acids, respectively. Lines A′ and B′ were series of glucosylceramides containing C18-sphingosine with normal fatty acids and hydroxy fatty acids, respectively. Lines C and C′ corresponded to galactosylceramide with C18-trihydroxy long chain base/normal fatty acid and glucosylceramide with the same ceramide composition. Lines D and D′ were series of galactosylceramide and glucosylceramide containing trihydroxy C18-long chain base/hydroxylated saturated fatty acid, respectively. HPTLC analysis showed that peaks 18, 29, 34, 39 and 44 contained at least three kinds of molecular species (Fig. 7). These peaks contained glucosylceramide carrying C18-trihydroxysphingosine/one more normal fatty acid as ceramide components (line C′) in addition to the galactosylceramides on line B. The component analysis of peak 39, 44 and 48 by GC/MS suggested that the galactosylceramides on line B were eluted together with galactosylceramides with one less carbon hydroxy fatty acid/C18-dihydrosphingosine as a minor component. Although Japanese quail intestinal monohexosylceramide contained C18-dihydrosphingosine as a minor sphingosine-base composition, we could not get any positive demonstration of its presence in each peak except 39, 44 and 48 by GC/MS analysis.

As shown in Table 1, C20-long chain bases were also components of the quail intestinal glycolipids. Peaks 43 and 48 contained 13.8 and 5.4% of 22:0 hydroxylated acid together with the major 24:0 hydroxy acid, although their FAB/MS analysis gave none of the ions corresponding to h22:0/t18:0 or h22:0/d18:1. This might indicate the

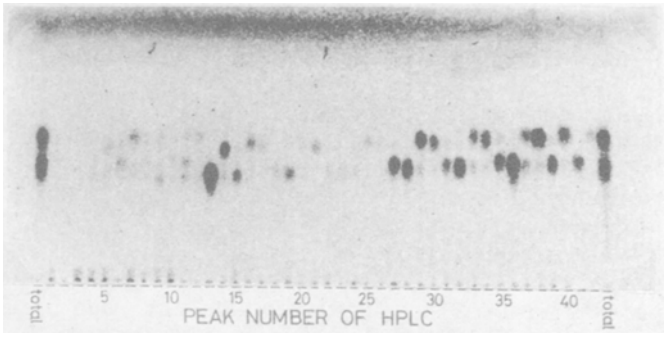

FIG. 3. HPTLC of bovine brain cerebrosides eluted from HPLC column of ERC-ODS-1272 at 26 C. The conditions of HPLC analysis were the same as for Fig. 2B. Each peak shown in Fig. 2B was analyzed by HPTLC with the solvent system II. Spots were visualized by α-naphthol/H_2SO_4 reagent.

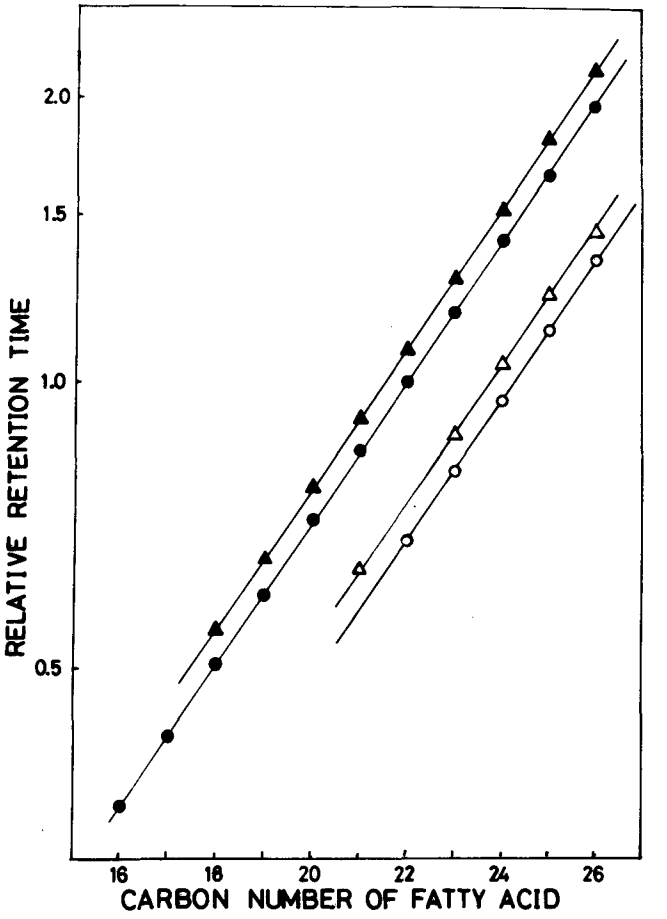

FIG. 4. Relationship between relative retention time and carbon number of fatty acid on HPLC of bovine brain cerebroside. Gal-cer (d18:1/n:0) (▲); Gal-cer (d18:1/hn:0) (●); Gal-cer (d18:1/n:1) (△); Gal-cer (d18:1/hn:1) (○).

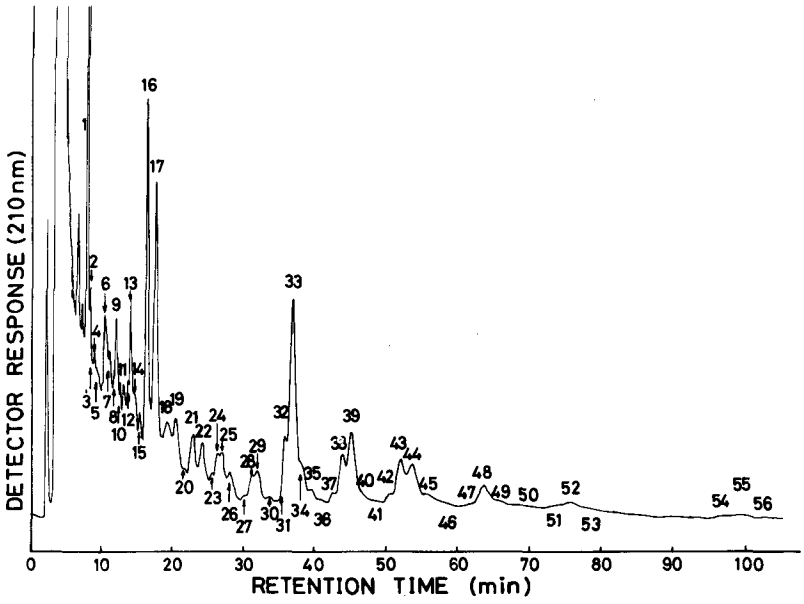

FIG. 5. HPLC quail small intestine monohexosylceramide. HPLC was performed as described in Fig. 2B, except for column temperature at 21 C.

TABLE 3

Molecular Species of Monohexosylceramides Isolated from Adult Quail Intestine

Line A				Line B				Line C				Line D			
Peak number	Sugar	LCB	FA	Peak number	Sugar	LCB	FA	Peak number	Sugar	LCB	FA	Peak number	Sugar	LCB	FA
17	Gal	d18:1	16:0	16	Gal	d18:1	h16:0	14	Gal	t18:0	16:0	12	Gal	t18:0	h16:0
				18	Gal	d18:1	h17:0								
22	Gal	d18:1	18:0	21	Gal	d18:1	h18:0								
				29	Gal	d18:1	h20:0	26	Gal	t18:0	20:0	24	Gal	t18:0	h20:0
				34	Gal	d18:1	h21:0	30	Gal	t18:0	21:0	28	Gal	t18:0	h21:0
				39[a]	Gal	d18:1	h22:0	35	Gal	t18:0	22:0	33	Gal	t18:0	h22:0
				44[a]	Gal	d18:1	h23:0	40	Gal	t18:0	23:0	38	Gal	t18:0	h23:0
50	Gal	d18:1	24:0	48[a]	Gal	d18:1	h24:0	45	Gal	t18:0	24:0	43	Gal	t18:0	h24:0
				52	Gal	d18:1	h25:0					48	Gal	t18:0	h25:0
				54	Gal	d18:1	h26:0					52	Gal	t18:0	h26:0

Line A′				Line B′				Line C′				Line D′			
Peak number	Sugar	LCB	FA	Peak number	Sugar	LCB	FA	Peak number	Sugar	LCB	FA	Peak number	Sugar	LCB	FA
17	Glc	d18:1	16:0	16	Glc	d18:1	h16:0	13	Glc	t18:0	16:0	11	Glc	t18:0	h16:0
22	Glc	d18:1	18:0					18	Glc	t18:0	18:0				
								25	Glc	t18:0	20:0	23	Glc	t18:0	h20:0
								29	Glc	t18:0	21:0	27	Glc	t18:0	h21:0
								34	Glc	t18:0	22:0	32	Glc	t18:0	h22:0
								39	Glc	t18:0	23:0	37	Glc	t18:0	h23:0
50	Glc	d18:1	24:0					44	Glc	t18:0	24:0	42	Glc	t18:0	h24:0
												47	Glc	t18:0	h25:0
												51	Glc	t18:0	h26:0

[a]These fractions contains dihydrosphingosine as a minor component of long chain base (see Results and Discussion).

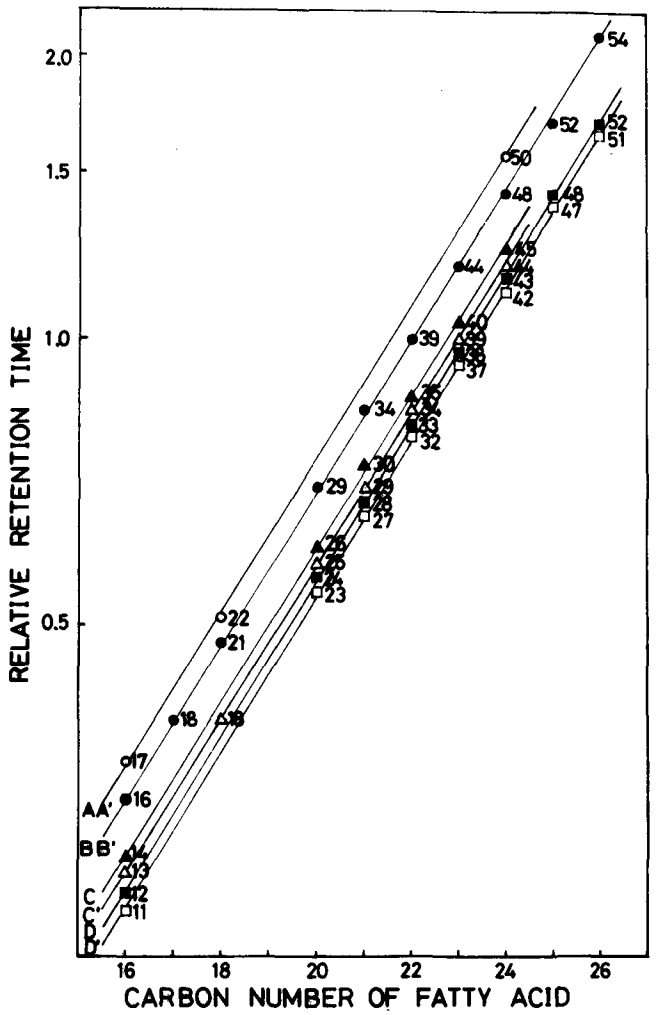

FIG. 6. Relationship between relative retention time and carbon number of fatty acid on HPLC of quail intestinal monohexosylceramide. A, gal-cer (d18:1/n:0) (○); B, gal-cer (d18:1/hn:0) (●); C, gal-cer (t18:0/n:0) (▲); D, gal-cer (t18:0/hn:0) (■); A′, glc-cer (d18:1/n:0) (○); B′, glc-cer (d18:1/hn:0) (●); C′, glc-cer (t18:0/n:0) (△); D′, glc-cer (t18:0/hn:0) (□).

presence of h22:0 fatty acid attached to t20:0 and d20:1 bases in peaks 43 and 48, respectively.

Interestingly, galactosylceramide and glucosylceramide containing C18-trihydroxysphingosine could be separated, although they have the same fatty acid composition (see lines C and C′ and lines D and D′).

The molecular species of monohexosylceramides isolated from Japanese quail intestine are summarized in Table 3. The intestinal tissues contained more than 49

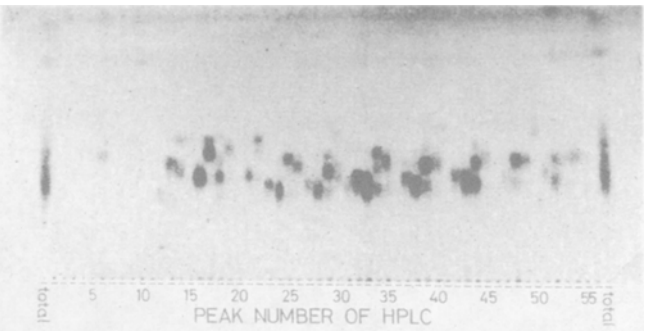

FIG. 7. HPTLC of quail intestinal monohexosylceramide eluted from HPLC column. Each peak from HPLC column as shown in Fig. 5 was analyzed by HPTLC with the solvent system II. Spots were detected by α-naphthol/H_2SO_4 reagent.

molecular species of monohexosylceramides that were identified from 56 peaks by HPTLC and TC/MS (or FAB/MS). HPLC data under conditions as specified in this study offered helpful information on their hydrophobic moiety.

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COMMUNICATIONS

Rice Bran Oil and Hypocholesterolemia in Rats

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Rats fed rice bran oil at 10% level for a period of eight weeks showed significantly lower levels of total cholesterol, low density lipoprotein and very low density lipoprotein cholesterol, both on cholesterol-containing and cholesterol-free diets. High density lipoprotein cholesterol was increased but triglyceride showed a decrease that was not statistically significant. Liver cholesterol and liver triglycerides were also reduced. Fecal excretion of neutral sterols and bile acids was increased after ingestion of rice bran oil.
Lipids 21, 715-717 (1986).

The great scarcity of edible oil in India has led to heavy reliance on edible oil imports. Recently, efforts have been made to exploit other vegetable oils, such as cottonseed and rice bran oils. Rice bran is a rich source of vegetable oil that has not been exploited to the fullest. On the basis of 55 million tons of rice production and 7.5% polish, the theoretical yield of bran is about 4 million tons and that of oil, 0.8 million tons. Our actual production is, however, 0.13 million tons, of which only 15% is edible grade. The rest is used in industry for making soap, paints and lubricants, etc. Crude rice bran oil contains high percentages of free fatty acids (15-56), wax (3.8-5.2), gum (0.4-0.5) and pigments (21 Y + 2.7 R-25 Y + 3.5 R), all of which can be removed easily following modern techniques (1,2). The constraint in the use of rice bran oil (RBO) as an edible oil is its high content of unsaponifiable matter (4.1%), which is above the permissible limit (3%) fixed for edible oil by the Central Committee of Food Standards, Government of India (2,3). Recently, antiatherogenic property of plant steroids, present in unsaponifiable matter, has been recognized (4-6). RBO is undergoing rigorous toxicological evaluation to clear it for human consumption as it is a new source of oil. Nutritional evaluation carried out in weanling rats at this institute showed good growth performance, absorption and utilization of the oil without any adverse effects (Rukmini, C., submitted for publication). These studies also indicated a hypocholesterolemic effect of the oil. We therefore planned a study to determine the hypocholesterolemic activity of RBO in rats, with a view that such studies would help in promoting it as an edible oil, in spite of its high unsaponifiable matter.

MATERIALS AND METHODS

RBO as well as groundnut oil (GNO) were purchased in bulk quantity from Food Fats and Fertilizers Limited (Tadepallegudem), Andhra Pradesh, India, and stored in sealed containers at 4 C.

The physicochemical characteristics like refractive index, iodine value, acid value, saponification value and unsaponifiable matter were determined using standard techniques (7). Fatty acids of RBO and GNO were methylated and methyl esters were analyzed in a Varian Model 3700 gas chromatograph fitted with flame detector on a column of 15% diethylene glycol succinate (DEGS) coated on chromosorb W. Nitrogen was used as a carrier gas at a flow rate of 25 ml/min. The oven temperature was 200 C (Table 1).

Animal experiment. Thirty-two male albino rats weighing between 100-120 g from this institute's colony were used. They were divided into four groups of eight animals each and were fed cholesterol diet (CD) and cholesterol-free diet (CFD) as shown in Table 2. Rats were housed in individual screen-bottomed cages, and a record of daily food intake was maintained. Animals were weighed once a week. This dietary regimen was continued for eight weeks. At the end of the experiment, animals were killed after an overnight fast and 4 ml of blood was withdrawn by cardiac puncture. The livers were excised.

Serum cholesterol was estimated by the method of Zlatkis et al. (8) and triglycerides were estimated by the method of Van Handel and Zilversmith (9). Prior to determination of high density lipoprotein (HDL), cholesterol, chylomicron, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) were precipitated by heparin + manganese chloride from serum samples according to the procedure of Burstein and Samillie (10). Liver lipids

TABLE 1

Composition of Diets (w/w%)

Dietary component	Cholesterol diet		Cholesterol-free diet	
	I Control	II Experimental	III Control	IV Experimental
Casein	15.0	15.0	15.0	15.0
Starch	66.3	66.3	67.8	67.8
Groundnut oil	10.0	—	10.0	—
Rice bran oil	—	10.0	—	10.0
Salt mixture[b]	4.0	4.0	4.0	4.0
Vitamin mixture[c]	1.0	1.0	1.0	1.0
Cellulose	2.0	2.0	2.0	2.0
Choline chloride	0.2	0.2	0.2	0.2
Cholesterol	1.0	1.0	—	—
Cholic acid	0.5	0.5	—	—

[a]There were eight animals in each dietary group.

[b]Salt mixture contained (% in the mixture) NaCl, 4.6; $Na_2HPO_4 \cdot H_2O$, 9.3; K_2HPO_4, 25.6; $CaH_4(PO_4)2 \cdot H_2O$, 14.5; $Fe(C_6H_5O_7) \cdot 5H_2O$, 3.3; $Ca(C_3H_5O_3)2 \cdot 5H_2O$, 34.9; $MgSO_4$, 7; $ZnSO_4 \cdot 7H_2O$ 0.05, KI, 0.9 and $Cr(C_2H_3O_2)3$, 0.02.

[c]Vitamin mixture contained (mg) riboflavin, 150; thiamine, 100; nicotinic acid, 1000; pyridoxin, 100; cyanocobalamine, 1; pantothenic acid, 500; folic acid, 50; ascorbic acid, 3750; vitamin K, 100; vitamin E, 100; vitamin A, 250,000 IU; vitamin D_2, 20,000 IU and sucrose to 100 g.

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were estimated by the method of Folch (11) and cholesterol and triglycerides were estimated by the same methods used for serum.

Feces were collected from each animal for three consecutive days before it was killed. Steroids were isolated quantitatively and freed from contaminants by column and thin layer chromatography as described by Grundy et al. (12). Twenty-four-hour excretions of bile acids and neutral sterols were estimated by colorimetric methods of Snell and Snell (13) and Zlatkis et al. (8), respectively. Statistical analysis was carried out by using Student's t-test.

RESULTS AND DISCUSSION

Except for high unsaponifiable matter (4.1% compared to 0.3% in GNO), physical parameters and fatty acid composition of RBO were similar to those of GNO (Table 1).

Daily diet consumption in different groups of rats was essentially similar. Rats fed the cholesterol diet with RBO showed slightly lower body weight gain compared to those fed the control diet. However, differences in body weight gain between the two groups were not statistically different. In another short-term (three-month) study designed to evaluate the safety of RBO, the growth of rats on RBO was not different from that on GNO (Rukmini, C., unpublished data).

As shown in Table 3, the mean total cholesterol levels in rats fed the cholesterol-containing diet alone with GNO were elevated to 287 ± 19.5 mg/dl. When RBO was substituted for GNO in the cholesterol-containing diet of rats, elevation of serum cholesterol as well as LDL + VLDL cholesterol was significantly lower ($P < 0.01$). On the other hand, HDL cholesterol was significantly elevated ($P < 0.05$). As a result, its ratios with total cholesterol as well as the LDL + VLDL cholesterol were raised significantly ($P < 0.05$). Triglycerides showed a tendency to fall. Liver cholesterol and liver triglycerides were also lowered. A similar trend was observed in rats fed the cholesterol-free diet supplemented with RBO. Thus, dietary RBO has a cholesterol-lowering effect both on exogenous and endogenous cholesterol. There are hardly any data available in the literature on the hypocholesterolemic effect of RBO. Epidemiological data suggest that the way in which cholesterol is distributed between lipoproteins is of significance in the development of atherosclerosis (14–19) and that a lowering of lipids convincingly reduces the occurrence of fatal or non-fatal events in subjects free of or with manifest coronary heart disease. Thus, RBO can be considered as a useful source of fat in the diet of man. The hypocholesterolemic activity

TABLE 2

Physicochemical Characteristic of GNO and RBO

Characteristics	GNO	RBO
Refractive index (40 C)	1.467	1.460
Iodine value (Wij's)	98.4	91.3
Saponification value	190	212
Acid value	2.0–6.0	1.18
Unsaponifiable matter (%)	0.3–1.0	4.1
Saponifiable matter (%)	99.0	95.9
Fatty acid (1%)		
16:0	14.35	21.36
18:0	3.10	0.81
18:1	42.63	42.04
18:2	35.95	35.19
18:3	1.25	—
20:0	2.71	—
Total EFA content[a]	37.20	35.79

Mean of three determinations.

[a]EFA, essential fatty acids.

TABLE 3

Food Intake, Gain in Weight, Serum and Liver Lipids in Rats (Mean ± SEM)

	Cholesterol diet		Cholesterol-free diet	
Groups	I Control GNO	II Experimental RBO	III Control GNO	IV Experimental RBO
Daily diet intake (g/day)	12.8 ± 0.2	12.5 ± 0.1	12.3 ± 0.2	12.4 ± 0.3
Gain in body weight (g/day)	2.25 ± 0.09	2.3 ± 0.02	2.05 ± 0.2	1.87 ± 0.21
Liver weight (g)	11.4 ± 0.6	9.7 ± 0.6	7.2 ± 0.4	7.4 ± 0.3
Serum lipids (mg/dl)				
Total cholesterol	287 ± 19	182 ± 18**	72.7 ± 4.0	62.3 ± 4.4
HDL cholesterol	62 ± 3	73 ± 2.5*	60.8 ± 5.0	58.7 ± 4.7
LDL + VLDL cholesterol	225 ± 20	109 ± 20**	11.9 ± 3.8	3.6 ± 1.6
$\frac{\text{HDL cholesterol}}{\text{Total cholesterol}}$	21.6 ± 2.4	40.1 ± 5.4*	83.6 ± 3.8	93.9 ± 4.3
$\frac{\text{HDL cholesterol}}{\text{LDL + VLDL cholesterol}} \times 100$	27.5 ± 4.7	66.9 ± 11.8*	511 ± 376	1540 ± 357**
Triglycerides	225 ± 16	188 ± 11	35 ± 3.6	28 ± 1.6
Liver lipids (mg/g)				
Total cholesterol	24.3 ± 3.4	19.0 ± 2.6	6.1 ± 0.9	5.2 ± 0.4
Triglycerides	9.4 ± 1.8	6.4 ± 2.8	4.9 ± 0.7	3.7 ± 0.5

There were eight animals in each dietary group. Significantly different from respective control: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 4

Fecal Excretion of Bile Acids and Neutral Sterols

Fecal excretion of cholesterol end-products (mg/day)	Cholesterol diet		Cholesterol-free diet	
	I Control GNO	II Experimental RBO	III Control GNO	IV Experimental RBO
Neutral sterols	26.1 ± 6.4[a]	61.5 ± 5.3***	2.8 ± 0.5	4.5 ± 0.3**
Total bile acids	58.6 ± 2.2	86.4 ± 15.6*	13.2 ± 1.6	15.4 ± 1.4

There were eight animals in each dietary group. Significantly different from respective controls. *, $P < 0.05$; **, $P < 0.01$; ***$P < 0.001$.
[a]Mean ± SE.

of RBO is unlikely to be due to polyunsaturated fatty acid alone, as both RBO and GNO have similar linoleic acid content. Plant sterols are known to have antiatherogenic property (4-6), but due to their lower content in the diets of experimental rats, it seems unlikely that the sterol fraction could cause the observed effect. Sitosterol is usually effective at a dose about twice the level of dietary cholesterol. Some fraction of the unsaponifiable matter may be more effective or may have some other effect on cholesterol metabolism. A detailed study of the chemistry of the unsaponifiable matter is underway.

As shown in Table 4, addition of RBO to the cholesterol diet enhances the excretion of bile acids and neutral sterols. However, this increase was found statistically significant in both neutral sterols and bile acids. However, addition of RBO to the cholesterol-free diet significantly increased only the neutral sterol excretion. The possible explanation for the effects on bile acid excretion is that a large amount of unsaponifiable matter of RBO in the small intestine may create an environment unsuitable for micelle formation or has an effect on the reabsorption of bile acids. A decreased influx of absorbed steroids reaching the liver promotes the conversion of liver cholesterol into bile acids. In the process, lipid reserves of liver are depleted, which we have observed in the present study. A significant increase in hepatic cholesterol synthesis may occur by feeding plant sterol with and without cholesterol (20). It is apparent, however, that any increase in bile acid production as a result of increased synthesis is not sufficient to balance a decrease that may have been caused by the lower cholesterol absorption (21).

We note that the fecal steroid excretion was greater during feeding of the high cholesterol diet than of the basal diet in this study. These results suggest that rats are able to increase bile acid output when fed cholesterol. Lin and Connor (22) concluded from their long-term studies that cholesterol feeding increased bile acid excretion in man. This shows that our experimental results in rats can be extended to man.

In summary, RBO consumed in moderate amounts lowers serum cholesterol. A three-generation study carried out at our institute with RBO did not reveal any deleterious effect. Hence, its use as a source of edible oil could be considered in spite of its high content of unsaponifiable matter which, in fact, as the present study shows, may confer some benefit.

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Desaturation of Fatty Acids in *Trypanosoma cruzi*

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Uptake and metabolism of saturated (16:0, 18:0) and unsaturated [18:1(n-9), 18:2(n-6), 18:3(n-3)] fatty acids by cultured epimastigotes of *Trypanosoma cruzi* were studied. Between 17.5 and 33.5% of the total radioactivity of [1-^{14}C]labeled fatty acids initially added to the culture medium was incorporated into the lipids of *T. cruzi* and mostly choline and ethanolamine phospholipids. As demonstrated by argentation thin layer chromatography, gas liquid chromatography and ozonolysis of the fatty acids synthesized, exogenous palmitic acid was elongated to stearic acid, and the latter was desaturated to oleic acid and 18:2 fatty acid. The 18:2 fatty acid was tentatively identified as linoleic acid with the first bond in the Δ9 position and the second bond toward the terminal methyl end. Exogenous stearic acid was also desaturated to oleic and 18:2 fatty acid, while oleic acid was only converted into 18:2. All of the saturated and unsaturated fatty acids investigated were also converted to a small extent (2–4%) into polyunsaturated fatty acids. No radioactive aldehyde methyl ester fragments of less than nine carbon atoms were detected after ozonolysis of any of the fatty acids studied. These results demonstrate the existence of Δ9 and either Δ12 or Δ15 desaturases, or both, in *T. cruzi* and suggest that Δ6 desaturase or other desaturases of the animal type are likely absent in cultured forms of this organism.
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Trypanosoma cruzi is a parasitic protozoan that causes Chagas' disease in man. Previous work from this laboratory demonstrated that [1-^{14}C]acetate was actively incorporated into fatty acids and other lipid fractions of cultured epimastigotes of *T. cruzi* (1–3). Both the pattern of incorporation of labeled acetate into saturated (16:0 and 18:0), monounsaturated (18:1) and diunsaturated (18:2) fatty acids and the decarboxylation ratios showed that "de novo" synthesis of fatty acids had occurred (3).

These studies also suggested that elongation of palmitic to stearic acid and Δ9 desaturation of stearic to oleic acid as well as further desaturation of oleic acid were likely to occur in *T. cruzi.* However, elongation of endogenous short chain saturated and unsaturated fatty acids by labeled acetate could not be entirely excluded. The existence of specific desaturases in *T. cruzi* was not demonstrated. Furthermore, although no 18:3 or higher polyunsaturated fatty acids were synthesized from [1-^{14}C] acetate, the absence of the corresponding desaturases could not be proven solely on the basis of the acetate studies. Biosynthesis of long chain polyunsaturated fatty acids has been demonstrated by Korn et al. (5) and Dixon et al. (6) for *Trypanosoma lewisi* and *Trypanosoma rhodesiense* and by Meyer and Holz (7) in Kinetoplastid flagellates.

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This investigation describes the uptake and metabolism of [1-^{14}C]-labeled fatty acids by *T. cruzi* in culture.

MATERIALS AND METHODS

Organisms. Epimastigotes of *T. cruzi* (Tulahuen strain) were cultured as previously described (2). *T. cruzi* epimastigotes, Tulahuen strain, were cultivated on a biphasic medium consisting of 33 g of tryptose blood agar base (Difco, Detroit, Michigan); 3 g of agar (Difco) and distilled water to make 1000 ml of solid phase; and 11.5 g of tryptose phosphate broth (Difco), 10 g of glucose, 14 g of brain heart infusion (Difco) and distilled water to make 1000 ml of liquid phase.

The cultures were maintained in this medium plus 0.5 ml of rabbit blood (defibrinated) for each 5 ml of solid phase. The experimental cultures were carried in 10 ml of liquid medium without blood, which consisted of 14 g of brain heart infusion (Difco), 11.5 g of tryptose phosphate broth (Difco), 10 g of glucose, 18.2 g of tryptose (Difco), 5.5 g of lab lemco powder, 9.1 g of sodium chloride and distilled water to make 1000 ml. All incubations were done at 28 C under a continuous stream of sterile air.

Uptake and metabolic studies. [1-^{14}C]Fatty acids (51–60 mCi/mmol) (Amersham, Des Plaines, Illinois) complexed to essentially fatty acid-free bovine serum albumin were added after Millipore filtration to 5 ml of the autoclaved medium (0.2 μCi/ml) at the time of inoculation. Inoculum densities were between 7.5 and 8.6 × 10^6 organisms/ml. Cultures were harvested after 96 hr of incubation with radioactive precursor. At this stage, the growth was exponential. The parasites were collected, and cellular lipids were extracted. Thin layer chromatography (TLC) and radioactivity determinations were carried out as previously described (2,3).

Gas liquid chromatography (GLC) and TLC of methyl esters of fatty acids. Fatty acid methyl esters were prepared from the total lipid extracts with 14% boron trifluoride in methanol (8). GLC of methyl esters and aldehyde methyl esters was carried out in a Packard Gas Chromatograph 7400 at 185–190 C. A 4 mm × 2 m coiled glass column packed with 10% Silar 10 C on 100–120 mesh Gas Chrom Q was used for the analysis. The Packard 894 Gas Proportional Counter was employed for radioactivity determinations. Identification of the radioactive peaks after GLC, argentation TLC and ozonolysis of unsaturated methyl esters of fatty acids was done as previously described (3). Peaks were identified by chromatography with mixtures of standard fatty acid methyl esters. When standards were unavailable, peaks were identified by their relative retention times.

Separation and radioactivity determinations of the methyl esters of fatty acids on the basis of their degree of unsaturation was carried out by $AgNO_3$-TLC as described by Cook (4).

Recovery of radioactivity after ozonolysis was measured by triangulation of the radioactivity peak areas obtained by GLC of known aliquots of the radioactive samples before and after ozonolysis.

RESULTS AND DISCUSSION

For [1-^{14}C]palmitic acid 17.5% ± 1.3 (mean ± half the range of two experiments) of the radioactivity added to the culture medium was incorporated into the lipids of epimastigotes of *T. cruzi*. For stearic, oleic, linoleic [18:2(n-6)] and linolenic [18:3(n-3)]-labeled acids, the percentages of the radioactivity incorporated were 20.0% ± 1.5, 33.5% ± 1.4, 29.9% (only one experiment) and 23.5% ± 0.5, respectively. Most of the label was found in the phospholipid fraction: 85.8% ± 8.1 of total lipid radioactivity for palmitic acid, 83.7% ± 6.2 for stearic, 70.3% ± 8.2 for oleic, 83.1% for linoleic and 86.7% ± 4.9 for linolenic acid (mean ± half the range of two experiments; only one experiment for linoleic acid). The choline phospholipids were preferentially labeled. When the labeled fatty acid was palmitic, 37.5% ± 0.6 of the radioactivity was incorporated. For stearic, oleic, linoleic and linolenic acids, the radioactivity incorporated was 40.8% ± 3.2, 39.7 ± 3, 63.3% and 66.9% ± 3, respectively. Other trypanosomatids have been shown to incorporate fatty acids from the culture medium (5–7), and some also synthesize mainly phosphatidylcholine from polyunsaturated fatty acids (6), contrary to what is known to occur in higher organisms, which incorporate polyunsaturated fatty acids mostly into phosphoglycerides of the ethanolamine, serine and inositol types. Some of these trypanosomatids, such as *T. lewisi* and *T. rhodesiense* (6,9) and *T. cruzi* (10,11), may be able to exert control over their lipid composition. Such control might be exerted by specific absorption mechanisms or at the biosynthetic level (6). These mechanisms could be characteristic for each species and be relatively independent of the lipid composition of the environment. Total lipids of *T. cruzi*, Tulahuen strain, represent 3.07% of wet weight. Concentration of glycerides is 2.1 mg/g of wet tissue; sterols, 1.92 mg/g; and phospholipids, 9.65 mg/g (10).

The metabolic products of each labeled fatty acid precursor are shown in Table 1. There was generally good agreement between the results obtained by argentation and GLC. Palmitic acid was readily elongated to stearic acid and desaturated further to 18:1 and 18:2 fatty acids. No elongation of stearic acid or any other unsaturated fatty acid was detected. Stearic acid was also desaturated to 18:1 and 18:2, whereas oleic acid was only converted into an 18:2 fatty acid. All of the three precursors as well as linoleic [18:2(n-6)] and linolenic [18:3(n-3)] acids used as substrates showed a small conversion into more highly unsaturated fatty acids (2–4%), as demonstrated by argentation chromatography. Synthesis of these higher polyunsaturated fatty acids could not be detected by radioactivity analysis of the synthesized products by radio-GLC, although it is possible that small radioactivity levels of these fatty acids were obscured by the radiochromatograph detector noise.

An attempt was made to characterize further the unsaturated 18:1 and 18:2 fatty acids synthesized by *T. cruzi* from the labeled precursors. These two unsaturated fatty acids were isolated by argentation chromatography. The position of their first double bond counting from the carboxyl end of the molecule was determined by radio-GLC analysis of the radioactive aldehyde methyl ester fragments formed after ozonolysis (12). For both 18:2 (Fig. 1A and 1B) and 18:1 (not shown), only one [1-^{14}C]aldehyde ester peak was found and identified as a C_9-fragment by comparison with a fatty acid mixture of C_{18}-monoene positional isomers and with ozonolysis aldehyde ester products of [1-^{14}C]oleic [18:1(n-9)] and linoleic [18:2(n-6)] standard fatty acids. No radioactive aldehyde methyl ester fragments of less than nine carbon atoms were detected after ozonolysis with any of the fatty acids investigated.

TABLE 1

Distribution of Radioactivity in Methyl Esters of Fatty Acids of *T. cruzi* after Incubation with [1-^{14}C]Fatty Acids

Method	Fatty acids	^{14}C-Fatty acid substrate				
		16:0	18:0	18:1(n-9)	18:2(n-6)[a]	18:3(n-3)
$AgNO_3$-TLC	Saturated	16.7 ± 3.3	13.3 ± 0.9	0.5 ± 0.3	0.4	0.6 ± 0.1
	Monounsaturated	23.1 ± 4.6	18.1 ± 2.2	40.9 ± 1.5	1.8	1.1 ± 1.0
	Diunsaturated	57.7 ± 6.3	65.3 ± 7.6	56.0 ± 4.9	94.0	1.7 ± 0.9
	Polyunsaturated[b]	2.4 ± 2.1	3.1 ± 1.8	2.5 ± 1.3	3.7	96.5 ± 4.0
GLC	16:0	9.3 ± 2.9	ND	ND	ND	ND
	18:0	8.1 ± 3.5	12.0 ± 2.6	ND	ND	ND
	18:1	18.7 ± 6.1	23.6 ± 5.9	41.3 ± 2.6	ND	ND
	18:2	63.7 ± 5.0	64.1 ± 6.3	58.6 ± 5.5	>99.0	ND
	18:3	ND[c]	ND	ND	ND	>99.0

Epimastigotes of *T. cruzi* were incubated for 96 hr with [1-^{14}C] fatty acid-containing medium (0.2 μCi/ml). Methyl esters of fatty acids were prepared and analyzed by argentation chromatography and GLC as described in Materials and Methods. Values are given as percentage of total radioactivity in methyl esters of fatty acids and represent the mean ± half the range of two experiments. Recovery of radioactivity after $AgNO_3$-TLC was 82.6% ± 2.1 (mean ± S.D., n = 5), and after GLC, 88.6% ± 4.3 (n = 5).

[a]Results from only one experiment.

[b]Three or more double bonds.

[c]ND, not detected.

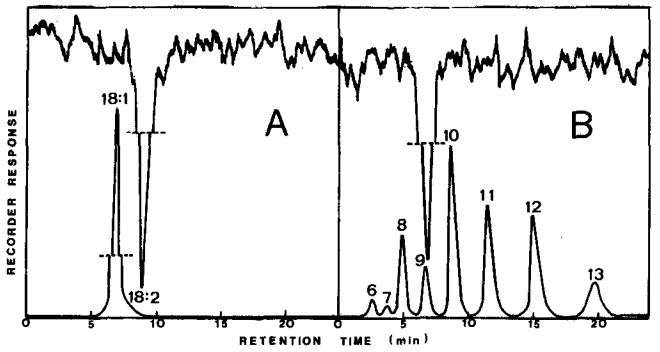

FIG. 1. Radio-GLC analysis of an 18:2 fatty acid methyl ester synthesized by *T. cruzi* after 96 hr of incubation with a [1-^{14}C] fatty acid precursor (palmitic acid-, stearic acid- or oleic acid-labeled) before and after ozonolysis. (A) Mass and radioactivity response of an unlabeled C_{18}-monoene fatty acid methyl ester mixture containing all of the positional isomers between position 6 and 14 added to a radioactive 18:2 fatty acid methyl ester sample synthesized by *T. cruzi*; (B) mass and radioactivity response of the same sample in (A) after ozonolysis. The superscript designates the number of carbon atoms in the aldehyde methyl esters formed by triphenylphosphine reduction of the ozonides. Recovery of radioactivity after ozonolysis was 85.8% ± 5.3 (mean ± S.D., n = 7).

These results demonstrate the presence of Δ9 desaturase if we start with 16:0 or 18:0 fatty acids. Also, it would suggest the presence of Δ12 and/or Δ15 desaturases (of the plant or plant-organism types) in *T. cruzi* and the absence of Δ6 or other desaturases of the animal type. These results also substantiate our previous findings regarding the biosynthesis of fatty acids from acetate in *T. cruzi* (3) and virtually eliminate the possibility that unsaturated fatty acids in this organism are synthesized by elongation of shorter chain unsaturated fatty acids, as in anaerobic bacteria (13). However, our results are different from those of Korn et al. in *T. lewisi* (5), Dixon et al. in *T. lewisi* and *T. rhodesiense* (6) and Meyer and Holz in several species of kinetoplastid flagellates (7); those workers showed significant synthesis of 18:3 and higher polyunsaturated fatty acids by blood or culture forms of these organisms. Our data agree with the known fatty acid composition of *T. cruzi*, characterized by a high content of linoleic acid, ca. 50% of total lipid fatty acids (11,14,15) and only small amounts of linolenic acid (less than 3.1% of total lipid fatty acids) or other polyunsaturated fatty acids. The presence of Δ9, Δ12 or Δ15 desaturases and the absence of the animal type desaturases may represent a strain characteristic and not a species characteristic. Finally, the presence of lipids in the culture medium could influence lipid synthesis by these organisms, as has been observed in cultured mammalian cells (16), and selectively inhibit synthesis of polyunsaturated fatty acids.

Lipid biosynthesis investigation in blood trypomastigotes and intracellular amastigote forms of *T. cruzi*, cultured epimastigotes in a lipid-free chemically defined media and in other trypanosomatic species would elucidate these important aspects of lipid metabolism in *T. cruzi*.

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Effect of Malonyl-CoA on Δ6 Desaturation Activity of Rat Liver Microsomes

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The effect of malonyl-CoA on linoleic acid desaturation and elongation reactions of rat liver microsomes was studied. Under strict desaturation conditions, the in vitro microsomal conversion of linoleic acid to γ-linolenic acid is time-dependent. When malonyl-CoA was added to the aforementioned incubation medium, linoleic acid was desaturated to γ-linolenic acid and elongated to its higher homologues. Under these conditions, Δ6 desaturation activity, calculated by adding γ-18:3, 20:3 and 20:4 acids, was neither inhibited nor activated by malonyl-CoA. These results indicate that the elongation of γ-linolenyl-CoA coupled to the desaturation of linoleic acid did not modify Δ6 desaturase activity.
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The biosynthesis of arachidonic acid from linoleic acid is produced in the endoplasmic reticulum of rat hepatocytes by an initial Δ6 desaturation of linoleic acid to γ-linolenic acid, an elongation to eicosa-8,11,14-trienoic acid and a final Δ5 desaturation to arachidonic acid. The desaturases apparently play relevant regulatory functions in this biosynthesis (1), and it has been shown experimentally in vivo that pharmacological doses of epinephrine, glucagon, glucocorticoids and dibutyryl cyclic AMP inhibit the desaturases (2-4). Besides, malonyl-CoA is required for the elongation reaction and its intracellular concentration is also modified by the hormones. The production of malonyl-CoA depends on the activity of acetyl-CoA carboxylase, an enzyme that is stimulated by insulin (5-8) and deactivated by glucagon, epinephrine or dibutyryl cyclic AMP (6,8-13). Therefore, the aim of the present work was to study the effect of malonyl-CoA on Δ6 desaturase activity in isolated rat liver microsomes.

MATERIALS AND METHODS

Animals and their treatment. Five adult female Wistar rats weighing 150-180 g and maintained on a commercial diet (Cargill type C) were used. The animals were killed by cervical dislocation. Livers were immediately removed and placed in ice-cold homogenizing solution (1:3, w/v) containing 0.25 M sucrose, 62 mM phosphate buffer pH 7.0, 0.15 M KCl, 5 mM $MgCl_2$ and 0.1 mM EDTA. After homogenization, liver microsomes were separated by differential centrifugation at 100,000 × g as described previously (14).

Incubation procedure for oxidative desaturation. The oxidative desaturation of linoleic acid to γ-linolenic acid by liver microsomes was measured by estimation of the percentage of conversion of 100 nmol of [1-^{14}C]linoleic acid (52.6 mCi/mmol) to γ-linolenic acid. Three nmol of labeled acid and 97 nmol of unlabeled acid were incubated with 2.5 and 5 mg microsomal protein in a metabolic shaker at 37 C for 5, 10, 20 and 40 min in a total volume of 1.5 ml of 0.15 M KCl-0.25 M sucrose solution. The medium contained 4 μmol ATP, 0.1 μmol CoA, 1.25 μmol NADH, 5 μmol $MgCl_2$, 2.25 μmol glutathione, 62.5 μmol NaF, 0.5 μmol nicotinamide and 62.5 μmol phosphate buffer pH 7.0. The [1-^{14}C]linoleic acid desaturation to γ-linolenic acid was also measured in the presence of 0.2 μmol per tube of malonyl-CoA using the procedure described above.

Incubation procedure for chain elongation. The elongation of linoleic acid by the liver microsomal preparation was measured by estimating the conversion of [1-^{14}C]-linoleic acid to eicosa-11,14-dienoic acid. The basic incubation medium was similar to that of the desaturation reaction except for the addition, in this case, of 1.5 μmol KCN and 0.2 μmol malonyl-CoA. ATP was added at a concentration of 10 μmol per tube. Five nmol labeled linoleic acid and 135 nmol unlabeled acid were incubated with 2.5 and 5 mg microsomal protein in tubes fitted with rubber stoppers and under nitrogen atmosphere. The incubations were carried out at 37 C for 5, 10, 20 and 40 min.

Fatty acid analysis. Incubations were stopped by the addition of 2 ml of 10% KOH in methanol. The fatty acids were recovered by saponification of the incubation mixture. The acids were esterified with 3 M HCl in methanol (3 hr at 68 C). The distribution of the radioactivity between linoleic and γ-linolenic acids or the elongation products was determined by gas liquid radiochromatography as described previously (14). The labeled methyl esters were identified by equivalent chain length determination and comparison with authentic standards.

RESULTS AND DISCUSSION

Under strict desaturation conditions used in this experiment, linoleic acid was converted to γ-linolenic acid by rat liver microsomes in a time-dependent reaction (Fig. 1). When malonyl-CoA was added to the incubation medium and the desaturation reaction was prevented by oxygen elimination and cyanide inhibition, the microsomes elongated linoleic acid not only to eicosa-11,14-dienoic (20:2) but also to docosa-13,16-dienoic (22:2) and tetracosa-15,18-dienoic (24:2) acids (Table 1). The elongation reaction was also time- and microsomal protein-dependent, but the increase of this reaction with time occurred exclusively in the 20:2 acid, suggesting that the conversion of 20:2 to acids of longer chain had already reached an equilibrium at the lowest times and at any concentration of the microsomal protein measured. Moreover, the elongation of linoleic acid to 20:2 acid did not reach a plateau even after 40 min of incubation.

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TABLE 1

Chain Lengthening of [1-^{14}C]Linoleic Acid

Min	Microsomal protein (mg)	18:2 (%)	20:2 (%)	22:2 (%)	24:2 (%)	Total chain lengthening (%)
5	2.5	96.4 ± 0.4	1.5 ± 0.2	1.6 ± 0.2	0.5 ± 0.3	3.6
10	2.5	95.9 ± 0.5	1.9 ± 0.4	1.3 ± 0.2	1.0 ± 0.2	4.2
20	2.5	94.6 ± 0.6	2.5 ± 0.2	1.8 ± 0.6	1.1 ± 0.2	5.4
40	2.5	92.5 ± 0.3	5.1 ± 0.2	1.6 ± 0.1	0.7 ± 0.2	7.5
5	5.0	92.7 ± 0.6	3.8 ± 0.6	1.8 ± 0.6	1.7 ± 0.7	7.3
10	5.0	93.4 ± 2.0	4.4 ± 1.6	1.3 ± 0.1	0.9 ± 0.4	6.6
20	5.0	85.6 ± 0.9	12.1 ± 0.9	1.4 ± 0.1	1.0 ± 0.1	14.4
40	5.0	82.4 ± 0.7	14.6 ± 0.5	1.6 ± 0.1	1.4 ± 0.7	17.6

One-hundred-forty nmol of linoleic acid were incubated at 37 C with the cofactors detailed in Materials and Methods. Analyses were performed in triplicate. Mean ± 1 SEM.

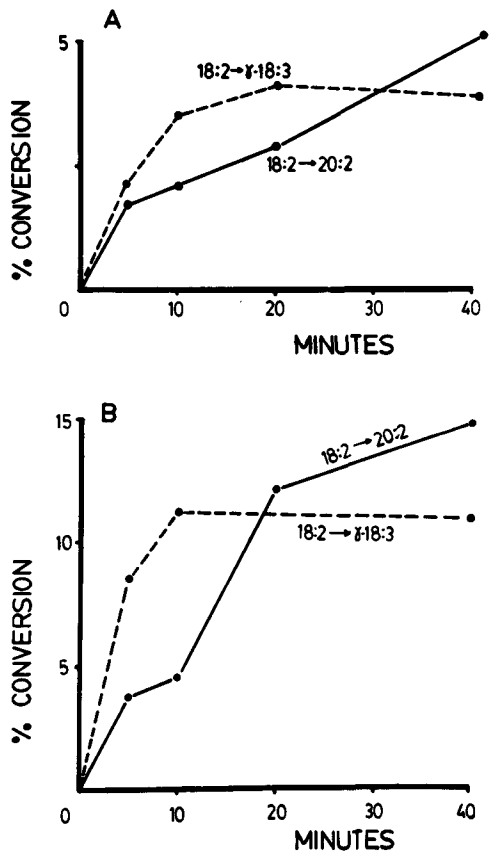

FIG. 1. Linoleic acid desaturation to γ-linolenic acid (– – – –) and linoleic acid elongation to eicosa-11,14-dienoic acid (——) after different times of incubation, in the presence of 2.5 mg (A) and 5 mg (B) of liver microsomal protein. Results are the mean of three determinations made for each point. Technical details are described in the text.

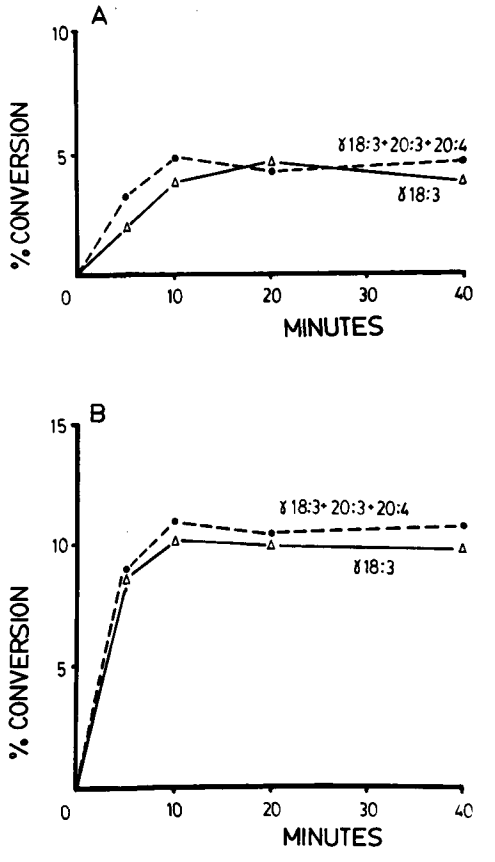

FIG. 2. Desaturation of linoleic acid to γ-linolenic acid (——) and conversion of linoleic acid into its desaturation products, γ-18:3 + 20:3 + 20:4 (– – – –), in the presence of malonyl-CoA after different times of incubation. (A) 2.5 mg of microsomal protein; (B) 5 mg of microsomal protein. Results are the mean of three determinations made for each point. Technical details are described in the text.

TABLE 2

Desaturation and Chain Lengthening of [1-^{14}C]Linoleic Acid

Min	Microsomal protein (mg)	18:2 (%)	18:3 (%)	20:2 (%)	20:3 (%)	20:4 (%)	22:2 (%)
5	2.5	94.1 ± 1.1	1.3 ± 0.3	1.4 ± 0.1	0.4 ± 0.2	1.4 ± 0.5	1.6 ± 0.2
10	2.5	93.3 ± 0.4	2.6 ± 0.2	1.8 ± 0.4	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.1
20	2.5	91.8 ± 0.9	2.4 ± 0.3	2.6 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	1.3 ± 0.1
40	2.5	89.0 ± 2.0	2.0 ± 0.2	4.5 ± 0.5	1.2 ± 0.3	1.3 ± 0.2	1.7 ± 0.2
5	5.0	88.5 ± 0.8	5.0 ± 0.6	1.8 ± 0.1	2.0 ± 0.1	1.7 ± 0.2	1.0 ± 0.2
10	5.0	85.7 ± 0.4	4.9 ± 0.2	3.1 ± 0.4	2.2 ± 0.2	3.1 ± 0.1	1.0 ± 0.1
20	5.0	81.7 ± 1.9	1.5 ± 0.5	7.1 ± 0.3	2.3 ± 0.1	5.9 ± 0.3	1.5 ± 0.1
40	5.0	79.1 ± 0.8	3.0 ± 0.2	8.8 ± 0.6	2.2 ± 0.4	5.3 ± 0.2	1.6 ± 0.4

One hundred nmol of linoleic acid were incubated at 37 C with 0.2 μmol of malonyl-CoA and other cofactors in aerobiosis; see Materials and Methods for details. Analyses were performed in triplicate. Mean ± 1 SEM.

The comparison of the elongation and desaturation reactions (Figs. 1A and 1B) shows similar but higher conversions for the elongation at the longest periods. However, the speed of the reaction measured at the shortest time (5 min) shows that the Δ6 desaturation was faster than the elongation (0.34 and 0.29 nmol/min/mg protein, respectively). These results agree with those of Bernert and Sprecher (15) who found a desaturation/elongation ratio of 2.3 after 3-min incubation of liver microsomes of rats raised on a fat-free diet. The higher ratio found by these authors is likely due to the fat-free diet; it has been shown on several occasions that such a diet increases the Δ6 desaturation and the Δ6 desaturation/elongation ratio (16).

When the two microsomal systems (desaturation and elongation) were allowed to react together in the presence of malonyl-CoA, linoleic acid was desaturated to γ-linolenic acid, elongated to 20:3 and desaturated to 20:4 acids, but also directly elongated to 20:2 and 22:2 (Table 2).

Although the structure of 20:3 was not checked in this experiment, it is possible to assume from findings of Ullman and Sprecher (17) and Marcel et al. (16) that it is mainly constituted by the 8,11,14-20:3 acid isomer derived from the γ-18:3 acid and a small amount of 5,11,14-20:3 acid derived from the 11,14-20:3 acid. Therefore, due to the low label found in the 20:3 chromatographic peak, the overall Δ6 desaturation conversion may be calculated by the addition of 18:3, 20:3 and 20:4 acids, and the elongation by the 20:2 and 22:2 addition. From these calculations (Figs. 2A and 2B), it is possible to deduce that the presence of malonyl-CoA neither inhibits nor activates linoleic acid desaturation to γ-linolenic acid in the conditions of the experiment. Therefore, it is difficult to admit that factors enhancing acetyl-CoA carboxylase activity might modify arachidonate biosynthesis by an increase of malonyl-CoA concentration. The direct effect of malonyl-CoA on the isolated microsomes is shown, as expected, by the presence of labeled 20:3 and 20:4 acids.

In conclusion, neither malonyl-CoA addition nor the elongation reaction of γ-linolenyl-CoA coupled to the Δ6 desaturation of linoleic acid modify the speed of linoleic acid desaturation.

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Selective Use of Palmitic Acid over Stearic Acid for Synthesis of Phosphatidylcholine and Phosphatidylglycerol in Lung

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The incorporation of [^{3}H]palmitic acid and [^{14}C]stearic acid into phospholipids in rabbit lung tissue was studied. Under equal molar concentrations of palmitate and stearate, palmitate was incorporated to the 1- and 2-positions of phosphatidylcholine (PC) and phosphatidylglycerol (PG) 2–3 times more than stearate. By contrast, palmitate was 30% less than stearate in phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine. These results suggest that preferential utilization of palmitate over stearate, rather than substrate availability, determines the high content of palmitoyl at the 1- and 2-positions of PC and PG in lung.
Lipids 21, 724–725 (1986).

The unique function of the lipid-lipoprotein surfactant complex layered on the air-water interface of pulmonary alveoli is to stabilize the air sac by lowering the surface tension at the alveolar lining layer, thus preventing the collapse of the lung during expiration. The most abundant component of lung surfactant is dipalmitoyl phosphatidylcholine (PC) (1).

Since palmitic acid and stearic acid are the two major saturated fatty acids in the free fatty acid pool in lung and palmitate pool is about two times greater than stearate (2), it is not known whether more dipalmitoyl PC being synthesized is due to the substrate availability or specific selection as compared with stearate homolog. The present study tests the utilization of equal molar palmitate and stearate as the substrates for the synthesis of phospholipids in lung to elucidate whether substrate availability or specific selection determines the synthesis of saturated PC and other phospholipids.

MATERIALS AND METHODS

Incubation of [^{3}H]*palmitic acid and* [^{14}C]*stearic acid with lung tissue slices.* A fatty acid suspension was prepared by sonicating 30 μCi (0.6 μmol) [9,10-^{3}H]palmitic acid and 30 μCi (0.6 μmol) [1-^{14}C]stearic acid (New England Nuclear, Boston, Massachusetts) in 6 ml 0.9% NaCl saline for 1 min in an ice water bath under N_2. The pH of the solution was adjusted to 7.4. Lung slices were prepared as described before (3). One g of lung slices was first rinsed with 4 ml Krebs-Ringer buffer (pH 7.4) in a 25-ml Erlenmeyer flask and then preincubated at 37 C in another 10 ml Krebs-Ringer buffer for 5 min in a shaking water bath followed by addition of 1 ml fatty acid suspension. The incubation was continued for another 60 min. At the end of the reaction, the flask was placed on ice, and the incubation medium was quickly withdrawn with a Pasteur pipette. The slices were washed with 30 ml cold saline three times, and the washes were discarded. The slices were homogenized in 3 volumes of saline. The homogenate was centrifuged at 500 × g for 10 min to remove cell debris and connective tissue, and the supernatant was used for lipid extraction.

Determination of ^{3}H- and ^{14}C-labeled lipids. Lipids in the 500 × g supernatant were extracted by the method of Bligh and Dyer (4). Phospholipids were separated by two dimensional thin layer chromatography (TLC) (5) on a precoated Silica Gel 60 plate (E. Merck, Darmstadt, Federal Republic of Germany) and identified with iodine vapor. Each phospholipid spot was scraped off into a scintillation vial for radioactivity determination.

Radioactivity of a sample was determined in 10 ml scintillation fluid (3.3 g 2,5-diphenyloxazole in 400 ml toluene and 200 ml ethylene glycol monoethyl ether) with a Beta-Trac 6895 liquid scintillation counter (Tracor Analytic, Elk Grove Village, Illinois) and was expressed as dpm derived via the external standard ratio.

Saturated PC was isolated by the method of Masson et al. (6) and further purified by one-dimensional silica gel TLC in a developing solvent of chloroform/methanol/water (65:45:5, v/v/v).

The distributuion of ^{3}H and ^{14}C at the 2- and 1-positions of PC was measured from the liberated fatty acids and lysoPC, respectively, after PC was hydrolyzed by *Croalus adamanteus* venom phospholipase A_2 (7).

RESULTS AND DISCUSSION

Under the assay conditions, about 10% of total radioactivity of each labeled fatty acid in the incubation media was found in lung homogenate after 1 hr incubation at 37 C. The amount of radiolabeled palmitate and stearate in lung homogenate was approximately the same.

As stated by Holub and Kuksis (8), selectivity and availability of substrate are two factors controlling the molecular species of glycerolipids. It is possible that substrate availability might determine the high content of palmitate over stearate in lung PC because of the larger pool of free palmitic acid than stearic acid, observed in rat lung (2) and in rabbit lung in this study (data not shown). However, the present results show that [^{3}H]palmitate was incorporated into total PC about two times more than [^{14}C]stearate at both the 1- and 2-positions, and the incorporation of [^{3}H]palmitate was even greater in the saturated PC, although both palmitate and stearate are saturated (Table 1). Since, in general, a large pool of substrate would lower the specific radioactivity and decrease the amount of radiolabeled substrate incorporated into the product, the results suggest that palmitate was preferentially incorporated into PC compared to stearate.

Phosphatidylglycerol (PG) is the second abundant component of surfactant phospholipids that also has a high percentage of palmitate (9). These results again show that palmitate compared with stearate was preferentially incorporated into PG (Table 1). The preferential incorporation of palmitoyl-CoA over oleoyl-CoA into PG in the homogenate of the whole lung

TABLE 1

Incorporation of [^{3}H]Palmitate/[^{14}C]Stearate into Phospholipids in Lung Slices

Phospholipids	[^{3}H]Palmitate/[^{14}C]stearate
Phosphatidylcholine	2.22 ± 0.79
1-Position	2.30 ± 0.15
2-Position	3.17 ± 0.38
Saturated	5.23 ± 0.37
Phosphatidylethanolamine	0.70 ± 0.03
Phosphatidylglycerol	2.35 ± 0.90
Phosphatidylinositol	0.76 ± 0.19
Phosphatidylserine	0.71 ± 0.07

The ratio of ^{3}H-dpm/^{14}C-dpm (mean ± SD of six experiments) of phospholipids was calculated based on the assumption that the ratio of ^{3}H-dpm/^{14}C-dpm of lung homogenate was designated as 1.00.

or type II cells has been previously observed (10), but the selective use of palmitate over stearate for PG synthesis has not been reported.

Unlike PC and PG, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine in lung all have a higher content of stearoyl than palmitoyl (9). Likewise, the incorporation of palmitate into these phospholipids was about 30% less than stearate. This study cannot explain whether there is a selective utilization of stearate over palmitate for the synthesis of these phospholipids, since the specific radioactivity of intracellular stearate would be higher than that of palmitate due to the smaller pool size of stearate. Nevertheless, the selection between palmitate and stearate for the syntheses of major and minor surfactant phospholipids is different.

Although no experiment in this study was designed to identify the pathway of the incorporation of palmitate and stearate into PC and PG, the results clearly indicate that selectivity plays a major role for the incorporation of palmitoyl to the 1- and 2-positions of PC and to PG in lung among the saturated fatty acid substrates.

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REVIEW

Biological Effects of Fish Oils in Relation to Chronic Diseases[1]

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The low incidence of cardiovascular disease in Greenland Eskimos appears to be due to their high intake of seal, whale and fish. The lipids of these marine animals lower serum triglyceride and cholesterol levels and help to prevent blood clotting. The latter effect has been related to a change in the balance of prostacyclin and thromboxane as a result of replacing n-6 polyunsaturated fatty acids in the body by n-3 polyunsaturated fatty acids present in marine lipids. Dietary fish oils have also been shown to inhibit development of mammary, pancreatic, intestinal and prostatic tumors in experimental animals. This effect may likewise be due to changes in the production of prostaglandins or related compounds. The involvement of prostaglandins and leukotrienes in immune responses has led to studies on the effects of fish oil on various chronic diseases associated with abnormalities of the immune system. Some of these diseases, such as multiple sclerosis and psoriasis, are also relatively uncommon in Eskimos. Preliminary results of these studies are encouraging, but more work is required to assess the usefulness of dietary fish oils in treatment of these diseases. In addition to their apparent therapeutic value, n-3 fatty acids are considered essential dietary components since they cannot be synthesized in the body and appear necessary for normal vision and probably other body functions.
***Lipids 21,* 731-732 (1986).**

Current interest in the biological effects of fish oils developed largely from studies on Greenland Eskimos (1-3). The main stimulus for such studies was the realization that these people show very little evidence of cardiovascular disease although they eat a diet that is high in fat and animal protein. Marine animals, including seals, whales and fish make up a relatively large proportion of this diet.

The Japanese have also traditionally had a relatively high consumption of fish, and this may help to account for their low incidence of cardiovascular disease (3). Furthermore, a recent report from the Netherlands has aroused considerable interest by providing epidemiological evidence of an inverse correlation between fish consumption and mortality from coronary heart disease (4). This led the authors to conclude "that the consumption of as little as one or two fish dishes per week may be of preventive value in relation to coronary heart disease."

MARINE LIPIDS AND CARDIOVASCULAR DISEASE

Much of the work stimulated by the studies on Eskimos has focused on the lipid portion of the diet because of earlier evidence that dietary fat can significantly influence serum cholesterol levels and atherosclerosis. The recent studies have disclosed two important effects of marine lipids that may explain why Eskimos have so little cardiovascular disease.

Polyunsaturated fish oils are very effective in lowering serum triglyceride levels and also can lower serum cholesterol levels (5-7). In addition, diets containing fish oils have an inhibitory effect on blood clotting (2,3,6-8), and this reduces the risk of thrombosis, often a major factor in heart attacks and strokes.

The reduction in serum triglycerides and cholesterol may be due to decreased production of very low density lipoproteins by the liver, possibly as a result of a decrease in triglyceride synthesis (3,7). The effects on blood clotting are most likely related to alterations in the production of different prostanoids from polyunsaturated fatty acids (3,7,8). The main polyunsaturated fatty acid in vegetable oils is linoleic acid [18:2(n-6)], which is converted in the body into arachidonic acid [AA; 20:4(n-6)]. AA in turn is converted by blood platelets into the thromboxane TXA_2, which causes constriction of blood vessels and aggregation of platelets leading to blood clotting. AA is also converted in blood vessel walls to the prostacyclin PGI_2, which relaxes blood vessels and prevents aggregation of platelets. It is thought that formation of these substances is balanced to allow clotting to occur after wounds or other injuries to blood vessel walls but to prevent clotting under normal physiological conditions (8).

Most fish oils and other marine lipids contain very little linoleic acid or other n-6 fatty acids (9). The main polyunsaturated fatty acids in such oils are eicosapentaenoic acid [EPA; 20:5(n-3)] and docosahexaenoic acid [22:6(n-3)]. EPA has the same structure as AA except for one additional double bond at the 3-position relative to the methyl end of the chain. EPA is thus a potential substrate for conversion to the thromboxane TXA_3 by platelets and to the prostacyclin PGI_3 by blood vessel walls. This could alter the tendency for blood to clot, since TXA_3 does not aggregate platelets as effectively as TXA_2, while PGI_3 seems to prevent aggregation about as effectively as PGI_2. In actual fact, EPA does not seem to be a very good substrate for TXA_3 synthesis but may compete with AA to decrease TXA_2 synthesis while allowing normal or increased synthesis of prostacyclin (3,7,8). Polyunsaturated fatty acids also serve as substrates for the formation of leukotrienes and other lipoxygenase products. Leukotrienes formed from EPA differ in their biological properties from those derived from AA, and this may also help to account for some of the observed effects of dietary fish oils (3).

FISH OILS AND CANCER

Another recent line of research has provided evidence that high-fat diets promote the development of certain types of cancer. Essential fatty acids seem to be required for this effect (10), and promotion by high-fat diets can be

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prevented by inhibitors of prostanoid biosynthesis (11,12). These observations and the knowledge that Eskimo populations have a relatively low incidence of cancer led to experiments in our own and other laboratories to investigate the effects of dietary fish oils on tumorigenesis in animals. Although small amounts of fish oil appeared to promote mammary tumorigenesis (10,13,14), diets containing higher levels failed to stimulate tumor growth and development and may even have had an inhibitory effect (13-16). More recent experiments have given similar results with pancreatic (17), intestinal (18) and prostatic tumors (19).

It is tempting to conclude that the differing effects of polyunsaturated vegetable oils and fish oils are due to differences in prostanoid formation, but other explanations may be possible. The unsaturated fatty acids in fish oils are more susceptible to oxidative changes than those in vegetable oils because the former have a higher degree of unsaturation and are perhaps less protected by naturally occurring antioxidants such as tocopherols. Oxidation products other than prostanoids may thus be acting to inhibit tumorigenesis. Fish oils contain only small amounts of the n-6 fatty acids that appear to be necessary for tumor promotion in animals, and this deficiency may also be a factor in their failure to increase tumor yields. Further studies with diets containing mixtures of fish oils and vegetable oils should help to clarify the mechanisms involved (20).

The low incidence of cancer in Eskimos is not necessarily related to diet. It has been suggested, for example, that the practice of suckling infants for relatively long periods may help to reduce the risk of breast cancer in Eskimos (21). It is also difficult to say whether consumption of fish by populations such as the Japanese is a factor in their low incidence of breast and colon cancer compared to that in other countries.

FISH OILS AND OTHER CHRONIC DISEASES

Evidence that polyunsaturated fatty acids and prostaglandins and leukotrienes derived from them can affect immune function and inflammatory reactions has led to interest in effects of fish oils on other chronic diseases such as hypertension (22), lupus (23,24), multiple sclerosis (25,26), rheumatoid arthritis (27) and psoriasis (28). Beneficial results have been reported in a number of these preliminary studies, but the role of prostaglandins and leukotrienes in immune reactions is complex and poorly understood, and alterations produced by feeding fish oils may exacerbate rather than ameliorate disease symptoms (29,30).

The fact that Eskimos appear relatively free of some of the diseases noted (31) provides a good rationale for further studies, but until much more information is available, advertising claims of widespread benefits from supplements of fish oil or other fish products should be viewed with caution.

ESSENTIAL NATURE OF n-3 FATTY ACIDS

It has long been recognized that n-3 fatty acids are less effective than n-6 fatty acids in preventing the classical symptoms of essential fatty acid deficiency (32). The presence of large amounts of n-3 fatty acids in lipids of tissues such as the retina and the central nervous system has led to the idea that these fatty acids serve specific functions distinct from those of n-6 fatty acids. It has proved difficult to demonstrate a deficiency of n-3 fatty acids (33), but recent studies of Neuringer et al. (34) have demonstrated loss of visual acuity in infant monkeys deprived of dietary n-3 fatty acids. It thus appears that n-3 fatty acids are essential dietary components, even though the benefits of increased intake are still unclear.

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ARTICLES

Comparative Study of the Blood Pressure Effects of Four Different Vegetable Fats on Young, Spontaneously Hypertensive Rats

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Following the suckling period, four groups of male four-week-old spontaneously hypertensive rats (SHR) were fed semisynthetic diets with 14% (by weight) of either sunflower seed oil [46% 18:2(n-6); linoleic acid (LA)-rich], linseed oil [62.5% 18:3(n-3) + 12.9% 18:2(n-6); α-linolenic acid (LNA)-rich], evening primrose oil [9.2% 18:3(n-6) + 71% 18:2(n-6); γ-linolenic acid (LNA)-rich] or hydrogenated palm kernel fat [1.5% 18:2(n-6); polyunsaturated fatty acid (PUFA)-deficient], respectively, up to an age of 18 wk. All diets enriched with PUFA provoked an attenuation of hypertension development. The effect was lowest in the LA-rich group and highest in the γ-LNA-rich group. Differences in fatty acid composition of renal phospholipids between groups reflect the fatty acids present in the respective dietary fats. Renomedullary production of $PGF_{2\alpha}$ was significantly reduced in α-LNA-rich and slightly diminished in γ-LNA-rich fed rats. Aortic formation of 6-keto-$PGF_{1\alpha}$ and TXB_2 was increased in animals fed the γ-LNA-rich diet. Thus, the attenuation of hypertension development cannot be explained only by changes in prostanoid formation. Other mechanisms possibly involved should be pursued.

Lipids 21, 733–737 (1986).

Diets enriched with polyunsaturated fatty acids (PUFA) have been shown to reduce blood pressure in salt-loaded rats (1,2), in renally hypertensive rats (3) and in man (4). In spontaneously hypertensive rats (SHR), a model closely related to human essential hypertension, no influence of dietary linoleic acid on blood pressure could be found using adult animals (5,6). But when the dietary period was prolonged, i.e., it was begun in the pregnant mothers, a distinct attenuation of hypertension development after feeding linoleic acid (LA)-rich or α-linolenic acid (LNA)-rich diets could be observed (5). Because one could argue that this dietary regimen is of little relevance for the clinical situation, we performed the present feeding experiments beginning with young male SHR in the prehypertensive period.

The hypotensive effects of dietary PUFA in salt-loaded rats and prenatally fed SHR have, at least in part, been ascribed to alterations in the endogenous production of prostaglandins (PG), which induce antihypertensive alterations to vascular and renal functions resulting in a depression of blood pressure (7–11).

The bioconversion of dietary C18-PUFA into C20-PG precursor fatty acids proceeds in mammals through desaturation and prolongation steps. This pathway is rate-limited by the activity of the Δ6-desaturase. Genetic, hormonal and nutritional factors control the activity of this enzyme and thereby contribute to the regulation of the synthesis of immediate precursors of PG production.

Evening primrose oil contains 18:3(n-6), and it was of interest to investigate the potency of this vegetable oil to modulate hypertension in SHR. Therefore, we investigated the hypotensive effect of evening primrose oil as compared to diets enriched with 18:2(n-6) (sunflowerseed oil) or 18:3(n-3) (linseed oil), including the question of whether the blood pressure-lowering effects of the different diets correlate with alternations in ex vivo prostanoid formation.

METHODS

Following the suckling period, 60 four-week-old male SHR of the Okamoto-Aoki strain were randomly divided into four subgroups and fed four semisynthetic diets which differed only in the fat composition. We used an LA-rich (sunflowerseed oil), an α-LNA-rich (linseed oil), a γ-LNA-rich (evening primrose oil) and a PUFA-deficient (hydrogenated palm kernel fat) diet. The composition of the diets is summarized in Table 1. Diets were given ad libitum up to an age of 18 wk. The animals received tap water ad libitum.

Systolic blood pressure of prewarmed (by infrared light for 20 min), conscious rats was measured in the morning by the tail cuff plethysmographic method. The size of the cuff was adapted to the growing animals. Heart rate was calculated from the recorded oscillations.

At 18 weeks, the rats were decapitated in the morning (8–11 a.m.). Kidneys, aortas, hearts, livers and brains were quickly removed, cleaned of blood and fat and stored at −20 C for a maximum of one month.

The kidney was cut into small pieces, placed immediately into a mixture of chloroform/methanol (2:1, v/v) and homogenized. Lipids were extracted according to Folch et al. (12). Phospholipids were separated by thin layer chromatography. The fatty acid composition was estimated by gas liquid chromatography (GLC) after forming methyl esters using our recently described method (13).

The kidney medulla was carefully removed, homogenized in KH_2PO_4/NaOH (1:10, v/v) buffer at 4 C and incubated 30 min at 37 C. After addition of 1 μg dihomo-$PGF_{2\alpha}$ as internal standard, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ were estimated by GLC (14). Intact aortas (Arcus aortae to Aorta abdominalis) were incubated in 10 ml Tyrode's solution for 60 min at 37 C. 6-Keto-$PGF_{1\alpha}$ and TXB_2 (the stable products from PGI_2 and TXA_2, respectively) were

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TABLE 1

Composition of LA-Rich, α-LNA-Rich, γ-LNA-Rich and PUFA-Deficient Diets[a]

	Diet			
	LA-rich	α-LNA-rich	γ-LNA-rich	PUFA-deficient
Ingredients (wt%)				
Casein	20	20	20	20
Starch	58	58	58	58
Cellulose	5	5	5	5
Salt mixture[b]	3	3	3	3
Vitamin mixture[c]	+	+	+	+
Fat	14 (sunflowerseed oil)	14 (linseed oil)	14 (evening primrose oil)	14 (hydrogenated palm kernel oil)
Main fatty acid composition (%)				
12:0	1	—	—	57
14:0	5	0.1	—	8.5
16:0	7	8.5	8.3	6
16:1	1	—	—	6
18:0	7.5	1.8	1.9	19
18:1(n-9)	29	14	9.4	2
18:2(n-6)	46	12.9	71	1.5
18:3(n-3)	3.5	62.5	—	—
18:3(n-6)	—	—	9.2	—

[a]LA, linoleic acid; LNA, linolenic acid; PUFA, polyunsaturated fatty acid.

[b]Salt mixture, McCollum (g/kg): 50 g NaCl; 75 g $MgSO_4$; 100 g NaH_2PO_4; 210 g KH_2PO_4; 160 g $Ca(H_2PO_4)_2$; 375 g Ca-lactate; 3 g Fe-citrate; 0.9 g KJ; 0.3 g $CuSO_4$; 0.15 g $ZnSO_4$; 0.2 g $MnSO_4$; 0.03 g NaF.

[c]Vitamin mixture (per kg): 5000 IU A; 4 mg B_1; 5 mg B_2; 6 μg B_6; 5 μg B_{12}; 12 mg nicotinic acid amide; 200 mg cholinic chloride; 15 mg panthenol; 46 mg folic acid; 300 IU D; 100 mg E.

estimated in the incubation fluid by radioimmunoassay, using antibodies produced from rabbits in our laboratory (15).

Results are expressed as mean values ± SEM with the number of observations in parentheses. A $p < 0.05$ was accepted as the criterion of significance. Differences between the groups were tested by the one-way analysis of variance. Subsequent comparisons of the individual means were performed using Scheffe's test. Alterations of blood pressure and heart rate were calculated by the two-way repeated measurement ANOVA designs and subsequent planned orthogonal contrasts.

RESULTS

Body weight, organ weights, fluid and food intakes. Body weights at the beginning of the feeding period were 123 ± 3 g, 124 ± 3 g, 124 ± 2 g and 124 ± 2 g in rats fed LA-rich, α-LNA-rich, γ-LNA-rich and PUFA-deficient diets, respectively. Food and water intake did not differ significantly between dietary groups during the feeding period. Body weight gain was highest in rats fed the γ-LNA-rich diet and lowest in those fed the PUFA-deficient one (Table 2). Relative weights of heart, kidneys, livers and brains were not significantly different among animals of the four experimental groups (Table 2).

Blood pressure and heart rate. All PUFA-rich diets provoked an attenuation of hypertension development compared to PUFA-deficient diets. The effect was most pronounced in SHR fed a γ-LNA-rich diet and lowest in those fed an LA-rich diet (Fig. 1). There were only slight differences in heart rate between the dietary groups during the experimental period (Fig. 2).

Fatty acid patterns and prostanoid formation. Feeding of PUFA-rich vegetable oils resulted in an accumulation of 18:2(n-6) in renal phospholipids compared to PUFA-deficiency (Table 3). In the renal phospholipids of SHR fed the γ-LNA-rich diet, we did not observe a significant accumulation of 18:3(n-6) and its C_{20}-homologues, 20:3(n-6) and 20:4(n-6), in contrast to animals on an LA-rich diet.

In the SHR on the α-LNA-rich diet, there was an increase in 18:3(n-3) and its C_{20}-homologue, 20:5(n-3). The 20:4(n-6) content in this group was lowered compared to the SHR fed the LA-rich and γ-LNA-rich diets. Renomedullary production of $PGF_{2\alpha}$ was significantly reduced in animals fed α-LNA-rich and γ-LNA-rich diets (Table 4). We did not detect $PGF_{1\alpha}$ formation in any of the dietary groups (detection limit 0.1 μg/g kidney medulla).

In aortic strips from SHR fed a γ-LNA-rich diet, we observed a significant increase in the formation of 6-keto-$PGF_{1\alpha}$ and TXB_2 (Table 4). In this dietary group, the 6-keto-$PGF_{1\alpha}$/TXB_2 ratio was decreased in comparison to all other dietary groups (Fig. 3).

DISCUSSION

In previous experiments using six-month-old adult SHR, no influence of dietary PUFA on blood pressure regulation could be found (5). But a blood pressure-lowering effect was observed when the dietary PUFA feeding was started prenatally, i.e., in the pregnant mothers (5).

The present experiments clearly demonstrate that SHR are also vulnerable to dietary manipulations during their growth and the development of hypertension. The three PUFA-rich diets attenuate the development of high blood pressure compared to PUFA-deficient diets. The effect

TABLE 2

Body Weight (g) and Relative Organ Weight (g/kg Body Wt) of Spontaneously Hypertensive Rats Fed LA-Rich, α-LNA-Rich, γ-LNA-Rich and PUFA-Deficient Diets[a]

	Diet				
	LA-rich (1)	α-LNA-rich (2)	γ-LNA-rich (3)	PUFA-deficient (4)	Significance[b]
Body wt	294 ± 5	305 ± 11	319 ± 9	280 ± 7	1/3 and 2/4, $p < 0.05$ 3/4, $p < 0.01$
Heart	3.1 ± 0.1	3.2 ± 0.1	3.5 ± 0.3	3.4 ± 0.2	ns
Kidney	3.2 ± 0.1	3.4 ± 0.1	3.3 ± 0.2	3.4 ± 0.1	ns
Liver	36.0 ± 3.0	33.4 ± 0.9	35.5 ± 1.2	35.3 ± 1.5	ns
Brain	5.6 ± 0.3	5.4 ± 0.1	5.5 ± 0.2	5.3 ± 0.5	ns

[a]Rats aged 18 wk; n = 15 for each group. See Table 1 for abbreviations.
[b]ns, Not significant.

TABLE 3

Primary Individual Fatty Acids in Phospholipids of Kidneys of Spontaneously Hypertensive Rats Fed LA-Rich, α-LNA-Rich, γ-LNA-Rich and PUFA-Deficient Diets[a]

	Diet				
Fatty acid (%)	LA-rich (1)	α-LNA-rich (2)	γ-LNA-rich (3)	PUFA-deficient (4)	Significance[b]
18:1(n-9)	8.5 ± 0.4	11.6 ± 1.2	6.9 ± 0.6	11.2 ± 1.0	1/4, $p < 0.05$ 1/2, $p < 0.01$ 2/3 and 3/4, $p < 0.001$
18:2(n-6)	16.1 ± 0.4	16.0 ± 0.6	13.4 ± 0.9	11.0 ± 0.9	1/3, 2/3 and 3/4, $p < 0.05$ 1/4 and 2/4, $p < 0.001$
18:3(n-6)	0	0	traces	0	ns
18:3(n-3)	0.1 ± 0.1	3.8 ± 1.5	0.3 ± 0.3	0.1 ± 0.1	1/2, 2/3 and 2/4, $p < 0.001$
20:3(n-9)	traces	0.5 ± 0.2	0.1 ± 0.1	1.0 ± 0.3	1/4 and 3/4, $p < 0.01$
20:3(n-6)	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.3	1.9 ± 1.1	ns
20:4(n-6)	28.7 ± 2.9	21.5 ± 0.6	29.5 ± 2.0	23.9 ± 1.9	1/2 and 2/3, $p < 0.05$
20:5(n-3)	traces	9.6 ± 2.5	1.8 ± 1.2	traces	1/2, 2/3 and 2/4, $p < 0.01$

[a]Rats aged 18 wk; n = 6–8. See Table 1 for abbreviations.
[b]ns, Not significant.

TABLE 4

Formation of Prostanoids in Kidney Medulla Homogenates (μg/g) and Aortic Strips (ng/g) of Spontaneously Hypertensive Rats Fed LA-Rich, α-LNA-Rich, γ-LNA-Rich and PUFA-Deficient Diets[a]

		Diet				
	Prostanoid	LA-rich (1)	α-LNA-rich (2)	γ-LNA-rich (3)	PUFA-deficient (4)	Significance
Kidney medulla	$PGF_{2\alpha}$ (n = 11–13)	2.09 ± 0.08	0.78 ± 0.08	1.71 ± 0.08	2.27 ± 0.27	3/4, $p < 0.05$ 1/2, 2/3 and 2/4, $p < 0.001$
Aorta	6-keto-$PGF_{1\alpha}$ (n = 8–11)	36.15 ± 2.44	30.36 ± 2.85	49.04 ± 3.99	36.48 ± 3.24	1/3, 2/3 and 3/4, $p < 0.001$
	TXB_2 (n = 8–11)	2.96 ± 0.19	3.35 ± 0.41	7.08 ± 0.61	3.77 ± 0.56	1/3, 2/3 and 3/4, $p < 0.001$

[a]Rats aged 18 wk.

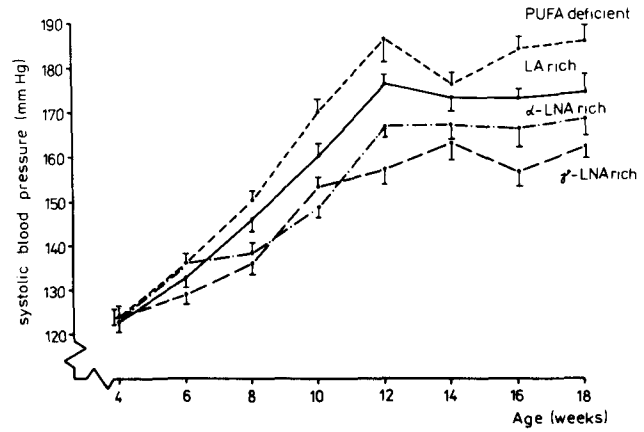

FIG. 1. Influence of linoleic acid (LA)-rich, α-linolenic acid (LNA)-rich, γ-LNA-rich and polyunsaturated fatty acid (PUFA)-deficient (PUFAd) diets on systolic blood pressure in young male spontaneously hypertensive rats. Diets were begun immediately after suckling. Systolic blood pressure was measured by the tail cuff plethysmographic method. The bars indicate SEM for n = 15 in each group. Statistically significant differences ($p < 0.05$) were observed in rats fed the PUFAd diet compared to those fed LA-rich, α-LNA-rich and γ-LNA-rich diets at weeks 10, 12, 16 and 18, as well as in rats fed the γ-LNA-rich diet compared to those fed the LA-rich and α-LNA-rich diets at weeks 12 and 16.

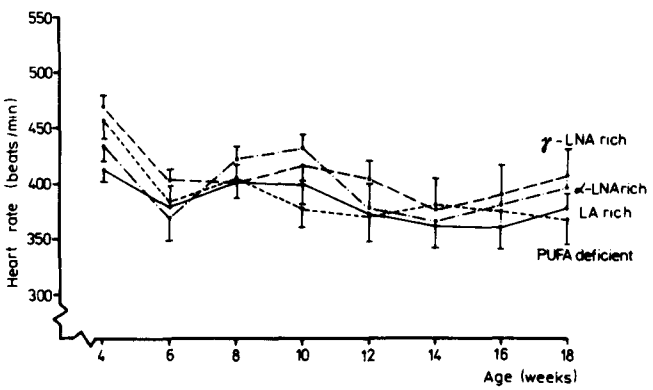

FIG. 2. Influence of LA-rich, α-LNA-rich, γ-LNA-rich and PUFA-deficient diets on heart rate in young male SHR. For details and abbreviations, see Fig. 1 legend. There were no statistically significant differences ($p > 0.05$) between dietary groups.

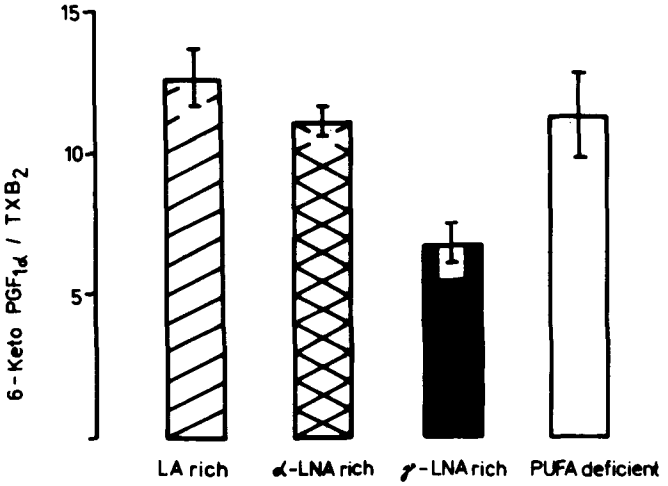

FIG. 3. Ratio of 6-keto-$PGF_{1\alpha}$/TXB_2 produced from aortic strips of 18-wk spontaneously hypertensive rats fed LA-rich, α-LNA-rich, γ-LNA-rich and PUFA-deficient diets. 6-Keto-$PGF_{1\alpha}$ and TXB_2 (stable products from PGI_2 and TXA_2, respectively) were estimated in the incubation fluid by radioimmunoassay. For abbreviations and further details, see Fig. 1 legend. Statistically significant difference ($p < 0.01$) was registered in rats fed the γ-LNA-rich diet compared to other groups.

was most pronounced in SHR fed the γ-LNA-rich diet and lowest in those fed the LA-rich one.

The present study relies entirely on systolic blood pressure, because the tail cuff method does not permit the exact measurement of diastolic blood pressure. Other studies in this field also have used systolic blood pressure measurements (1-7). But we must consider that systolic blood pressure is less variable and diastolic blood pressure could be a better indicator of hypertension.

In nonhypertensive rats, dietary PUFA has no effect on blood pressure (16). Dietary PUFA reduces blood pressure only if the regulating system is somehow "stressed," e.g., by salt-loading or a genetically determined disorder of blood pressure regulation in SHR (8,16).

Although food and water intake did not significantly differ between dietary groups during the feeding period, there were significant differences in weight gain. The increased weight gain in SHR on the γ-LNA-rich diet was unexpected, because evening primrose oil given as a nutrition supplement caused significant weight loss in placebo-controlled, double blind clinical trials, suggesting that, at least in patients with a family history of obesity, γ-LNA-supplementation may be helpful in reducing body weight (16). It is beyond the scope of this study to find out the underlying mechanisms for the weight differences among the four dietary groups of SHR. It is assumed that the effect might be caused by influences on the function of the pituitary gland and related mechanisms.

Blood pressure-attenuating effects of the diets seem to be inversely proportional to their effects on weight gain. Since reduced body weight is associated with improvement of hypertension in SHR (17,18) other mechanisms must play a role in the blood pressure-lowering effects of the three PUFA-rich diets.

In our experiments, changes in fatty acid pattern of renal phospholipids reflect the fatty acids present in the respective dietary fats. Dietary PUFA and their homologous long chain fatty acids are accumulated in the tissues of SHR fed a PUFA-rich diet. But it is important to note that feeding evening primrose oil did not result in an enrichment of 18:3(n-6) or 20:3(n-6) in renal phospholipids (Table 3). In this respect, it is of interest that Δ5-desaturase is very active in rat tissues (19), resulting in a fast conversion of 20:3(n-6) to 20:4(n-6). In agreement with these findings, we could not observe a significant formation of 20:3(n-6)-derived $PGF_{1\alpha}$ in kidney medulla homogenates of SHR fed the γ-LNA-rich diet, and renomedullary synthesis of 20:4(n-6)-derived $PGF_{2\alpha}$ was not different between animals on the LA-rich and γ-LNA-rich diets.

In kidney medulla homogenates of animals fed the α-LNA-rich diet, we observed a marked reduction of $PGF_{2\alpha}$ formation compared with all other experimental groups; this is a well-known effect of dietary n3-PUFA on the formation of prostanoids of the 2-series.

In accordance with previous experiments (8), we could not find a reduction of renomedullary $PGF_{2\alpha}$ formation after feeding a PUFA-deficient diet. These findings can

be explained by a deficit of the substrate, which provokes an increased activity of prostanoid-synthesizing enzymes (8,20,21).

In addition to the renal PG with a modulating role in water and electrolyte homeostasis (22), the vasodilator PGI_2 and the vasoconstrictor TXA_2 might be assumed as metabolites derived from arachidonic acid involved in blood pressure regulation. Several studies have shown that aortic tissue from SHR generates significantly more PGI_2 than tissue from normotensive controls (23,24). Thus, it has been suggested that PGI_2 might play a compensatory role in this model of hypertension.

The hypotensive action of dietary PUFA is likely to be mediated by a reduction of the vascular tone, which has been shown under in vitro and in vivo conditions (10,25,26). The results presented demonstrate that, in comparison to SHR fed an LA-rich or α-LNA-rich diet, the stronger hypotensive effect in SHR fed a γ-LNA-rich diet is associated with a marked increase in aortic PGI_2 formation. These findings confirm and extend results obtained by Schölkens et al. (25), who observed an increased formation of antiaggregatory activity of rat aortas treated with evening primrose oil. From these results, it is tempting to assume that an increased formation of the potent vasodilator PGI_2 may, at least in part, be involved in the hypotensive effect of γ-LNA-rich diet in SHR. The increase in PGI_2 formation could be explained by the higher availability of 20:4(n-6). Unfortunately, the fatty acid pattern could not be estimated in the aortic tissue during the present experiments.

Recently, our group (15) and others (27-28) found that vascular tissue can produce the potent vasoconstrictor TXA_2. Consequently, it was postulated that enhanced TXA_2 formation in the vessel walls of SHR of the Okamoto-Aoki strain could contribute to the development of hypertension. In the present experiments, vascular TXB_2 formation was markedly increased in the group fed the γ-LNA-rich diet compared to the other dietary groups. When we calculated the ratio between 6-keto-$PGF_{1\alpha}$ and TXB_2 produced by the aortic tissue, there was a distinct shift from 6-keto-$PGF_{1\alpha}$ toward TXB_2 after the γ-LNA-rich diet, whereas no differences could be found in the 6-keto-$PGF_{1\alpha}$/TXB_2 ratio among SHR fed LA-rich, α-LNA-rich or PUFA-deficient diets. So far, our results of ex vivo prostanoid formation cannot fully explain the effects of dietary PUFA on blood pressure, although they are consistent with previous dietary trials in SHR in which the feeding was begun prenatally (5).

Further experiments are being done to clarify whether an antisympathetic action is responsible for the hypotensive potency of dietary PUFA in the SHR model. In prenatally fed SHR as well as in salt-loaded rats, it has been shown that the reduction of sympathetic tone after a PUFA-rich diet is involved in the attenuation of hypertension development (29-31).

In conclusion, the results of the present study show that dietary sunflowerseed oil, linseed oil and evening primrose oil attenuate the development of hypertension in SHR when the regimen is begun immediately after suckling, during the prehypertensive period. The effect was most pronounced in SHR treated with evening primrose oil. From the data presented, we conclude that the decrease in blood pressure of SHR cannot be explained only by changes in prostanoid formation. Therefore, other mechanisms possibly involved should be pursued.

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Effects of Cholestanol Feeding and Cholestyramine Treatment on the Tissue Sterols in the Rabbit

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Rabbits were fed diets enriched with cholestanol or cholesterol (3.5 g/wk) for 4–12 weeks. During cholestanol feeding, the concentration of cholestanol in blood serum, liver, heart and aorta increased 15–30 times. In serum and liver, the concentration of cholesterol also increased. Cholestanol-fed rabbits developed inflammatory changes in the liver, with proliferation of small bile ducts. Liver tests were only slightly abnormal. Morphological atherosclerosis of the aorta was only occasionally seen in rabbits receiving cholestanol for eight weeks or less.

During cholesterol feeding, the amounts of cholesterol in different tissues increased dramatically, most in the aorta. Morphological atherosclerosis in the aorta was found in all rabbits fed cholesterol-enriched diets for more than four weeks. Brain cholestanol was doubled in rabbits fed cholestanol for eight weeks, whereas brain sterols did not change significantly during cholesterol feeding. After an additional regression period with cholestyramine for eight weeks, the increased content of cholestanol in the brain was unchanged in cholestanol-fed rabbits. These observations are discussed in relation to the cholestanolosis of the brain that develops in the rare inherited human disease cerebrotendinous xanthomatosis.
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In the rare hereditary disease cerebrotendinous xanthomatosis (CTX), the degradation of the cholesterol side chain is impaired. Recently, evidence has been presented by studies in vivo (1) and in vitro (2) for a lack of mitochondrial C_{27}-steroid-26-hydroxylase (EC 1.14.13.15) in this disease. This block leads to the accumulation of early intermediates of the bile acid synthesis (3). In a recent work, we have presented evidence for a novel pathway for the synthesis of cholestanol from 7α-hydroxy-4-cholesten-3-one (4). This pathway is accelerated in patients with CTX (5). As a result, they have higher concentrations of cholestanol in their blood plasma. Previously, accumulation of cholestanol has been found in all examined tissues from patients with CTX (6). These patients have tendinous xanthomas and accumulation of cholestanol in the brain, with progressive ataxia, spasticity and mental deterioration. Several patients with CTX have developed premature atherosclerosis.

It has not been clarified whether increased concentrations of cholestanol precursors or increased levels of cholestanol itself lead to the accumulations in CTX. The present study aims to clarify the causal role of cholestanol in sterol accumulations in different tissues, particularly in brain and aorta. We performed feeding experiments to compare cholestanol with cholesterol. We also performed studies to elucidate the reversibility of the sterol accumulations.

MATERIALS AND METHODS

Chemicals. Cholesterol CH-USP, lot, no. 14F-0258, epicoprostanol (5β-cholestan-3α-ol) and cholestanol (5α-cholestan-3β-ol) were obtained from Sigma Chemical Co. (St. Louis, Missouri). Analyses of epoxidized samples of cholesterol by high performance liquid chromatography (HPLC) and gas chromatography (GC) as described below showed that this lot contained 0.07% of cholestanol. Cholestyramine (Questran®) was obtained from Bristol Lab (Syracuse, New York). M-chloroperbenzoic acid was obtained from Fluka AG (CH9470, Buchs, Switzerland). All other chemicals and solvents used were standard commercial high purity materials. [1,2-^{3}H]Cholestanol was prepared by H_2/PtO_2 reduction in ethyl acetate/acetic acid (98:2, v/v) from [1,2-^{3}H]cholesterol (Amersham, United Kingdom) (7). The product was purified by HPLC as described below.

Chow. Ordinary pelleted rabbit chow was obtained from Astro-Ewos (Södertälje, Sweden). The chow contained 14–16% protein, 3–4% fat, 18–21% fiber and all necessary vitamins at recommended levels. Cholesterol or cholestanol, when required, was dissolved in chloroform to constitute 2% of the weight and given in daily portions of 25 g (0.5 g sterol/animal/24 hr). The same amount of chloroform was added to the control chow. Pellets were dried at room temperature under a stream of nitrogen until the chloroform was completely removed. The chow with added cholestyramine was prepared by suspending the powder (1.0 g/25 g chow/animal) in ethanol. Pellets were soaked in this suspension and dried at room temperature under a stream of nitrogen. The powder then stuck to the pellets. Some loss of this powder from the pellets during feeding was unavoidable; loss was roughly estimated at 20–30%.

Animals, feeding and exsanguination. Forty-six male Dutch rabbits (Hylyne Commercial Rabbits, Cheshire, England) were used in four separate series. The results in Tables 1 and 2 are from one representative series, and values for each animal are shown separately. At the start of the feeding period, they were ca. two months old, and weighed ca. 1000 g. They were kept in separate cages during the experiment. The cholesterol or cholestanol feeding (induction) period lasted 4–8 weeks, and the cholestyramine treatment (regression) period lasted another eight weeks. In the induction period, four control rabbits received ordinary stock diet treated with chloroform but with no other supplements. Four rabbits received 25 g of pellets containing 2% (w/w) cholesterol or cholestanol per day, as well as ordinary pellets ad libitum during each 24-hr period. Weight gain was similar in the three groups. At the end of the induction period, two rabbits from each group were exsanguinated in pentobarbital anesthesia. Blood serum, brain, heart, aorta and liver were immediately removed for further analysis. The rest of the animals received ordinary chow supplemented with cholestyramine (1.0 g/animal/24 hr) during the next eight weeks. Rabbits then were exsanguinated, and organs and blood serum were analyzed as for the first group.

Absorption of sterols. The absorption study was designed to compare the extent of absorption of cholesterol and cholestanol. It was performed at the end of the feeding

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period. As we were interested not in the amount absorbed during one passage through the intestinal tract but in the total amount absorbed, rabbits were not put in restraining cages to prevent coprophagy. The animals were given a single dose of [1,2-^{3}H]cholesterol or [1,2-^{3}H]cholestanol together with [4-^{14}C]sitosterol added as internal standard according to Borgström (8). The dosage of each sterol was ca. 1 μCi with a specific activity of 44.0 Ci/mmol (114 Ci/mg). The labeled compounds were given by gastric tube, and no extra food was given in 4 hr.

It has been shown that in rats most activity is excreted during the first 24 hr (9), and after four days there is almost no activity in the feces. Recirculation of the isotope is minor during this period, although working with coprophagous animals may alter this situation. Assuming that the isotope might be found in feces for a longer period than in noncoprophagous animals, we collected feces in daily portions for eight days. The sterols were extracted as described below.

Passage of cholestanol across the blood-brain barrier. A 22.5-μCi quantity of [1,2-^{3}H]cholestanol, prepared as described above, was dissolved in 0.5 ml ethanol and added to 2 ml Intralipid®. The suspension was subjected to ultrasonic vibrations and injected intravenously into an ear vein of each of two rabbits. The animals were killed after 12 days, and the brains were removed and hydrolyzed.

Isolation and analysis of sterols. Cholesterol and cholestanol were isolated from different tissues and blood serum by the following procedures: tissue samples were subjected to hydrolysis and lipid extraction as described previously (10) by refluxing at 80 C for 1 hr with KOH/ethanol (5:100, w/v), followed by extraction twice with n-hexane. The hexane extracts were blown to dryness, and the sterols were separated by HPLC. Epicoprostanol was used as internal standard and added to the hexane extract. A Supelcosil-LC 18 column (250 × 4.6 mm, 5 μm particle size, obtained from Supelco, Bellefonte, Pennsylvania) was used with methanol/water (95:5, v/v) as the mobile phase at a flow rate of 1 ml/min. The chromatograph was equipped with a constant flow pump (ConstaMetric III, Laboratory Data Control, Milton Roy Co., St. Petersburg, Florida) and a differential refractometer (R-401, Waters Associates, Milford, Massachusetts). To get satisfactory reproducibility of the quantitative analyses, the lower limit of sterol was about 50 μg. The retention times (min) were cholesterol-5,6-epoxide, 18; epicoprostanol, 27; cholesterol, 36; cholestanol, 42.

Quantitation by gas chromatography. Due to the great difference in concentration of cholesterol and cholestanol in brain, we had to remove cholesterol from the samples. Quantitative conversion of cholesterol to epoxides was performed by incubating aliquots of the hexane extract, blown to dryness with N_2 and containing epicoprostanol as internal standard, with 20 mg of m-chloroperbenzoic acid in 2 ml of chloroform at 45 C for 30 min. Purification by HPLC was performed, and the fractions containing epicoprostanol and cholestanol were combined. Quantitation of cholestanol was then performed by GC. A Hewlett Packard 5880A gas chromatograph with a 26-m capillary column (id 0.32 mm) CP-TM-WAX I, 57 CB was used at isothermic conditions at 185 C. The lower limit of detection was about 0.09 μg.

Quantitations of cholestanol in aorta and serum in the control group were performed by gas chromatography, but without preceding epoxidation.

Quantitation of cholestanol in the commercial cholesterol. The purity of the cholesterol was assessed by the following procedure: 3 mg of cholesterol was added with 300 μg of epicoprostanol as internal standard. The cholesterol was converted to epoxides and removed from the sample by HPLC as described above. Cholestanol was quantitated by GC in pooled HPLC fractions containing all epicoprostanol and cholestanol. The contamination by cholestanol was 0.07% in the CH-USP cholesterol and 0.1% in the cholesterol Sigma grade 99+%.

Histological examination. Specimens for histological examination were taken from the proximal, mid and distal parts of the aorta, from coronary and brain arteries and from the liver. The tissues were immediately fixed in 4% formalin, embedded in paraffin, cut in 5-μm-thick sections and stained with haematoxyline-eosin. Selected samples were embedded in gelatine for staining for fat with oil red.

RESULTS

Table 1 shows the content of sterols in liver and blood serum in rabbits fed diets enriched with cholestanol or cholesterol (3.5 g/wk) for eight weeks and in rabbits fed for an additional eight weeks a diet of ordinary chow with cholestyramine (1.0 g/day). In two rabbits that received cholestanol, the total sterol content of the liver increased from ~2 mg/g to ~8 mg/g. The content of cholestanol in the liver increased by 4–5 mg/g (25–35 times), but the concentration of cholesterol also increased. In blood serum, the concentration of cholestanol increased more than 30 times, and cholesterol was approximately doubled. In three other series, rabbits were fed 2% cholestanol for 4, 8 and 12 weeks, respectively (results not shown). In all these series, liver and serum cholestanol also increased. By histological examination, prominent changes were found in the liver of cholestanol-fed animals: portal inflammation, edema, proliferation of small bile ducts and scattered necrosis of hepatocytes.

Table 1 further shows that when rabbits were given cholesterol, hepatic sterol concentration increased to about 15 mg/g. In serum, cholesterol increased to a level up to 25 times that of the control. Hepatic and serum cholestanol also increased in these animals.

After an additional eight weeks with cholestyramine treatment, serum and liver cholesterol were normalized in the cholestanol-fed animals. In the cholesterol-fed animals, serum cholesterol and cholestanol were normalized during the cholestyramine treatment, whereas the increase of cholesterol in the liver was not completely reversed. Histologically, no liver abnormalities were observed during cholesterol feeding.

Table 2 shows the changes of sterol concentration in aorta, heart and brain of control animals and rabbits fed sterol-enriched chow. In the controls, only small amounts of cholestanol were present in the heart and the aortic wall. In the rabbits fed a cholesterol-enriched diet, the cholestanol content increased both in the heart and the aorta. In aorta, the rise of cholestanol was 14–18 times the control level; in heart, it was 2 times that of control. After cholestyramine treatment, the amount in aorta fell to 7–10 times that of control and in heart 1–2 times that of control.

TABLE 1

Concentrations of Sterols in Liver and Blood Serum of Rabbits Fed Diets Enriched in Cholestanol or Cholesterol[a]

Additions to pellets during first weeks	Induction group	Regression group	Liver		Blood serum	
			Cholesterol (mg/g wet wt)	Cholestanol (mg/g wet wt)	Cholesterol (mg/ml serum)	Cholestanol (mg/ml serum)
None	1		2.1	0.16	0.34	0.03
	2		2.0	0.14	0.38	0.03
		3	2.0	0.12	0.12	0.01
		4	2.1	0.12	0.18	0.01
Cholestanol (2%)	5		4.0	5.44	0.79	1.01
	6		3.9	3.83	0.73	0.84
		7	2.6	0.31	0.18	0.02
		8	2.7	0.25	0.37	0.03
Cholesterol (2%)	9		14.5	0.41	6.00	0.16
	10		14.6	0.44	8.90	0.25
		11	3.9	0.20	0.35	0.03
		12	2.6	0.14	0.19	0.02

[a]Dutch male rabbits, weighing ~1000 g at the start of the experiment, were fed ordinary pelleted food or pellets with added 2% (w/w) cholestanol or cholesterol until exsanguination after eight weeks (induction group). Another group (regression group) was treated identically, but all rabbits were kept for an additional eight weeks. In this period, they were given the stock diet with cholestyramine added (1.0 g/day). Sterols were analyzed by high performance liquid chromatography, except for serum, for which gas chromatography was used (cf. Materials and Methods).

TABLE 2

Concentrations of Sterols in Aorta, Heart and Brain of Rabbits Fed Diets Enriched in Cholestanol or Cholesterol[a]

Additions to pellets during first 8 wk	Induction group	Regression group	Aorta		Heart		Brain	
			Cholesterol (mg/g wet wt)	Cholestanol (mg/g wet wt)	Cholesterol (mg/g wet wt)	Cholestanol (mg/g wet wt)	Cholesterol (mg/g wet wt)	Cholestanol (mg/g wet wt)
None	1		1.4	0.04	1.2	0.06	21.8	0.16
	2		1.8	0.06	1.3	0.06	19.4	0.16
		3	1.6	0.04	1.0	0.05	24.2	0.13
		4	1.0	0.03	1.2	0.08	21.7	0.18
Cholestanol (2%)	5		1.6	0.94	0.8	0.89	22.5	0.36
	6		1.3	0.61	1.0	0.85	18.8	0.31
		7	1.6	0.24	1.3	0.10	20.7	0.37
		8	1.3	0.22	1.2	0.08	19.2	0.32
Cholesterol (2%)	9		20.6	0.72	3.1	0.12	21.4	0.12
	10		20.8	0.84	4.0	0.13	23.1	0.12
		11	8.6	0.28	2.6	0.13	16.5	0.14
		12	7.4	0.31	1.4	0.07	18.4	0.19

[a]Conditions as described in Table 1.

Some of the cholestanol-fed animals showed histological changes in the aorta after eight weeks, i.e., atherosclerosis with focal fibrinoid necrosis and infiltration of monocytes and multinucleated giant cells of the intima and inner third of the media. The changes were most prominent in the proximal part of the aorta. Coronary and brain arteries were morphologically unaffected.

Table 2 shows that in rabbits fed cholesterol-enriched diets the total sterol concentration in aorta and heart increased to a large extent. In the cholesterol-fed animals, histological examination showed atherosclerosis of the aorta. Such changes were visible already after four weeks of cholesterol-feeding.

After the regression period, 1/3 to 1/4 of the amount of cholestanol accumulated in the aortic tissue was still present in the cholestanol-fed rabbits. Also, in the rabbits fed cholesterol, the amounts of both cholesterol and cholestanol remaining in the tissue after the regression period were larger than in the controls.

Table 2 shows that in the cholestanol-fed rabbits the accumulation of cholestanol was approximately the same in heart and aorta. In the cholesterol-fed rabbits, on the

TABLE 3

Concentration of Cholestanol in Brain of Rabbits Fed Diets Enriched in Cholestanol or Cholesterol[a]

Group	Number	Addition to pellets during first 4–8 wk	Cholestanol in brain (mg/g wet wt): *mean;* SEM (range)	
			Induction group	Regression group
1	8	None	*0.15;* 0.012 (0.10–0.19)	
2	6	None		*0.16;* 0.014 (0.12–0.20)
3	7	Cholestanol (2%)	*0.31;* 0.022 (0.28–0.41)	
4	8	Cholestanol (2%)		*0.34;* 0.030 (0.24–0.51)
5	8	Cholesterol (2%)	*0.14;* 0.014 (0.08–0.21)	
6	8	Cholesterol (2%)		*0.15;* 0.014 (0.14–0.20)

[a]Conditions as described in Table 1, but within each feeding group results for rabbits fed for 4–12 wk were pooled. The additional regression period was 8–12 wk. Cholestanol was quantitated by gas chromatography after removal of cholesterol by epoxidation followed by high performance liquid chromatography. Statistical differences between groups were calculated by Student's t-test: 3 vs 1, $p < 0.0005$; 4 vs 2, $p < 0.0005$; 3 vs 5, $p < 0.0005$; 3 vs 4, $0.35 > p > 0.30$; 5 vs 1, $0.25 > p > 0.20$; 5 vs 6, $0.30 > p > 0.25$.

other hand, the accumulation of cholesterol was much more pronounced in aorta than in heart. Also, the cholestanol content of the aorta increased during feeding with cholesterol. In the aorta, the reversal of the accumulations of both sterols was incomplete.

Table 2 shows that in rabbits fed cholestanol, the content of cholestanol in the brain doubled compared to the control group. No significant increase of cholesterol was seen in cholesterol-fed rabbits. After the regression period, cholestanol-fed rabbits still had the same high concentration of cholestanol as after the feeding period.

Table 3 shows the mean cholestanol concentration in the brain of rabbits from four different experimental series (a total of 46 animals) and the statistical differences between the groups. The accumulation of cholestanol in the cholestanol-fed group is highly significant compared to both the control and the cholesterol-fed group. Further, there is no statistically significant difference in brain cholestanol between the cholestanol induction and regression groups.

DISCUSSION

Shefer et al. (11) recently found that when rats fed a stock diet were given trace amounts of ^{3}H-cholesterol, 45% of the isotope was absorbed; when they were given ^{3}H-cholestanol, only 14% was absorbed. In the rabbit strain we studied, however, both cholesterol and cholestanol were absorbed to a higher degree, about 80–90% and 50–60%, respectively.

In our study, equivalent amounts of cholestanol and cholesterol were given. The concentration of cholesterol in the organs and blood serum increased much more during cholesterol feeding than did cholestanol during cholestanol feeding, however. Since the aim of this study was to compare different organs with respect to their sterol content during sterol feeding, we only performed preliminary studies on excretion of the two steroids and their metabolites. Less efficient absorption probably contributes to the more limited accumulation of cholestanol. In humans, unchanged cholestanol is more easily excreted in bile than is cholesterol (12). One may speculate whether this is also the case in rabbit. It has also been shown that cholestanol is a quite efficient substrate for 7α-hydroxylation, at least in vitro (7,13).

In cholestanol-fed rabbits, the highest concentrations of cholestanol were found in the liver. Morphological changes were also seen, with portal inflammation and proliferation of small bile ducts. "Liver enzymes" in serum were only slightly increased, however. Concrements in the gall bladder were also found in all animals. Previously, biliary concrements in cholestanol-fed rabbits have been shown to consist of deoxycholic acid and allodeoxycholic acid (14). Inflammatory changes of the gall bladder and bile ducts have been shown also in previous studies on cholestanol-fed rabbits (15,16). Also, the cholesterol content of the liver was increased in cholestanol-fed rabbits. Recently, Shefer et al. (11) have reported a 2.6-fold rise of HMG-CoA reductase activity in rat liver during cholestanol feeding. During the regression period, the morphology and the sterol content of the liver was completely normalized. The reversibility of the changes may be of interest in connection with the successful arrest of pathology in CTX during treatment with chenodeoxycholic acid (17,18).

In the present study, the absolute increase (as mg/100 ml) of serum cholestanol in cholestanol-fed rabbits was much less than the rise of cholesterol in cholesterol-fed rabbits, as discussed above. In cholesterol-fed rabbits, there is a direct proportionality of the cholesteryl ester influx in aorta and the lipoprotein concentration in the plasma (19). If this is also the case for cholestanol, it should be expected that less steroid was deposited in the aorta in our cholestanol-fed rabbits than in the cholesterol-fed animals. This was found to be true. We cannot conclude from our study which of the two steroids is most easily deposited and/or removed from the aortic wall. We have demonstrated a rather extensive deposition of cholestanol in the arterial wall, however, with morphological atherosclerosis in some animals. The total sterol concentration in the cholestanol-fed animals is less than in the cholesterol-fed ones, so the development of atherosclerosis seems to occur at a lower sterol serum concentration. Previously, atherosclerosis has been observed in cholestanol-fed birds (16).

In the animals fed cholesterol, all tissues had increased cholestanol content. In aorta, the cholestanol content (mg/g wet wt) was approximately the same in cholesterol- and cholestanol-fed rabbits, significantly higher in both groups than in the control group. However, due to the high content of cholesterol in the cholesterol-fed group, the cholestanol concentration was only 3.8% of the total sterols, compared to 53.4% in the cholestanol-fed group. The cholestanol content of the cholesterol added to the chow was 0.07%. Even if all contaminating cholestanol had been absorbed and none of it excreted, a maximum of 0.01 mg/g tissue weight could have accumulated. This is apparently too little to cause accumulations of the present magnitude in all organs examined. A significant contribution to the cholestanol content of an organ such as the intestine may have occurred, however. Therefore, the endogenous synthesis of cholestanol from cholesterol was obviously increased in the situation when the animal was loaded with cholesterol. It might be speculated that the increased substrate concentrations could activate either the "direct" route (20) or the "7α-hydroxylation/dehydroxylation" route (4,5) from cholesterol to cholestanol.

The most striking observation on the sterol deposition in the cholestanol-fed rabbits was the deposition of cholestanol in the brain. We also found labeled cholestanol in the brain of two rabbits killed 12 days after the injection of [1,2-^{3}H]cholestanol (cf. Materials and Methods). We could demonstrate that the cholestanol fraction contained about 3000 dpm/g wet weight, corresponding to the passage of 0.05% of the labeled cholestanol across the blood-brain barrier. Thus, both methods clearly demonstrate passage of cholestanol across the blood-brain barrier. It has recently been stressed (21) that the passage of this steroid through the blood-brain barrier had previously not been shown. In our study, no reduction of the increased brain cholestanol was seen after an additional eight weeks on a regression regimen. This is strikingly different from all other organs studied.

Possible significance of the present observations for the pathogenesis of CTX. In CTX, cholestanol and cholesterol are deposited, particularly in the central nervous system (6,22). In some patients with this disorder, premature atherosclerosis has been reported (12). Characteristic abnormalities of the blood chemistry in CTX are increased serum cholestanol (12) and increased concentrations of bile acid intermediates in serum (3). These intermediates accumulate because the degradation of the cholesterol side chain is deficient in CTX patients. Increased levels of such intermediates might even be obligatory for the depositions to develop. In the present study, we show that accumulation of cholestanol in rabbit organs can occur in animals when serum cholestanol is increased by feeding cholestanol.

Previous studies have shown deposition of labeled cholestanol in brain tissue of CTX-patients at autopsy, several years after administration of labeled cholesterol (23-25). It was not determined in these studies if the labeled sterol which passed the blood-brain barrier was cholesterol or cholestanol.

The species difference necessitates great caution in interpreting the present results in relation to CTX. It is interesting that cholestanol increased so rapidly and so much in brain, and that no reversal occurred. This may point to a rather rapid passage of cholestanol across the blood-brain barrier. In most patients with CTX, the onset of cerebral symptoms occurs in the third to fourth decade. This points to an accumulation during years when patients have increased serum cholestanol concentrations.

In the rabbit, there were differences among tissues concerning the reversibility of the cholestanol depositions. Our study indicates that cholestanol can be easily removed from heart and liver. Reversal of the depositions in aorta was much slower. The depositions in the brain were not removed to any significant extent on the regression regimen used in the present study.

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Intestinal Absorption and Fecal Excretion of 5,6α-Epoxy-5α-Cholesta-3β-ol by the Male Wistar Rat

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The intestinal absorption of 5,6α-epoxy-5α-cholesta-3β-ol, an oxysterol formed by cholesterol autoxidation, has been evaluated in the male Wistar rat. Measurement of the $^{14}C/^{3}H$ ratio in the serum (by the method of Zilversmit and Hugues) and in the feces showed that a large proportion of the epoxide was absorbed. Epoxide clearance from the blood was very rapid, but its excretion in the stool continued for several days, corresponding to the fraction of the epoxide stored in the animal.
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In the context of a study of the risks of atheromatous disease arising from the presence of "oxysterols" in foods, we evaluated the intestinal absorption of 5,6α-epoxy-5α-cholesta-3β-ol ("α-oxide") in a group of male Wistar rats. ^{14}C-Labeled α-oxide and tritiated α-oxide absorption was followed by measuring the $^{14}C/^{3}H$ ratio in serum and in feces (1).

α-Oxide is formed by cholesterol autoxidation at the time its epimeric 5,6β-epoxide-5β-cholesta-3β-ol (β-oxide) and many other oxysterols (2) are formed. These cholesterol derivatives are considered potentially angiotoxic (3). They have been directly correlated with atherogenesis (4) and indirectly with their cytotoxic (5,6), mutagenic (7,9) and enzyme-inhibiting properties (10,11). These oxysterols can be formed from free or esterified cholesterol (12) during the cooking or storage of foods. Because of the relatively large quantities of these compounds found in the animal food fats used in deep-fat friers (100 to 300 ppm; unpublished results), the impregnation of foods by these heated fats should be considered a nutritional risk.

Moreover, α-oxide is present in the serum of patients with hypercholesterolemia (13). The epimers α-oxide and β-oxide have recently been isolated from normal human serum (14).

MATERIALS AND METHODS

Cholesterol was purchased from Sigma Chemical Co. (St. Louis, Missouri); [4-^{14}C]cholesterol (50 mCi/mM) and [1,2-^{3}H]cholesterol (50 Ci/mM) were from C.E.A. (Gif-sur-Yvette, France). Kieselgel $60F_{254}$ chromatographic plates were provided by Merck (Darmstadt, Federal Republic of Germany). Solvents of the grade Normapur came from Prolabo (Paris, France).

Unlabeled α-oxide was prepared according to the method of Djerassi (15).

Preparation of tritiated α-oxide. Forty-five mg of highly purified cholesterol (recrystallized several times in MeOH) and dissolved in 2 ml ethyl ether was added to 45 mg paranitroperbenzoic acid. At the same time, 250 μCi of [1,2-^{3}H]cholesterol (50 Ci/mM) dissolved in 1 ml anhydrous ether was added. The mixture was kept for 20 hr at room temperature, then extracted with ether, washed in 0.5 NaOH and dried in sodium sulfate. The dry residue was chromatographed on silica gel. The α-oxide was first eluted in a benzene/sulfuric ether mixture (8:2, v/v to 5:5, v/v). Pure fractions of the α-isomer (~100 μCi) were combined and diluted in an ethanol solution of cold α-oxide to a specific activity of 1.4 mCi/mmol. The purity of the sample was evaluated by gas liquid chromatography-mass spectrometry on an SE-54 capillary column at 275 C and was found to be higher than 95%. The order in which the epoxides emerged in GLC was the opposite of that observed with column chromatography.

Preparation of [4-^{14}C]α-oxide. The preparation protocol was identical to that used for the synthesis of tritiated α-oxide, but [4-^{14}C]cholesterol was used as the starting reagent. Specific radioactivity was then adjusted to 1.4 mCi/mM with an ethanol solution of unlabeled α-oxide.

Animal experiments. Five μCi of each α-oxide (^{14}C and ^{3}H) was dissolved in 50 μl ethanol, and 450 μl of physiological saline was added. The mixture was then sonicated for 15 min. The suspension containing the tritiated compound was injected into the caudal vein, and the suspension containing the [4-^{14}C] compound was administered by forced feeding (per os) with a catheter. The injection and the forced feeding were carried out simultaneously.

The male Wistar rats (300 g; Cesal, Montmedy, France) were then placed in individual metabolic cages to collect feces and prevent coprophagy. The feces of each rat were collected every day. The animals were given a daily 20-g ration of a semisynthetic diet of 8% peanut oil, 17% casein, 69% starch, 4% cellulose, 1% mineral mixture and 1% vitamins.

Blood samples of 1–1.5 ml were collected in EDTA from the caudal vein on days 2, 3, 4 and 7. The blood was centrifuged (3000 rpm) and the plasma removed. On day 7, the animals were decapitated, and their blood was collected.

Treatment of the plasma before counting radioactivity. The plasma was isolated by centrifugation at 3000 rpm for 15 min at 4 C. The nonsaponifiable fraction (obtained after saponification by 5 ml of KOH-MeOH [N] for 15 min) was extracted by three washes (15 ml) in ethyl oxide. The organic phases were combined and evaporated under a nitrogen stream, and the nonsaponifiable fraction was then weighed.

Treatment of feces. The feces were oven-dried for 12 hr at 100 C. The lipids were extracted by ethanol for 48 hr, using a Kumagawa microextractor. The total fecal lipid extract obtained by filtration (0.5–1 g) was extracted with 50 ml of ethanol at 100–120 C for 48 hr. The ethanol was then filtered and vacuum-evaporated. The lipid extract was collected, dried and weighed. Radioactivity was directly measured on this nonsaponifiable lipid extract.

Measurement of sample radioactivity. The nonsaponifiable (in the case of plasma) or total fecal lipid extract (in the case of feces) was deposited on a disk of filter paper (diameter 10 mm) and burned on an Oxymat apparatus.

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The radioactivities of the tritiated (^3H_2O) and ^{14}C ($^{14}CO_2$) fractions were measured with a Packard Tri-carb 360D counter. In the case of ^3H, the scintillation mixture consisted of 700 ml of dioxane, 300 ml of toluene, 20 g of naphthalene and 7 g of butyl PBD. Carbo-Max (Kontron-Roche, Trappes, France) was used in the case of ^{14}C.

Counting with the Tri-carb instrument was done 4 hr after recovery from the Oxymat. The counting time was 20 min. Measurements were performed as follows: control count (capsule + filter paper), sample count (capsule + filter paper + sample) and apparatus count (a cycle without capsule).

The final values consist of the difference between the number of dpm in a sample count and the mean value of the control counts, under these operating conditions.

The efficiency of the Oxymat apparatus with respect to both isotope separation and counting was 95%, compared to Tri-carb counting. The 5% correction was not applied since it was lower than the degree of measurement accuracy, which was about 20%.

Measurement of intestinal absorption. Intestinal absorption was evaluated by measuring the isotope ratio R in the plasma on days 2, 3 and 4 (1) and in the nonsaponifiable from the fecal matter: R = {[^{14}C radioactivity (sample)] ÷ [^3H radioactivity (sample)]} × {[^3H radioactivity (control)] ÷ [^{14}C radioactivity (control)]}.

The correction factor was obtained by measuring the value of the $^3H/^{14}C$ ratio for equal radioactivities of ^{14}C and ^3H α-oxide (control). For this purpose, the same number of dpm of [4-^{14}C]- and [1,2-^3H]α-oxide were deposited on the same filter-paper disk and measured in the same way as in the samples.

RESULTS

Radioactivity and $^3H/^{14}C$ ratio in the plasma. Radioactivities as a function of time (2, 3, 4 and 7 days) plotted on semilogarithmic coordinates give a straight line (Fig. 1). The slope is the same for the ^{14}C and ^3H samples, indicating an α-epoxide half-life of 40 ± 7 hr for this phase of observations.

The values of the corrected $^{14}C/^3H$ ratios are shown in Table 1. The mean value of this ratio, which defines the intestinal absorption of the α-oxide, is 93 ± 22.

These results show that α-oxide was substantially absorbed (about 90%) by the intestinal mucosa (Table 1) and that α-oxide radioactivity in the plasma was very low at 48 hr (Fig. 1) in terms of both ^3H and ^{14}C. This indicates the existence of a clearance phenomenon in the blood, beginning in the first hours after administration.

Radioactivity and the $^{14}C/^3H$ ratio in the fecal matter. The daily fecal excretion of ^{14}C and ^3H α-oxide and the cumulative fecal excretion as a function of time are shown in Figures 2 and 3, respectively. The maximum was observed at 3 days followed by a rapid decrease, which corresponded to the excretion of about 3.5×10^6 dpm of each of the epoxides after 4 days. Then the decrease slowed considerably and the pattern became nearly linear. After 3 days, ^{14}C excretion exceeded that of ^3H, whereas the opposite was true after 2 days.

The curve representing the radioactivity not excreted by the animals, i.e., the difference between the administered radioactivity and the fraction excreted in the stools, as a function of time is shown in Figure 4. The two patterns of decrease were once more observed. One was rapid between 0 and 3 days (excretion of about 3.5×10^6 dpm of each isotope). The second pattern was slow and almost linear, during which the α-oxide half-life was estimated to be 27 days. Extrapolation of this linear portion of the curve shows that the remaining radioactivity (7.5×10^6 dpm) would have been excreted in 66 days. The curves for ^{14}C and ^3H α-oxide are nearly identical.

The values of the isotope ratios in the fecal matter as a function of time are shown in Table 2. After a maximum on the third day, when ^{14}C was predominant, the values remained constant until the seventh day.

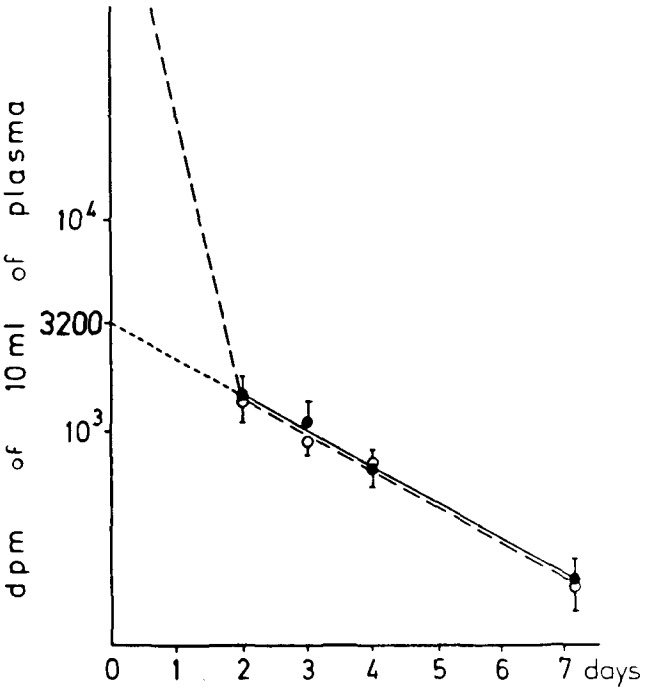

FIG. 1. Semilogarithmic representation of the corrected radioactivity in the nonsaponifiable lipids from plasma of rats fed ^{14}C α-oxide (●) and injected with ^3H α-oxide (○) as a function of time.

TABLE 1

Corrected Isotope Ratios in the Plasma of Rats Simultaneously Fed ^{14}C α-Oxide and Injected with ^3H α-Oxide

Time (days)	Number of animals					
	1	2	3	4	5	6
2	70	62	98	118	60	121
3	126	91	127	95	110	82
4	80	90	114	94	45	78
7	90	90	100	91	60	88

R = $10^2 \times {}^{14}C/^3H$ (plasma) × $^3H/^{14}C$ (controls). Mean value: 93 ± 20.

DISCUSSION

The values of the $^{14}C/^3H$ ratios measured in the plasma (93 ± 22%, Table 1) show that a large proportion of the

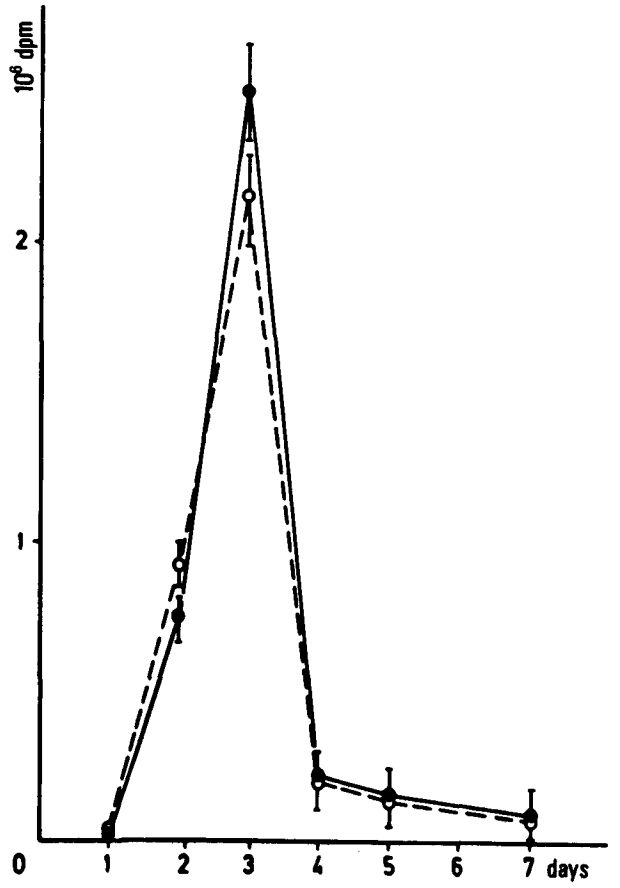

FIG. 2. Daily fecal excretion of ^{14}C (●) and ^{3}H (O) of rats fed ^{14}C α-oxide and injected with ^{3}H α-oxide.

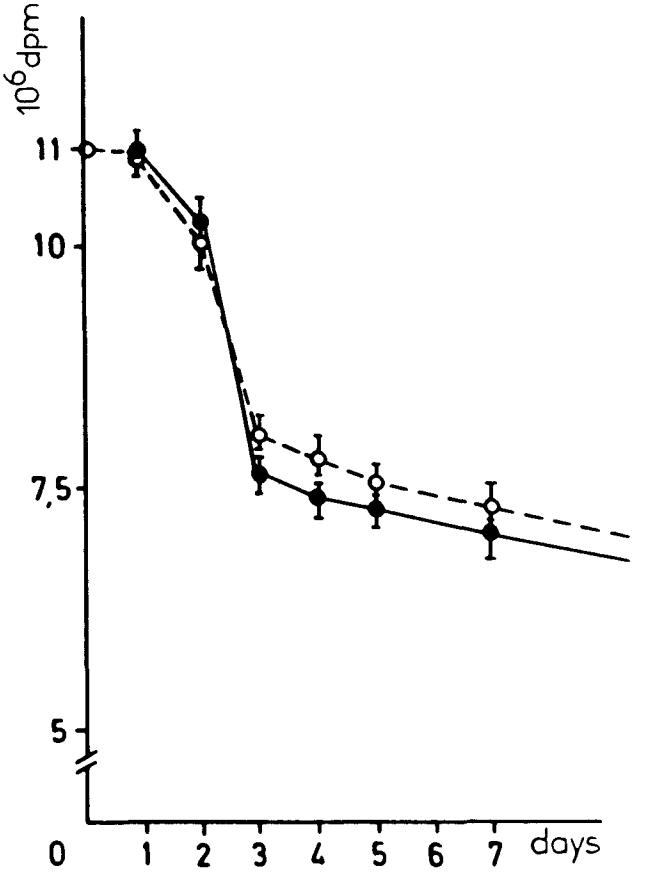

FIG. 4. Difference between total administered radioactivity to rats per os (^{14}C) (●) and by intravenous injection (^{3}H) (O) and total radioactivity excreted in stools.

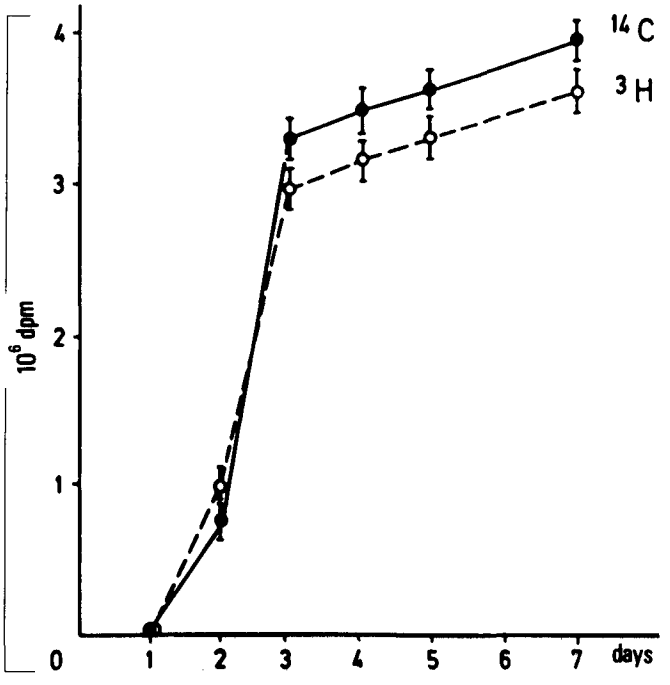

FIG. 3. Cumulative fecal excretion of ^{14}C (●) and ^{3}H (O) of rats fed ^{14}C α-oxide and injected with ^{3}H α-oxide.

TABLE 2

$^{14}C/^{3}H$ Ratio in Stools of Rats Fed ^{14}C α-Oxide and Injected with ^{3}H α-Oxide as a Function of Time

Time (days)	Number of animals						
	1	2	3	4	5	6	Mean
1	0.75	0.50	0.47	0.34	0.34	0.43	0.47 ± 0.1
2	—	0.45	—	0.72	—	0.62	0.60 ± 0.14
3	1.21	1.01	1.33	1.05	0.87	0.97	1.07 ± 0.17
4	1.00	0.87	0.87	0.89	0.91	0.60	0.86 ± 0.13
5	0.73	0.86	0.84	1.00	0.88	0.84	0.86 ± 0.09
6	0.66	0.78	0.83	0.98	0.84	0.77	0.81 ± 0.10

α-oxide orally administered to the rats was absorbed. The same isotope ratio measured in the feces (Table 2) had a constant value, on the order of 85% after 72 hr, after which it can be assumed that the unabsorbed fraction had been eliminated. This confirms the assumption that the orally administered α-oxide was absorbed by the rats' intestinal mucosa.

The constant values of the isotope ratios in the plasma and the fecal matter indicate that the labeled compounds followed identical metabolic pathways and were excreted

in the same way. The difference between the two methods of administering the α-oxide resulted in a lag between the periods in which each form appeared in the blood. ^{3}H α-oxide (injected) appeared sooner than the ^{14}C α-oxide, which was slowly absorbed. This phenomenon led to a predominance in the excretion of the orally administered ^{14}C over that of ^{3}H (injected), which is particularly evident in the curve representing cumulative fecal excretion (Fig. 3). The effect was a systematic exaggeration of the measurements of intestinal absorption by the method of Zilversmit and Hugues (1).

The results in Figure 1 show that after 48 hr there was very little residual radioactivity in the plasma. The rapid decrease in radioactivity in the blood has been demonstrated in other studies. Fredrickson and Ono (16) reported that 28×10^3 dpm of 25-hydroxy-cholesterol injected into rats passed very quickly into the bile (100% in 4 hr), essentially in the form of acidic sterols other than cholic acid. After 48 hr, the residual radioactivity in the serum was very low or nonexistent. The very rapid clearance of circulating oxysterols was evidenced in another series of preliminary experiments carried out in our laboratory. Our objective was to assay oxysterols in serum lipoproteins after oral administration. For six days, six male Wistar rats were given 3 μCi per day of a mixture of oxysterols (17) labeled with ^{14}C (specific activity 10 mCi/mM). Measurements on the seventh day (24 hr after the last feed) in the liver, feces and plasma showed that the residual radioactivity in the plasma was too low (~100 dpm) to allow an assay of oxysterols in VLDL, LDL or HDL, and a large part of the radioactivity was found in the liver and in the fecal matter in the form of acid compounds accompanied by a small population of neutral compounds.

In a similar experiment, Peng et al. (18) concluded that 25-hydroxy-cholesterol orally administered to monkeys (30 μCi in 0.1 mg) was absorbed. After 24 hr, the radioactivity found in the lipoproteins amounted to 1.5×10^3 dpm/mg, which was a small residual fraction compared to the 30 μCi injected; 86% of this radioactivity was found in VLDL and LDL and only 10% in HDL.

The pattern by which the tritiated isotope was excreted suggests the existence of a system in which α-oxide and/or its metabolites are stored: the ^{3}H radioactivity cleared from the plasma after two days was only partially excreted in the stools after three days (3×10^6 dpm). The difference between the quantity injected and the quantity excreted (8×10^6 dpm, 75%) was retained in the animal (radioactivity excreted in urine was negligible). The decrease in residual radioactivity of the tritiated isotope in the stools (Fig. 4) followed two patterns: a rapid excretion of 35% of the radioactivity followed by a very slow excretion of the remaining 65%.

If we assume there is a system for storing α-oxide, it must have a limited capacity, beyond which the excess fraction is rapidly excreted. In the present case, this would mean that 75% of the activity was stored (3.75 μCi, or 1 mg) and 25% was eliminated in 72 hr. This system could be an enterohepatic cycle in which α-oxide and/or its metabolites behave like bile acids.

The present work shows that, under our experimental conditions, α-oxide is absorbed by the intestinal mucosa of the rat. Although its clearance from blood is rapid, its excretion in feces is slow. This implies that after being absorbed, it is stored in the animal. Since α-oxide is formed at the same time as other oxysterols during cholesterol autoxidation and is present in many food products, and since oxysterols are known to be potentially angiotoxic and carcinogenic, the absorption of small but constant doses of this oxysterol constitutes a long-term toxic risk.

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Changes in Lipids in Liver and Serum of Rats Fed a Histidine-excess Diet or Cholesterol-supplemented Diets

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The influence of dietary excess (5%) L-histidine on serum and liver lipids was examined in rats. Feeding a histidine-excess diet for 3, 6, 14 or 30 days caused growth retardation, hepatomegaly and decreased liver lipids throughout the period of the experiment. Hypercholesterolemia was observed after feeding a histidine-excess diet for 6 days; then serum cholesterol continuously increased for 30 days. Serum triglyceride on day 30 in rats fed the histidine-excess diet showed a significant decrease compared to rats fed the basal diet. Serum phospholipids of rats fed the histidine-excess diet for 7 or 14 days showed a significant increase compared to rats fed the basal diet. When rats were fed a basal, histidine-excess or cholesterol-supplemented diet (0.5% and 1.0% cholesterol) for 6 days, the distribution of serum high density (HDL), low density (LDL) and very low density lipoprotein cholesterol in rats fed the histidine-excess diet was similar to that of rats fed the basal diet, whereas LDL-cholesterol increased and HDL-cholesterol decreased in rats fed the cholesterol-supplemented diet.
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Rats fed diets with high levels of a single amino acid showed growth retardation (1). Solomon and Geison (2), Harvey et al. (3) and Aoyama et al. (4–6) have reported that dietary supplementation with excess L-histidine (His) caused growth depression, hepatomegaly and an increase of plasma cholesterol in rats. Although the rats did not ingest much cholesterol with histidine feeding, serum cholesterol showed a significant increase. Recently, Aoyama et al. (5) reported a short-term (6-day) change in liver and serum components of rats fed a histidine-excess diet. The changes in liver and serum components in long-term feeding of a histidine-excess diet were not shown in detail. It is known that hypercholesterolemia by cholesterol feeding showed marked changes in distribution of lipoproteins (7), but the distribution of lipoproteins in rats fed a histidine-excess diet was undetermined. To compare the effect of excess His and cholesterol on serum lipoproteins, His or cholesterol was added to the basal diet. The present study was thus undertaken to examine the long-term (30-day) effect and the distribution of serum lipoproteins in rats fed a histidine-excess diet.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats, weighing an average of 100 g, were housed individually in screen-bottomed cages in an air-conditioned room at a temperature of ca. 23 C. The rats were allowed free access to food and water except in experiment 3. Composition of the experimental diets is shown in Table 1. For the histidine- and alanine-excess diets, 5% His and 8.6% L-alanine (Ala), respectively, were added to the basal diet. All rats were fed a stock diet (Clea Japan, Tokyo, Japan) for 4 days, followed by the basal diet for 2 days prior to the experimental diet. In experiment 1, rats were fed either the basal or the histidine-excess diet for 0, 3, 6, 14 or 30 days. In experiment 2, rats were fed either the basal or the histidine-excess diet for 0, 7 and 14 days. In experiment 3, rats were fed the basal, the histidine-excess or the alanine-excess diet for 7 days. The alanine-excess diet was isonitrogenous with the histidine-excess diet. The basal and the alanine-excess diets were fed either ad libitum or in pair-feeding. Pair-fed rats received the same amount of diet as rats fed the histidine-excess diet. In experiment 4, rats were fed either the basal diet or a cholesterol-supplemented diet containing 0.5% or 1.0% cholesterol with 0.125% or 0.25% cholic acid, respectively. All dietary changes in the content of His, Ala, cholesterol and cholic acid were compensated for by adjusting the amount of carbohydrate. Rats were fasted for 4 hr (0900–1300) after receiving the experimental diets and were anesthetized with ethyl ether. Serum was prepared from blood obtained by cardiac puncture of the rats.

Analytical methods. Liver lipids were extracted and purified by the method of Folch et al. (8) and used for the determination of cholesterol, phospholipids and total lipids. Liver total lipids were determined gravimetrically.

TABLE 1

Composition of Experimental Diets

Ingredient	Diet		
	Basal (%)	L-histidine-excess (%)	L-alanine-excess (%)
Casein[a]	25	25	25
L-Histidine[b]	0	5	0
L-Alanine[b]	0	0	8.6
Corn oil[c]	5	5	5
Vitamin mixture[d]	0.85	0.85	0.85
Choline chloride[a]	0.15	0.15	0.15
Mineral mixture[e]	3.5	3.5	3.5
Sucrose[f]	21.8	20.2	19.0
Starch[g]	43.7	40.3	37.9
Retinyl palmitate[h]	0.27 mg/100 g of diet		
Cholecalciferol[h]	2.5 μg/100 g of diet		
dl-α-Tocopheryl acetate[h]	10 mg/100 g of diet		

[a]Katayama Chemical Industries Co., Osaka, Japan.
[b]Ajinomoto Co., Tokyo, Japan.
[c]Nihon Shokuhin Kako Co., Fuji, Shizuoka, Japan.
[d]Oriental Yeast Co., Tokyo, Japan (see reference 28).
[e]AIN-76™ mineral mixture (see reference 29).
[f]Taito Co., Tokyo, Japan.
[g]Corn, Chuo Shokuryo Co., Inazawa, Japan.
[h]Eizai Co., Tokyo, Japan.

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Total lipid phosphorous was measured by the method of Bartlett (9), and values were multiplied by 25 to obtain phospholipid content. Liver triglyceride value was calculated from total liver lipid minus liver cholesterol and phospholipids. Liver glycogen was isolated and purified by precipitation with ethyl alcohol from a 30% potassium hydroxide digest of aliquots of the liver and was estimated by the phenol-sulfuric acid method (10). Cholesterol in liver and serum was measured by the method of Pearson et al. (11) in experiment 1 and by the enzymatic method of Siedel et al. (12) in experiments 2 and 3. Serum glucose was determined by the method of Werner et al. (13), and serum triglyceride was measured using a commercial kit (Trigly-quick-BMY, Boehringer Mannheim Yamanouchi, Japan). Serum phospholipids were determined colorimetrically (14) after precipitation by trichloroacetic acid and oxidation by sulfuric acid. High density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) cholesterol were separated by the method of Noma et al. (15) and measured by the o-phthalaldehyde method (16).

Statistical analysis. Statistical significance of differences between values was analyzed by Duncan's multiple-range test (17).

RESULTS

Experiment 1. Food intake, body weight gain and liver weight of rats fed either the basal or the histidine-excess diet for 0, 3, 6, 14 or 30 days are summarized in Table 2. Food intake and body weight gain were suppressed by the histidine-excess diet. Liver weight increased during the first six days on the histidine-excess diet and then plateaued. Histidine supplementation caused a significant enlargement of liver throughout the period of the experiment.

Tables 3 and 4 summarize serum cholesterol, serum triglyceride, serum glucose and liver glycogen, total lipids, phospholipids, cholesterol and triglyceride of rats fed either the basal or the histidine-excess diets. After feeding excess histidine for six days, a significant rise in serum cholesterol was observed, and the increase con-

TABLE 2

Food Intake, Body Weight Gain and Liver Weight of Rats Fed Either a Basal or a Histidine (His)-Excess Diet

Diet	Feeding period (days)	Food intake (g/feeding period)	Body weight gain (g/feeding period)	Liver weight (g/100 g body weight)
	0	—	—	4.78 ± 0.27^{c}
Basal	3	$42 \pm 1^{f,*}$	15 ± 1^{ef}	4.53 ± 0.07^{c}
His-excess	3	25 ± 1^{g}	2 ± 1^{g}	5.82 ± 0.29^{b}
Basal	6	83 ± 3^{e}	29 ± 2^{d}	4.77 ± 0.20^{c}
His-excess	6	52 ± 4^{f}	9 ± 4^{fg}	6.98 ± 0.19^{a}
Basal	14	186 ± 6^{c}	59 ± 4^{c}	4.53 ± 0.09^{c}
His-excess	14	131 ± 7^{d}	25 ± 7^{de}	7.10 ± 0.34^{a}
Basal	30	450 ± 2^{a}	154 ± 2^{a}	4.63 ± 0.09^{c}
His-excess	30	322 ± 5^{b}	89 ± 2^{b}	6.82 ± 0.19^{a}

*Means ± SEM for four rats. Means within a column not followed by the same superscript are significantly different ($p < 0.05$).

TABLE 3

Serum Cholesterol, Serum Triglyceride, Serum Glucose and Liver Glycogen of Rats Fed Either a Basal or a Histidine (His)-Excess Diet

Diet	Feeding period (days)	Serum cholesterol (mg/100 ml)	Serum triglyceride (mg/100 ml)	Serum glucose (mg/100 ml)	Liver glycogen (mg/g)
	0	$142.9 \pm 6.9^{cd,*}$	77.1 ± 8.0^{c}	127.9 ± 12.8^{b}	19.72 ± 2.14^{de}
Basal	3	153.8 ± 9.4^{cd}	88.9 ± 10.0^{c}	143.9 ± 8.0^{ab}	17.88 ± 2.62^{e}
His-excess	3	154.4 ± 8.8^{cd}	71.0 ± 11.3^{c}	134.9 ± 3.5^{ab}	30.03 ± 5.81^{bc}
Basal	6	137.1 ± 2.1^{d}	120.5 ± 18.3^{bc}	141.9 ± 9.4^{ab}	27.06 ± 3.61^{cd}
His-excess	6	176.2 ± 21.6^{c}	84.7 ± 22.9^{c}	130.9 ± 1.8^{b}	39.85 ± 2.85^{a}
Basal	14	145.9 ± 7.2^{cd}	193.8 ± 27.0^{b}	155.1 ± 3.1^{a}	34.25 ± 1.48^{abc}
His-excess	14	215.2 ± 14.4^{b}	115.2 ± 12.3^{bc}	138.2 ± 3.5^{ab}	40.70 ± 2.03^{a}
Basal	30	143.5 ± 3.7^{cd}	383.9 ± 69.3^{a}	149.5 ± 4.7^{ab}	34.51 ± 1.58^{abc}
His-excess	30	247.6 ± 9.9^{a}	196.7 ± 38.4^{b}	148.5 ± 4.8^{ab}	36.77 ± 1.51^{ab}

*Means ± SEM. Means within a column not followed by the same superscript are significantly different ($p < 0.05$).

tinued throughout the experiment. The serum cholesterol concentration in rats fed the basal diet was constant for 30 days. The serum triglyceride concentration continuously increased throughout the experiment. The liver glycogen content significantly increased during the first six days and then plateaued in rats fed a histidine-excess diet. In rats fed the basal diet, an increase of the liver glycogen content was observed, but the increase was lower than that of rats fed the histidine-excess diet. The total liver lipid content in rats fed the histidine-excess diet decreased for 6 days, slightly increased up to 14 days and then plateaued. Throughout the experiment, the liver lipid content in rats fed the histidine-excess diet was significantly lower than in rats fed the basal diet. Liver phospholipids and triglycerides showed the same tendency. Liver cholesterol in rats fed the histidine-excess diet decreased for 14 days, then appeared to increase, although not significantly. Feeding a histidine-excess diet resulted in a significant decrease in liver cholesterol.

Experiment 2. Serum cholesterol, triglyceride and phospholipids of rats fed either the basal or the histidine-excess diet for 0, 7 and 14 days are summarized in Table 5. Serum cholesterol was significantly higher and serum triglyceride significantly lower on days 7 and 14 in rats fed the histidine-excess diet than in rats fed the basal diet. These observations agree with the results of experiment 1. Serum phospholipids of rats fed the histidine-excess diet for 7 and 14 days were significantly higher than in rats fed the basal diet.

Experiment 3. Food intake, body weight gain, liver weight, total lipids and cholesterol of rats fed the basal, histidine-excess or alanine-excess diet for 7 days are summarized in Table 6. The rats fed the histidine-excess diet consumed less than rats fed the basal or alanine-excess diet and showed marked growth retardation. Basal or alanine-excess pair-fed rats showed body weight gains similar to the rats fed the histidine-excess diet. The liver weight of rats fed the histidine-excess diet was significantly higher than that of any other groups. The total liver lipids of rats fed the histidine-excess diet were significantly lower than in other groups and the rats pair-fed the basal or the alanine-excess diet showed lower liver lipids than rats fed either diet ad libitum. Liver cholesterol of rats fed the histidine-excess diet was significantly lower than of ad libitum-fed rats, but was not significantly different from the pair-fed rats; liver cholesterol of rats fed the alanine-excess diet ad libitum was significantly lower than of rats fed the basal diet ad libitum. Serum cholesterol, triglyceride and glucose and liver glycogen of rats fed the basal, histidine-excess or alanine-excess diet for 7 days are summarized in Table 7. Serum cholesterol of rats fed the histidine-excess diet was significantly higher than in other groups and the pair-fed rats showed lower serum cholesterol than ad libitum-fed rats.

TABLE 4

Liver Total Lipids, Phospholipids, Cholesterol and Triglyceride of Rats Fed Either a Basal or a Histidine (His)-Excess Diet

Diet	Feeding period (days)	Total lipids (mg/g)	Phospholipids (mg/g)	Cholesterol (mg/g)	Triglyceride (mg/g)
	0	$54.6 \pm 0.8^{abc,*}$	29.7 ± 0.6^{ab}	4.25 ± 0.35^{a}	20.7 ± 1.6^{abc}
Basal	3	58.4 ± 2.8^{a}	30.9 ± 0.1^{a}	3.96 ± 0.14^{ab}	23.5 ± 2.8^{ab}
His-excess	3	40.3 ± 1.1^{de}	25.0 ± 1.4^{c}	3.04 ± 0.21^{cd}	12.3 ± 2.0^{de}
Basal	6	50.1 ± 0.8^{c}	29.1 ± 0.8^{ab}	3.38 ± 0.15^{bcd}	17.6 ± 1.2^{bcd}
His-excess	6	35.9 ± 1.7^{e}	22.8 ± 0.7^{d}	2.87 ± 0.24^{d}	10.2 ± 3.1^{de}
Basal	14	52.3 ± 1.3^{bc}	28.7 ± 0.4^{b}	3.69 ± 0.24^{abc}	20.0 ± 1.7^{abc}
His-excess	14	42.7 ± 2.9^{d}	24.0 ± 0.7^{cd}	2.75 ± 0.17^{d}	15.9 ± 3.2^{cde}
Basal	30	57.3 ± 1.2^{ab}	28.6 ± 0.4^{b}	3.75 ± 0.28^{abc}	25.0 ± 1.0^{a}
His-excess	30	42.5 ± 0.6^{d}	24.0 ± 1.3^{cd}	3.31 ± 0.20^{bcd}	15.8 ± 0.7^{cde}

*Means ± SEM for four rats. Means within a column not followed by the same superscript are significantly different ($p < 0.05$).

TABLE 5

Serum Cholesterol, Triglyceride and Phospholipids of Rats Fed a Basal or Histidine (His)-Excess Diet

Diet	Feeding period (days)	Cholesterol (mg/100 ml)	Triglyceride (mg/100 ml)	Phospholipids (mg/100 ml)
	0	$107.3 \pm 4.8^{b,*}$	63.8 ± 8.7^{c}	216.4 ± 7.4^{b}
Basal	7	106.0 ± 2.7^{b}	95.7 ± 3.8^{b}	219.1 ± 5.9^{b}
His-excess	7	164.9 ± 12.2^{a}	53.1 ± 8.5^{c}	277.6 ± 23.2^{a}
Basal	14	113.7 ± 1.2^{b}	141.6 ± 15.0^{a}	234.8 ± 2.3^{b}
His-excess	14	174.9 ± 5.4^{a}	83.6 ± 10.2^{bc}	283.2 ± 8.9^{a}

*Means ± SEM for five rats. Means within a column not followed by the same superscript are significantly different ($p < 0.05$).

Serum triglyceride of rats fed the histidine-excess diet was significantly lower than in other groups, whereas serum triglyceride of pair-fed rats was higher than that of rats fed ad libitum. Serum glucose of rats fed the histidine-excess diet was significantly lower than in other groups, but there was no difference in serum glucose between the ad libitum-fed and pair-fed rats. Liver glycogen of rats fed the histidine-excess diet was significantly higher than of rats fed either the basal or the alanine-excess diet ad libitum; there was no difference compared to the pair-fed rats.

Experiment 4. Food intake, body weight gain, liver weight, total lipids and cholesterol of rats fed the basal, histidine-excess or one of two cholesterol-supplemented diets are summarized in Table 8. Food intake and body weight gain were suppressed by the inclusion of excess histidine in the diet. Cholesterol supplementation had no effect on food intake or body weight gain. Liver weight showed a significant increase in rats fed the histidine-excess diet and the 1.0% cholesterol-supplemented diet. Total liver lipids and cholesterol were significantly lower in rats fed the histidine-excess diet and higher in those fed the cholesterol-supplemented diets compared to the rats fed the basal diet.

Serum cholesterol, distribution of cholesterol among lipoprotein classes and serum triglyceride in rats fed the basal, histidine-excess or one of two cholesterol-supplemented diets are summarized in Table 9. Serum cholesterol significantly increased in rats fed the histidine-excess and cholesterol-supplemented diets. In cholesterol-supplemented groups, serum cholesterol rose as the amount of dietary cholesterol increased. The rats fed the histidine-excess diet showed a significant increase in HDL and LDL cholesterol. In cholesterol-supplemented groups, all fractions increased, especially LDL cholesterol, which rose in proportion to the amount of cholesterol supplementation. The percentage of three cholesterol fractions in rats fed the histidine-excess diet was similar to that fed the basal diet. In cholesterol-supplemented groups, the percentage of HDL cholesterol was significantly lower and that of LDL cholesterol was higher compared to the rats fed a basal diet. The percentage of VLDL cholesterol did not change in any group. Serum triglyceride was significantly lower in rats fed the histidine-excess diet and similar to those fed the basal diet in rats fed the cholesterol-supplemented diets.

DISCUSSION

Our observation that feeding a histidine-excess diet caused growth retardation, hepatomegaly and hypercholesterolemia is similar to that reported by Solomon and Geison (2) and Aoyama et al. (4–6). It was evident that hepatomegaly was not due to lipid accumulation in liver but was due partly to glycogen accumulation. These observations are similar to those by Aoyama et al. (5). Hepatomegaly caused by lipid accumulation was observed in choline-deficiency (18), orotic acid feeding (19) and arginine-deficiency (20). Therefore, hepatomegaly caused by excess His seems different from these other causes of hepatomegaly.

TABLE 6

Food Intake, Body Weight Gain, Liver Weight, Liver Lipids and Liver Cholesterol of Rats Fed a Basal, Histidine (His)-Excess or Alanine (Ala)-Excess Diet for Seven Days

Diet	Food intake (g/7 days)	Body weight (g/7 days)	Liver weight (g/100 g body weight)	Liver lipids (mg/g)	Liver cholesterol (mg/g)
His-excess, ad libitum	$60 \pm 1^{b,*}$	13 ± 1^{c}	6.90 ± 0.23^{a}	33.2 ± 2.0^{c}	2.91 ± 0.10^{c}
Basal, ad libitum	98 ± 2^{a}	36 ± 1^{a}	5.14 ± 0.11^{b}	43.6 ± 1.9^{a}	3.71 ± 0.11^{a}
Ala-excess, ad libitum	94 ± 2^{a}	31 ± 2^{b}	5.15 ± 0.12^{b}	43.0 ± 0.7^{a}	3.17 ± 0.03^{b}
Basal, pair-fed†	60	12 ± 1^{c}	4.76 ± 0.05^{b}	38.7 ± 0.5^{b}	2.98 ± 0.07^{bc}
Ala-excess, pair-fed	60	11 ± 1^{c}	4.98 ± 0.12^{b}	38.2 ± 1.1^{b}	2.88 ± 0.04^{c}

*Means ± SEM. Means within a column not followed the same superscript are significantly different ($p < 0.05$).

†Rats were fed the same amount of diet as the rats fed a His-excess diet.

TABLE 7

Serum Cholesterol, Triglyceride, Glucose and Liver Glycogen of Rats Fed a Basal, Histidine (His)-Excess or Alanine (Ala)-Excess Diet for Seven Days

Diet	Serum cholesterol (mg/100 ml)	Serum triglyceride (mg/100 ml)	Serum glucose (mg/100 ml)	Liver glycogen (mg/g)
His-excess, ad libitum	$158.7 \pm 5.0^{a,*}$	72.1 ± 4.8^{c}	134.8 ± 4.1^{b}	83.6 ± 5.8^{a}
Basal, ad libitum	121.6 ± 3.9^{b}	126.4 ± 12.9^{b}	155.9 ± 4.4^{a}	63.6 ± 4.8^{b}
Ala-excess, ad libitum	110.7 ± 2.7^{c}	133.7 ± 8.7^{b}	149.6 ± 5.7^{a}	65.9 ± 6.3^{b}
Basal, pair-fed	100.2 ± 2.1^{d}	176.2 ± 10.7^{a}	149.2 ± 4.2^{a}	83.2 ± 4.1^{a}
Ala-excess, pair-fed	100.4 ± 1.9^{d}	151.4 ± 9.8^{ab}	147.1 ± 2.1^{a}	86.6 ± 5.0^{a}

*Means ± SEM. Means within a column not followed by the same superscript are significantly different ($p < 0.05$).

In the 30-day time-course study, serum cholesterol increased continuously in rats fed the histidine-excess diet compared to rats fed the basal diet. This hypercholesterolemia is not explained just by the increase in transport from liver to serum, because in spite of the continuous increase of serum cholesterol for 30 days, liver cholesterol did not show the corresponding decrease. Solomon and Geison (21) and Qureshi et al. (22) using liver slices or homogenates indicated that in vitro incorporation of radioactive substrates into cholesterol increased in rats fed the histidine-excess diet. Therefore, hypercholesterolemia in rats fed the histidine-excess diet may be attributed to the stimulation of cholesterol synthesis in rat liver.

Harvey et al. (3) reported that a histidine-excess diet (8% His) produced a significant reduction in plasma and liver copper in rats. Copper-deficient diets have been shown to cause hypercholesterolemia and increased rates of precursor incorporation into cholesterol in rats (23-25). Harvey et al. (3) suggested that hypercholesterolemia caused by excess histidine was due to changes in copper status produced by the chelating action of histidine.

Hypotriglyceridemia induced by feeding a histidine-excess diet might not be due to a decreased food intake in histidine-fed rats. Eichelman et al. (26) reported that His-supplemented stock and fat-free diets depressed acetyl-CoA carboxylase and fatty acid synthetase in liver of fasted-refed rats. Our results are in agreement with their observation. Also, serum triglyceride increased continuously in rats fed either the basal or the histidine-excess diet. Since liver triglyceride did not change with the feeding periods, the stimulation in transport of triglyceride from liver to serum did not seem to be the cause.

It was found that a histidine-excess diet caused a significant increase in serum phospholipids. It is well known that phospholipids are the main constitutive lipids of HDL (27). HDL cholesterol was most of the total cholesterol in rat serum, and the intake of a histidine-excess diet did not change this distribution. Our results showed that in rats fed a histidine-excess diet, serum total cholesterol increased by more than 50% and serum phospholipids by 20-30% (calculated from Table 5). These data suggest that the increase in serum cholesterol is explained partly by the increase of lipoprotein concentration and partly by the change in lipid composition, especially the cholesterol content of each lipoprotein.

Rats fed the histidine-excess diet received a high level of nitrogen and showed lower food intake than rats fed the basal diet. Therefore, we examined the effect of excess nitrogen and restricted food intake by rats pair-fed an alanine-excess diet. Since Daniel and Waisman (1) reported that Ala had less toxicity than other amino acids at that rate of intake, we used Ala for the nitrogen source. Our data indicated that low concentrations of serum triglyceride and serum glucose as well as high concentrations of serum cholesterol and phospholipids in rats fed the histidine-excess diet were not due to high nitrogen or reduced food intake. On the other hand, the low content of lipids and cholesterol in the liver may be related to food intake. Liver lipids of rats fed the histidine-excess diet, however, were lower than in the pair-fed rats; therefore not only the lower food intake but a direct effect of histidine might have affected the lipids in rats fed the histidine-excess diet.

The percentage of each lipoprotein fraction in rats fed the histidine-excess diet was similar to that in rats fed the basal diet. On the other hand, in rats fed cholesterol-

TABLE 8

Food Intake, Body Weight Gain, Liver Weight, Liver Lipids and Liver Cholesterol of Rats Fed a Basal, a Histidine (His)-Excess or Cholesterol-Supplemented Diets for Six Days

Diet	Food intake (g/6 days)	Body weight gain (g/6 days)	Liver weight (g/100 g body weight)	Liver lipids (mg/g)	Liver cholesterol (mg/g)
Basal	$79 \pm 2^{a,*}$	28 ± 1^{a}	4.96 ± 0.14^{c}	54.0 ± 1.4^{b}	2.22 ± 0.07^{c}
His-excess	48 ± 2^{b}	12 ± 1^{b}	6.70 ± 0.14^{a}	37.4 ± 0.9^{c}	1.79 ± 0.08^{d}
0.5% cholesterol	80 ± 3^{a}	30 ± 2^{a}	5.16 ± 0.11^{bc}	107.2 ± 1.9^{a}	18.88 ± 0.24^{b}
1.0% cholesterol	74 ± 1^{a}	26 ± 1^{a}	5.45 ± 0.14^{b}	108.2 ± 1.8^{a}	19.97 ± 0.16^{a}

*Means ± SEM for six rats. Means within a column not followed by the same superscript are significantly different.

TABLE 9

Serum Total Cholesterol, HDL, LDL and VLDL Cholesterol and Triglyceride of Rats Fed a Basal, Histidine (His)-Excess or Cholesterol-Supplemented Diet for Six Days

Diet	Total cholesterol (mg/100 ml)	HDL cholesterol (% of total)	LDL cholesterol (% of total)	VLDL cholesterol (% of total)	Triglyceride (mg/100 ml)
Basal	$136.3 \pm 3.2^{d,*}$	61.8 ± 1.4^{a}	29.2 ± 1.4^{c}	9.0 ± 1.0^{a}	160.4 ± 14.7^{b}
His-excess	181.6 ± 5.6^{c}	62.3 ± 2.6^{a}	29.6 ± 2.4^{c}	8.1 ± 1.6^{a}	100.6 ± 8.8^{a}
0.5% cholesterol	206.5 ± 6.9^{b}	28.9 ± 0.7^{b}	60.6 ± 1.2^{b}	10.5 ± 0.8^{a}	184.4 ± 14.5^{b}
1.0% cholesterol	235.2 ± 4.7^{a}	21.7 ± 0.9^{c}	68.1 ± 0.8^{a}	10.2 ± 0.5^{a}	171.5 ± 13.1^{b}

*Means ± SEM for six rats. Means within a column not followed by the same superscript are significantly different ($p < 0.05$).

supplemented diets, the percentage of LDL cholesterol increased and that of HDL cholesterol decreased. Hypercholesterolemia in rats fed a high cholesterol diet has been studied in detail (27). It is characterized by the appearance of abnormal lipoproteins such as β-VLDL and HDL_c and by an increase in LDL. These lipoproteins are enriched in cholesteryl esters. We used a convenient method to separate the HDL, LDL and VLDL cholesterol (15). This method is clinically applied to separate human serum cholesterol and was used here to separate each fraction of cholesterol from rats, for which β-VLDL and HDL_c are likely to come with the LDL fraction. Therefore, in our data the LDL fraction showed the highest value of the three fractions in rats fed cholesterol-supplemented diets. In hypercholesterolemia of rats fed the histidine-excess diet, the appearance of these abnormal lipoproteins was not recognized. In this hypercholesterolemia, our data indicate the possibility that the metabolism of each lipoprotein may be nearly normal, but this assumption must be verified by further study.

Although the detailed mechanism for hypercholesterolemia induced by excess His was not determined, this hypercholesterolemia would be a new model for testing the effects of various dietary factors that influence cholesterol metabolism.

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Dominance of Δ^7-Sterols in the Family Caryophyllaceae

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The predominant 4-desmethylsterols from the leaves of 13 species in 11 genera of the family Caryophyllaceae are 24-ethyl-Δ^7-sterols. In eight species, *Scleranthus annus* L., *Paronychia virginica* Spreng., *Lychnis alba* Mill., *Silene cucubalus* Wibel, *Dianthus armeria* L., *Gypsophilia paniculata* L., *Saponaria officinales* L. and *Myosoton aquaticum* (L.) Moench, the major sterols are spinasterol (24α-ethylcholesta-7,22E-dien-3β-ol) and 22-dihydrospinasterol (24α-ethylcholest-7-en-3β-ol), with spinasterol at more than 60% of the desmethylsterol in the latter six species. Both 24α- and 24β-ethyl-Δ^7-sterols are present in two species, *Minuartia caroliniana* Walt. and *Spergula arvensis* L., which possess 24β-ethylcholesta-7,25(27)-dien-3β-ol and 24β-ethylcholesta-7,22E,25(27)-trien-3β-ol as well as spinasterol and 22-dihydrospinasterol. *Cerastium arvense* L., *C. vulgatum* L. and *Arenaria serpyllifolia* L. possess 24-alkyl-Δ^5- and Δ^7-sterols. These three species synthesize sitosterol (24α-ethylcholest-5-en-3β-ol), 24ξ-methylcholest-5-en-3β-ol, spinasterol, 22-dihydrospinasterol and the stanols, sitostanol (24α-ethyl-5α-cholestan-3β-ol) and 24ξ-methyl-5α-cholestan-3β-ol. Avenasterol (24-ethylcholesta-7,24(28)Z-dien-3β-ol) was also isolated from five species. Sterol biosynthetic capability may be a useful characteristic in examining the taxonomic relatedness of plants in the Caryophyllaceae.
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Although the majority of angiosperm plants are reported to produce 24-alkyl-Δ^5-sterols (1-4), species in several families, including Theaceae (tea) and Cucurbitaceae (cucumber), synthesize 24-alkyl-Δ^7-sterols as the dominant 4-desmethylsterols (1,3,5-8). Two characteristics of sterol biosynthesis, i.e., the cycloartenol-lanosterol bifurcation and the configuration at C-24 of the 24-ethylsterols, are suggested to be correlated with other evolutionary trends (1,3,9). From the 12 families of plants in the order Caryophyllales (10), the sterol composition of several species within four families—Amaranthaceae (amaranth), Cactaceae (cactus), Chenopodiaceae (goosefoot) and Phytolaccaceae (pokeweed)—is reported (6,11-22). In the Cactaceae, *Lophocereus schottii* produces six Δ^7-sterols, including lathosterol, spinasterol and 22-dihydrospinasterol (schottenol) (12-15), whereas *Stenocereus thurberi* produces three Δ^5-sterols with sitosterol as the major component (13). In the Phytolaccaceae, *Phytolacca esculenta* and *P. americana* synthesize exclusively Δ^7-sterols (21-23). From the mature photosynthetic tissue of the 14 species examined in the family Chenopodiaceae, three species produce exclusively Δ^7-sterols, *Beta vulgaris* produces a 7:3 ratio of Δ^7- to Δ^5-sterols, five species synthesize a 1:1 ratio of Δ^7- to Δ^5-sterols and five species synthesize Δ^5-sterols as their dominant sterols (16-20). Within the Amaranthaceae, of which 19 varieties from 10 species were examined, all species synthesized Δ^7-sterols as the dominant sterols with Δ^5-sterols present at low levels, i.e., 2-20% of the total 4-desmethylsterol (11).

We examined 13 species from 11 genera within five tribes of the family Caryophyllaceae (pink), which is in the order Caryophyllales, to determine if the predominance of Δ^7-sterols is a biosynthetic trait that may be correlated with other phylogenetic characteristics and, thus, could be a useful parameter in the analysis of the evolution of higher plants. Ten of the species in the family Caryophyllaceae produce exclusively Δ^7-sterols, whereas three species within one tribe synthesized relatively fixed mixtures of Δ^7- and Δ^5-sterols with low levels of stanols. The angiosperm classification system of Cronquist (10), the tribal arrangement of the Caryophyllaceae by Thomson (24), the subtribe arrangement of the Alsineae by McNeill (25) and the nomenclature of Gray's manual (26) are used in this paper.

MATERIALS AND METHODS

Plants were field-collected during the summer and fall of 1982 and 1983 as available. *Gypsophilia paniculata* L. (W. Atlee Burpee Co., Warminster, Pennsylvania) was grown in the greenhouse and harvested at the same stage of maturity as field-collected samples. The identity of plant species was independently confirmed by Albert List Jr. of Drexel University, Philadelphia, Pennsylvania.

The plants were washed and cleaned of all necrotic tissue, and the mature photosynthetic tissue (leaves and stems) was finely chopped and acetone-extracted in a soxhlet for 48 hr. The acetone extract was evaporated to dryness under reduced pressure and saponified in 5% KOH (w/v) in ethanol/water (7:3, v/v). This solution was diluted with an equal volume of water, and the neutral lipids were extracted with equal volumes of ether (4×). The neutral lipids were fractionated by alumina chromatography by eluting with two void volumes each of hexane, hexane/benzene (1:1, v/v), benzene, ether, ether/methanol (1:1, v/v) and methanol. The sterols eluted in the ether fraction.

The sterols were separated by preparative reverse-phase liquid chromatography (RPLC) on a Perkin-Elmer C_{18} High Efficiency Column by eluting with acetonitrile/methanol (9:1, v/v; 7.5 ml/min) at 35 C. The first 150 ml were eluted from the column, and then 20 ml fractions were collected for the next 600 ml. The sterols typically eluted in fractions 10-25. Fractions were evaporated under nitrogen and serially analyzed on gas liquid chromatography (GLC) to produce an elution profile as previously reported (16,17). Further purification of individual sterols was accomplished as previously described (16,17,27), depending on the complexity and composition of the sterol mixtures so isolated.

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GLC analysis of sterols was performed with a Perkin-Elmer Sigma 3B with either 1% XE-60 on Chromosorb Q (100–120 mesh) at 230 C or 0.75% SE-30 on Chromosorb Q at 235 C with He, 35 ml/min. Relative retention times (RRT) are to cholesterol. Analytical RPLC was performed with a Zorbax ODS column (30 cm × 3 mm) with acetonitrile/isopropanol (8:2, v/v; 1.5 ml/min) at 45 C in a Perkin-Elmer 3 liquid chromatograph equipped with a LC-75 UV detector. Sample peaks were detected at 205 nm and scanned between 200 and 300 nm. The α_c (K′ sample/K′ cholesterol) was calculated as previously described (28). Electron impact mass spectroscopy was performed at 70 eV on a Finnigan model 4000 equipped with a series 6000 data system. ^{1}H nuclear magnetic resonance spectroscopy (NMR) was performed at 360 MHz at ambient temperature on a Bruker model WH 360, in $CDCl_3$ with TMS as an internal standard. Authentic standards were obtained and purified as previously reported (16,17,27).

RESULTS

The dominant 4-desmethylsterols of the Caryophyllaceae are the 24-ethyl-Δ^7-sterols (Table 1). All species examined contain spinasterol (24α-ethylcholesta-7,22E-dien-3β-ol) and 22-dihydrospinasterol (24α-ethylcholest-7-en-3β-ol). Spinasterol had an RRT of 1.57 ± 0.01 on SE-30, 1.52 ± 0.03 on XE-60 and an α_c of 1.10 ± 0.01. The mass spectrum produced the following characteristic ions at m/e: 412 (M^+, 35%), 397 ($M\text{-}CH_3^+$, 27%), 379 ($M\text{-}CH_3\text{-}H_2O^+$, 19%), 369 ($M\text{-}C_3H_7^+$, 33%), 271 (M-side chain-2H$^+$, 98%), 255 (M-side chain-H_2O^+, 100%). The ^{1}H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.56, C-19(s) 0.80, C-21(d,J=6Hz) 1.03, C-26,27(d,d,J=6Hz) 0.80 and 0.86, C-29(t,J=7Hz) 0.81. The 22-dihydrospinasterol had an RRT of 1.81 ± 0.02 on SE-30, 1.71 ± 0.06 on XE-60 and an α_c of 1.26 ± 0.02. The mass spectrum produced the following characteristic ions at m/e: 414 (M^+, 99%), 399 ($M\text{-}CH_3^+$, 74%), 381 ($M\text{-}CH_3\text{-}H_2O^+$, 16%), 273 (M-side chain$^+$, 78%), 255 (M-side chain-H_2O^+, 100%). The ^{1}H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.54, C-19(s) 0.80, C-21(d,J=6Hz) 0.94, C-26,27(d,d,J=6Hz) 0.82 and 0.84, C-29(t,J=7Hz) 0.85. The chemical shifts for the protons on several carbons of the 24β-epimers, chondrillasterol and 22-dihydrochondrillasterol, respectively, are sufficiently different to confirm the 24α-configuration (7,8,16,17), i.e., the ^{1}H NMR spectra of the 24α-epimers were not superimposable on spectra of authentic 24β-epimeric standards.

Five species—*Lychnis alba* Mill., *Dianthus armeria* L., *Myosoton aquaticum* (L.) Moench., *Cerastium arvensis* L. and *Minuartia caroliniana* Walt.—also contained avenasterol (24-ethylcholesta-7,24(28)Z-dien-3β-ol) (Table 1). Avenasterol had an RRT of 1.87 ± 0.01 on SE-30, 1.88 ± 0.02 on XE-60 and an α_c of 0.93 ± 0.01. The mass

TABLE 1

Percent Composition of the 4-Desmethylsterols in the Caryophyllaceae

	24ξ-Methylcholesterol	Sitosterol	Avenasterol	Spinasterol	22-Dihydrospinasterol	24β-Ethylcholesta-7,25(27)-dien-3β-ol	24β-Ethylcholesta-7,22,25(27)-trien-3β-ol	24ξ-Methylcholestanol	Sitostanol
Scleranthea									
Scleranthus annuus				41.8	58.2				
Paronychieae									
Paronychia virginica				55.0	45.0				
Lychnideae									
Lychnis alba			6.5	61.9	31.6				
Silene cucubalus				72.2	27.8				
Diantheae									
Dianthus armeria			5.5	73.0	21.5				
Gypsophilia paniculata				63.2	36.8				
Saponaria officinales				80.2	19.8				
Alsineae									
subtribe Sabulininae									
Spergula arvensis				3.5	16.9	10.6	69.0		
Minuartia caroliniana			3.5	46.1	5.5	38.4	6.5		
subtribe Stellariinae									
Myosoton aquaticum			5.8	66.6	27.6				
Cerastium arvense	2.4	18.1	7.7	8.0	56.9			1.1	5.8
Cerastium vulgatum	1.1	21.7		10.1	42.5			1.8	22.8
Arenaria serpyllifolia	1.8	15.1		37.5	29.3			2.2	14.1

spectrum produced the following characteristic ions at m/e: 412 (M^+, 26%), 397 ($M-CH_3^+$, 24%), 379 ($M-CH_3-H_2O^+$, 25%), 314 ($M-C_7H_{14}^+$, 92%), 299 ($M-C_7H_{14}-CH_3^+$, 49%), 271 (M-side chain-$2H^+$, 100%), 255 (M-side chain-H_2O^+, 82%). The ion at m/e 314 with its high relative abundance is characteristic of a 24(28) double bond (29). The ^1H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.54, C-19(s) 0.80, C-21(d,J=6Hz) 0.95, C-26,27 (d,d,J=6Hz) 0.98 and 0.98, C-29(d,J=6Hz) 1.58. These chromatographic characteristics, mass spectra and ^1H NMR spectra are in agreement with previously published values (16,17,29).

Minuartia caroliniana Walt. and *Spergula arvensis* L. contained 38.4% and 10.6%, respectively, of 24β-ethylcholesta-7,25(27)-dien-3β-ol (Table 1). This sterol had an RRT of 1.76 ± 0.02 on SE-30, 1.74 ± 0.03 on XE-60 and an α_c of 0.91 ± 0.02. The mass spectrum produced the following characteristic ions at m/e: 412 (M^+, 9%), 397 ($M-CH_3^+$, 8%), 314 ($M-C_7H_{14}^+$, 5%), 299 ($M-C_7H_{14}-CH_3^+$, 5%) 271 (M-side chain-$2H^+$, 100%), 255 (M-side chain-H_2O^+, 24%). The ^1H NMR spectra displayed the following chemical shifts in ppm from TMS for each carbon designated: 3H at C-18(s) 0.53, 3H at C-19(s) 0.79, 3H at C-21(d,J=6Hz) 0.91, 3H at C-26(s) 1.56, 2H at C-27(s,s) 4.67, 4.76, 3H at C-29(t,J=7Hz) 0.80. The chromatographic characteristics, mass spectra and ^1H NMR of this sterol are in agreement with previously published values (7). We use the 25(27) rather than the 25(26) designation of the Δ^{25}-bond for natural 25-dehydrosterols, based on the work of previous investigators (3,7,30). These two species also contained 24β-ethylcholesta-7,22E,25(27)-trien-3β-ol (6.5% and 69.0%, respectively) (Table 1). This sterol had an RRT of 1.59 ± 0.02 on SE-30, 1.57 ± 0.03 on XE-60 and an α_c of 0.72 ± 0.02. The mass spectra produced the following major characteristic ions at m/e: 410 (M^+, 37%), 395 ($M-CH_3^+$, 13%), 381 ($M-C_2H_5^+$, 13%), 377 ($M-CH_3-H_2O^+$, 5%), 363 ($M-C_2H_5-H_2O^+$, 3%), 326 ($M-C_6H_{12}^+$, 8%), 300 (M-110$^+$, 22%), 285 (M-125, 9%), 273 (M-side chain$^+$, 30%), 272 (M-side chain-H^+, 32%), 271 (M-side chain-$2H^+$, 100%), 255 (M-side chain-H_2O^+, 41%). The ^1H NMR spectra displayed the following chemical shifts in ppm from TMS for each carbon designated: 3H at C-18(s) 0.54, 3H at C-19(s) 0.80, 3H at C-21(d,J=6Hz) 1.02, 3H at C-26(s) 1.65, 2H at C-27(s) 4.70, 3H at C-29(t,J=7Hz) 0.84. The chromatographic characteristics, mass spectra and ^1H NMR of this sterol are consistent with previously published values (7).

Cerastium arvensis L., *C. vulgatum* L. and *Arenaria serpyllifolia* L. also contained low levels (<22%) of sitosterol (24α-ethylcholest-5-en-3β-ol) (Table 1). Sitosterol had an RRT of 1.62 ± 0.01 on SE-30, 1.55 ± 0.02 on XE-60 and an α_c of 1.23 ± 0.01. The mass spectrum produced the following characteristic ions at m/e: 414 (M^+, 92%), 399 ($M-CH_3^+$, 61%), 396 ($M-H_2O^+$, 43%), 381 ($M-CH_3-H_2O^+$, 16%), 303 ($M-C_7H_9-H_2O^+$, 61%), 273 (M-side chain$^+$, 61%), 255 (M-side chain-H_2O^+, 100%). The ^1H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.68, C-19(s) 1.01, C-21(d,J=6Hz) 0.92, C-26,27(d,d,J=6Hz) 0.81 and 0.83, C-29(t,J=7Hz) 0.85. These values are in agreement with authentic sitosterol and previously published values (16,17,27). These three species also contained 24ξ-methylcholest-5-en-3β-ol (Table 1). This component had an RRT of 1.30 ± 0.01 on SE-30, 1.30 ± 0.02 on XE-60 and an α_c of 1.10 ± 0.01. The mass spectrum produced the following characteristic ions at m/e: 400 (M^+, 65%), 385 ($M-CH_3^+$, 18%), 382 ($M-H_2O^+$, 26%), 367 ($M-CH_3-H_2O^+$, 18%), 315 ($M-C_6H_{13}^+$, 26%), 289 ($M-C_7H_9-H_2O^+$, 49%), 273 (M-side chain$^+$, 46%), 255 (M-side chain-H_2O^+, 100%). The ^1H NMR spectra displayed broad signals for hydrogens on C-18 as well as a multiple of doublets in the 0.75–0.85 ppm region, indicative of an epimeric mixture of 24α-methylcholesterol (campesterol) and 24β-methylcholesterol (22-dihydrobrassicasterol) (30,31). Since plants are known to produce epimeric mixtures of 24α- and 24β-methylcholesterol (30,31), further separation was not attempted.

The three species that contain Δ^5-sterols also contain the saturated analogues, sitostanol (24α-ethyl-5α-cholestan-3β-ol) and 24ξ-methyl-5α-cholestan-3β-ol (Table 1). Sitostanol had an RRT of 1.64 ± 0.01 on SE-30 and 1.55 ± 0.02 on XE-60. The α_c was estimated at 1.41–1.44 as previously described (17). The mass spectrum produced the following characteristic ions at m/e: 416 (M^+, 31%), 401 ($M-CH_3^+$, 10%), 398 ($M-H_2O^+$, 2%), 383 ($M-CH_3-H_2O^+$, 4%), 344 ($M-C_4H_6-H_2O^+$, 1%), 275 (M-side chain$^+$, 2%), 257 (M-side chain-H_2O^+, 7%), 248 (M-168$^+$, 14%), 233 (M-183$^+$, 78%), 215 (M-201$^+$, 100%). The ^1H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.65, C-19(s) 0.80, C-21(d,J=6Hz) 0.91, C-26,27(d,d,J=6Hz) 0.81 and 0.83, C-29(t,J=7Hz) 0.84. The chromatographic characteristics, mass spectra and ^1H NMR spectra were identical to authentic sitostanol and previously published values (17,32). The planarity of the A/B ring juncture (5α-H) was demonstrated as previously described (17). The 24ξ-methylcholestanol had an RRT of 1.32 ± 0.01 on SE-30 and 1.30 ± 0.02 on XE-60. The α_c was estimated at 1.23–1.26 as previously described (17). The mass spectrum produced the following characteristic ions at m/e: 402 (M^+, 35%), 387 ($M-CH_3^+$, 17%), 369 ($M-CH_3-H_2O^+$, 6%), 257 (M-side chain-H_2O^+, 7%), 248 (M-154$^+$, 11%), 233 (M-169$^+$, 82%), 215 (M-187$^+$, 100%). This is in agreement with previously reported values (17,32). The ^1H NMR spectra displayed a broad signal for hydrogens on C-18 and a multiple of doublets between 0.77–0.85 indicating a similar epimeric mixture as found in the 24ξ-methylcholest-5-en-3β-ol component.

Cycloartenol (9β,19-cyclo-4,4,14α-trimethylcholest-24-en-3β-ol) was isolated and is reported as a percentage of the total sterol from *Scleranthus annuus* L. (13.9%), *Silene cucubalus* Wibel (51.2%), *D. armeria* L. (9.6%), *G. paniculata* L. (19.2%), *S. officinales* L. (26.6%), *M. aquaticum* (L.) Moench. (16.1%), *C. arvense* L. (10.8%), *C. vulgatum* L. (17.0%) and *A. serpyllifolia* L. (39.7%). Cycloartenol had an RRT of 1.81 ± 0.04 on SE-30, 1.71 ± 0.08 on XE-60 and an α_c of 0.90 ± 0.04. The mass spectrum produced the following characteristic ions at m/e: 426 (M^+, 27%), 411 ($M-CH_3^+$, 53%), 408 ($M-H_2O^+$, 16%), 393 ($M-CH_3-H_2O^+$, 29%), 365 ($M-C_3H_7-H_2O^+$, 7%), 339 ($M-C_5H_9-H_2O^+$, 5%), 314 (M-side chain$^+$, 14%), 299 (M-side chain-CH_3^+, 6%), 286 ($M-C_9H_{14}-H_2O^+$, 35%), 271 ($M-C_9H_{14}-CH_3-H_2O^+$, 100%), 259 ($M-C_{12}H_{23}^+$, 10%). The ^1H NMR spectra displayed the following chemical shifts in ppm from TMS for each carbon designated: 3H at C-18(s) 0.81, 2H at C-19(d,d,J=6Hz) 0.33 and 0.55, 3H at C-21(d,J=6Hz) 0.88, 3H at C-26(s) 1.60, 3H at C-27(s) 1.68,

3H at C-30(C-4α,s) 0.96, 3H at C-31(C-4β,s) 0.96, C-32 (C-14α,s) 0.89. The chromatographic characteristics, mass spectra and ^{1}H NMR spectra are in agreement with previously reported values (17) and are identical to an authentic cycloartenol standard.

Lanosterol [4,4,14α-trimethylcholesta-8(9),24-dien-3β-ol] was isolated and is reported as a percentage of the total sterol from *S. annuus* L. (24.2%), *C. arvense* L. (1.8%) and *A. serpyllifolia* L. (2.8%). Lanosterol had an RRT of 1.60 ± 0.02 on SE-30, 1.48 ± 0.04 on XE-60 and an α_c of 0.81 ± 0.01. The mass spectrum produced the following characteristic ions at m/e: 426 (M^+, 49%), 411 ($M-CH_3^+$, 100%), 393 ($M-CH_3-H_2O^+$, 39%), 273 ($M-C_{10}H_{15}-H_2O^+$, 15%), 259 ($M-C_{12}H_{23}^+$, 19%). The ^{1}H NMR spectra displayed the following chemical shifts in ppm from TMS for three protons at each carbon designated: C-18(s) 0.69, C-19(s) 1.00, C-21(d,J=6Hz) 0.91, C-26(s) 1.61, C-27(s) 1.67, C-30(C-4α,s) 0.98, C-31(C-4β,s) 0.81, C-32(C-14α,s) 0.88. The chromatographic characteristics, mass spectra and ^{1}H NMR spectra are in agreement with previously reported values (17) and are identical to an authentic lanosterol standard.

DISCUSSION

From the seven species examined in four of the five tribes (Scleranthea, Paronychieae, Lychnideae, Diantheae) of the Caryophyllaceae, the 24α-ethyl-Δ^7-sterols (Table 1) are the dominant sterols in the mature photosynthetic tissue. Spinasterol is the dominant sterol in six of these species (i.e., 55–80% of the total 4-desmethylsterol) with 22-dihydrospinasterol (20–45%) and, in two species, avenasterol (~5%) completing the 4-desmethylsterol complement. In *S. annuus,* 22-dihydrospinasterol (58%) is the dominant sterol. With respect to 4-desmethylsterol composition, these plants are, therefore, similar to several species in the Cucurbitaceae (1,5) and the Chenopodiaceae (17). The lack of peak broadening on the ^{1}H NMR spectra of spinasterol and 22-dihydrospinasterol and the inability to superimpose these spectra on the 24β-epimers (chondrillasterol and 22-dihydrochondrillasterol) run concurrently confirm that the 24α-epimers are the dominant sterols present in these species. If the 24β-epimers are present, they are present at less than 10% of each sterol component examined, which is the limit for our detection by ^{1}H NMR (5,31).

The sterol composition of the six species examined in the tribe Alsineae is diverse (Table 1) and may reflect a recapitulation of sterol biosynthetic evolution within one taxon of higher plants. Within the subtribe Sabulininae, both *S. arvensis* and *M. caroliniana* synthesize 24α- and 24β-ethyl-Δ^7-sterols. The dominant sterols produced by *S. arvensis* are the 24β-ethyl-Δ^7-sterols, 24β-ethyl-5α-cholesta-7,22E,25(27)-trien-3β-ol at 69% of the total 4-desmethylsterol composition and the 22(23)-dihydro analogue present at 10%. The 24α-ethyl-Δ^7-sterols are 22-dihydrospinasterol (16.9%) and spinasterol (3.5%), in which no 24β-epimers could be detected by ^{1}H NMR using the criteria described above. *M. caroliniana* possesses a qualitatively similar sterol profile (Table 1) with a 1:1 distribution of 24α- to 24β-ethyl-Δ^7-sterol ratio. The isolation of avenasterol from *M. caroliniana* supports the biosynthesis of the 24α-ethylsterols since avenasterol is a known intermediate in 24α-ethylsterol metabolism (5,30,33). The 24β-ethyl-Δ^7-sterols from both species possess the 25(27) double bond. We repeated the sterol isolation from the photosynthetic tissue of the evergreen perennial *M. caroliniana* at different times of the year (winter, spring, summer, fall), and all isolations produced similar sterol compositional profiles, which indicates that the 24α- and 24β-epimeric ratio remains constant and does not appear to shift during the growth of the mature plant. The 24β-ethyl-$\Delta^{7,25(27)}$-sterols in the seeds of Cucurbits are suggested to reflect a recapitulation of phylogeny (37) or a state of arrested sterol metabolism that is altered upon germination (34–39). *M. caroliniana* produces this unusual sterol profile in the mature plant. The presence of 24β-ethyl-$\Delta^{25(27)}$-sterols as the dominant sterols in higher plants, e.g., *Clerodendrum* (40 and references therein), is atypical.

Within the subtribe Stellariinae, the 24α-ethyl-Δ^7-sterols are the dominant sterols of the four species examined. *M. aquaticum* synthesizes only Δ^7-sterols, whereas *C. arvense, C. vulgatum* and *A. serpyllifolia* also synthesize the Δ^5-sterols sitosterol and 24ξ-methylcholesterol and the corresponding stanols, sitostanol and 24ξ-methylcholestanol. The latter three species are similar in sterol composition to five species in the Chenopodiaceae, three in the genus *Chenopodium* and two in *Salicornia* (17). The stanols all possess a *trans* A/B ring juncture as observed in the ^{1}H NMR spectra and are only detected in species synthesizing Δ^5-sterols. These 5α-stanols are presumably synthesized from the Δ^5-sterols as demonstrated in other organisms (41–43). No detectable levels of 24β-ethylsterols were identified from the four species in the subtribe Stellariinae.

The sterol composition of the mature photosynthetic tissue from plants appears potentially useful in the chemosystematics of various plant taxons. However, for this sterol data to be useful a complete characterization of the sterol profile from many related plants may be required. The organization and relatedness of species in the tribes Scleranthea, Paronychiae, Lychnideae and Diantheae are generally agreed upon (25,44), and the sterol composition of the seven species examined is consistent. Within the tribe Alsineae the five species examined are easily organized on the subtribe level if one assigns the plant *M. caroliniana* to the genus *Minuartia* rather than *Arenaria* (25,44). The 24β-ethyl-$\Delta^{25(27)}$-sterols appear to be useful compounds in establishing this taxonomic relatedness. The sterol composition of *M. caroliniana* would support the inclusion of this species in the genus *Minuartia* of the subtribe Sabulininae rather than its inclusion in the genus *Arenaria* of the subtribe Stellariinae. This genus assignment *(Minuartia)* was recently proposed for this species (25,44), and the sterol biosynthetic capacity of the plant is supportive of this assignment. Additional sterol profiles of species in *Minuartia* and *Arenaria* as well as other genera in this tribe will be required to determine the usefulness of sterol biosynthesis to the chemosystematics of the tribe. However, the predominance of Δ^7-sterols in several families in the order Caryophyllales (see Introduction) may itself be useful in determining the origin of the order and the origin of families within the order from the primal family Phytolaccaceae (10). The presence of 24β-ethylsterols and the apparent regulation of the synthesis of Δ^5-sterols in various subgroups may provide additional evidence on the origins and evolutionary development of species within families.

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Regulation of Cholesterol Synthesis in Isolated Epithelial Cells of Human Small Intestine

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We have investigated the regulation of cholesterol synthesis in isolated human small intestine epithelial cells (enterocytes). It was established that the amount of cholesterol synthesized increased linearly with the incubation time and the number of cells in the incubation mixture; the synthesis was suppressed by 7-ketocholesterol. Cholic, dehydrocholic, chenodeoxycholic, glycocholic, taurocholic, taurochenodeoxycholic and taurodeoxycholic acids inhibited cholesterol synthesis in enterocytes to different degrees in a dose-dependent manner. Lithocholic acid enhanced the rate of cholesterol synthesis. Deoxycholic acid, methyl ester of cholic acid and cholesterol did not affect the process. No bile acids tested, with the exception of taurodeoxycholic acid, affected fatty acid synthesis in enterocytes. Most bile acids also decreased cholesterol synthesis in cultured human skin fibroblasts. The results obtained make it possible to postulate that cholesterol synthesis in human enterocytes may be subject to a complex regulation by bile acids.
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Mucosa of the small intestine is one of the active sites of cholesterogenesis (1,2). It was shown that intestine ranked second only to liver with respect to cholesterol synthesis under normal lipidemic conditions and that it becomes the major site of cholesterol production in hypercholesteremia (3,4). The contribution of the small intestine to total body cholesterol synthesis normally amounts to about 10% (3) or more (1). However, in hypercholesterolemia, when cholesterol synthesis in most organs except the intestine is suppressed, its contribution can increase up to 50% (3). In spite of the important role of the small intestine in cholesterol metabolism, data on the regulation of cholesterol synthesis are scanty. It has been shown that cholesterol has a limited effect on cholesterol synthesis in the intestine (2,4–9). Diversion of bile or treatment with bile acid sequestrants leads to increased cholesterol synthesis in this organ (2,9–11). Another approach involving the study with small intestine organ cultures (2,5–8, 12) or isolated enterocytes (9,13–15) has not yet provided sufficient information about the regulation of cholesterol synthesis in these models.

In the present study, we investigated the effect of individual bile acids and cholesterol on cholesterol synthesis in isolated human enterocytes.

MATERIALS AND METHODS

Cells. The small intestine (middle jejunum) of children from several days to 10 years of age was taken at autopsy within 1 hr after death. Intestines from donors suffering from gut and blood diseases and metabolic disorders were not used. Enterocytes were isolated according to Carter et al. (16) with the modification described previously (17). Small intestine segments were everted over plastic rods, washed in minimum essential medium (MEM; Flow, Irvine, Scotland) and then in cold Dulbecco phosphate-buffered saline (PBS; Flow) without Ca^{++} and Mg^{++}. The everted segments were incubated for 10 min at 37 C and then for 1 hr at 25 C with shaking at 140 rpm in the medium containing 65.7% PBS without Ca^{++} and Mg^{++} and 34.3% three-times-distilled water (v/v), and supplemented with 1% (w/v) polyvinylpyrrolidone (M.W. 40,000; Sigma Chemical Co., St. Louis, Missouri). Released cells were sedimented and washed twice in Dulbecco's modified Eagle's medium (DMEM; Flow) by centrifugation at 500 × g for 10 min, and filtered through nylon mesh. The cell yield was $1–2 \times 10^7$ cells per 10 cm of intestine, with cell viability being 80–90% according to the trypan blue exclusion test. Detailed characterization of isolated enterocytes was reported previously (17).

Human skin fibroblasts were maintained in MEM containing 10% fetal calf serum (FCS), 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1% MEM-nonessential amino acids, 2 mM L-glutamine, 100 μg/ml kanamycin and 2.5 μg/ml fungisone (all reagents from Flow) at 37 C in a CO_2 incubator (5% CO_2, 95% air).

Cell protein content was determined according to Bradford (18).

Bile acids. The term "bile acid" was used to denote the class of compounds having a cholane nucleus, but does not specify the extent of ionization or physical properties (19). All bile acids were obtained from Sigma and were at least 98% pure. Conjugated bile acids were dissolved in DMEM to a final concentration of 20 mM. Cholic, dehydrocholic, chenodeoxycholic and deoxycholic acids were dissolved as follows: each bile acid was placed in DMEM at a final concentration of 20 mM, the pH was adjusted to about 9 and the suspension was incubated at 37 C with intensive shaking until completely dissolved (usually 1–2 hr). Then, the pH of the solution was adjusted to 7.4. No precipitate was found with microscopic examination of the solution. Lithocholic acid and methyl ester of cholic acid were dissolved in absolute ethanol (Merck, Darmstadt, Federal Republic of Germany) to final concentrations of 100 mM and 60 mM, respectively, by incubation at 37 C for 3 hr with intensive shaking.

Cholesterol (Sigma S-CH) and 7-ketocholesterol (Sigma) were dissolved in absolute ethanol to final concentrations of 50 mM and 25 mM, respectively.

Low density lipoprotein (LDL) ($1.019 < d < 1.050$ g/cm^3) was isolated from plasma of healthy donors by sequential preparative ultracentrifugation at 105,000 × g (20). The homogeneity of lipoproteins was checked by analytical ultracentrifugation.

Cholesterol synthesis. The incubation mixture contained 2×10^6 cells, 6 μCi [2-^{14}C]sodium acetate (Amersham, Buckinghamshire, England; specific radioactivity

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40-60 μCi/mmol), DMEM in a final volume of 0.5 ml and other substances as indicated. When substances were added in the form of ethanol solutions, the corresponding amount of ethanol was also added to the control samples. It was shown in preliminary experiments that ethanol at a concentration of 2% decreases cholesterol synthesis in enterocytes by 20%.

The incubation mixture was placed in the wells of a "Multidish" (Nunc, Roskilde, Denmark) and incubated for 3 hr at 37 C with shaking at 60 rpm in an orbital shaker in a CO_2 incubator (5% CO_2, 95% air). After incubation, the cells were sedimented by centrifugation at 500 × g for 10 min at 4 C, washed with 10 ml of DMEM and resuspended in the 50 μl of DMEM. The samples were saponified by incubation in 5 M KOH in 50% ethanol at 100 C for 2 hr. Cholesterol was precipitated with digitonin (Sigma) according to Sperry and Webb (21). Fatty acids were isolated by thin layer chromatography using Kieselgel plates (Merck); petroleum ether/ethyl ether/acetic acid (85:15:1, v/v/v) was used as developing solvent. The areas corresponding to sterols and fatty acids were identified by comparison with standards, scraped off and counted.

A total of 24 enterocyte preparations from 24 donors was used for experiments. A comparison of the results obtained on enterocytes from different donors gave the between-preparation coefficient of variation of about 100% and the within-sample coefficient of variation of about 10%. Effects of different substances were measured in each preparation of enterocytes and compared with appropriate controls. Results are expressed relative to the controls.

Human skin fibroblasts (fifth passage) were grown to confluency in the wells of a Multidish. The cells were preincubated for 24 hr in serum-free medium containing 1 mg/ml BSA (Sigma). Then the cells were incubated in the same medium with 6 μCi/ml [^{14}C]acetate and 2 mM bile acid for 24 hr at 37 C. After incubation, the cells were dissolved in 0.2 M NaOH, and the incorporation of [^{14}C]acetate into digitonin-precipitable sterols (DPS) was determined as described above.

Each experiment was done in triplicate and repeated 2-4 times on enterocyte preparations obtained from different donors. Representative experiments are shown in the figures.

RESULTS

The rate of cholesterol synthesis in isolated human enterocytes was evaluated by measuring the incorporation of [^{14}C]acetate into DPS. The rate of cholesterol synthesis was constant within the first 2 hr and decreased slightly between the second and third hours of incubation (Fig. 1A). The amount of cholesterol synthesized correlated with the number of cells in the incubation mixture in a linear fashion within the range of up to 3 × 10^6 cells (Fig. 1B). All the cholesterol synthesized remained inside the cells; no labeled sterols were found in the medium after 3 hr of incubation (not shown). 7-Ketocholesterol, a well-known inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (22), inhibited cholesterol synthesis in enterocytes by 70% in a dose-dependent manner (Fig. 2). Mevenoline (0.1 μg/ml) inhibited cholesterol synthesis in enterocytes by 40% (unpublished observation).

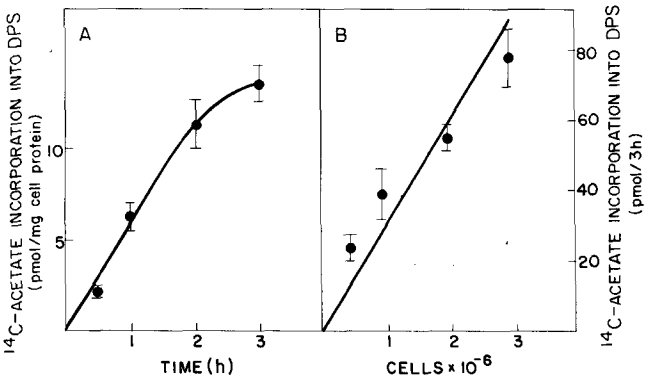

FIG. 1. Time (A) and dose (B) dependence of cholesterol synthesis in enterocytes. Cells were incubated for the indicated periods of time (A) or for 3 hr (B) at 37 C in the presence of 12 μCi/ml [^{14}C]acetate. The amount of [^{14}C]acetate incorporated into DPS was determined as described in Materials and Methods. Each point represents the mean ± SEM of triplicate determinations.

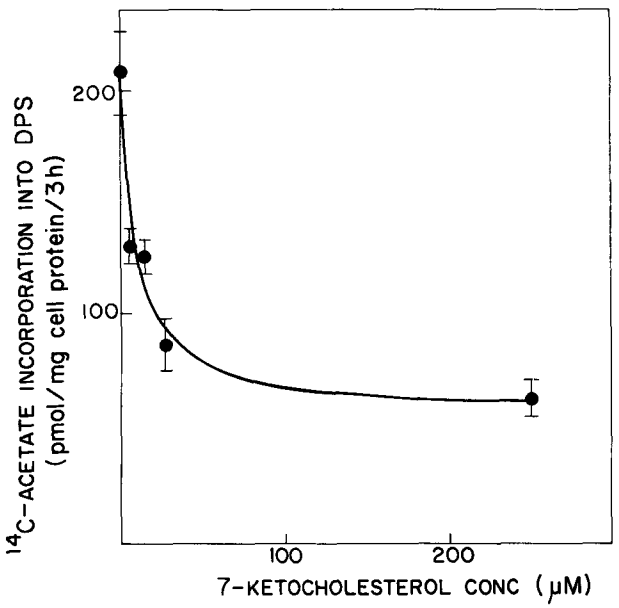

FIG. 2. Effect of 7-ketocholesterol on cholesterol synthesis in enterocytes. Cells (2 × 10^6) were incubated for 3 hr at 37 C in the presence of 12 μCi/ml [^{14}C]acetate at the indicated concentrations of 7-ketocholesterol. Amount of [^{14}C]acetate incorporated into DPS was determined as described in Materials and Methods. Each point represents the mean ± SEM of triplicate determinations.

The effect of nonconjugated bile acids on de novo cholesterol synthesis in isolated human enterocytes is shown in Figure 3. Cholic, chenodeoxycholic and dehydrocholic acids inhibited cholesterol synthesis in a dose-dependent manner by a maximum of 80, 60 and 45%, respectively ($p < 0.001$). Deoxycholic acid did not affect cholesterol synthesis. Methyl ester of cholic acid caused a 20% elevation of the cholesterol synthesis rate; the effect, however, was not statistically significant ($p > 0.05$). The most unexpected result was observed when cholesterol synthesis was measured in the presence of lithocholic acid; it brought about a fourfold increase in the incorporation of [^{14}C]acetate into DPS.

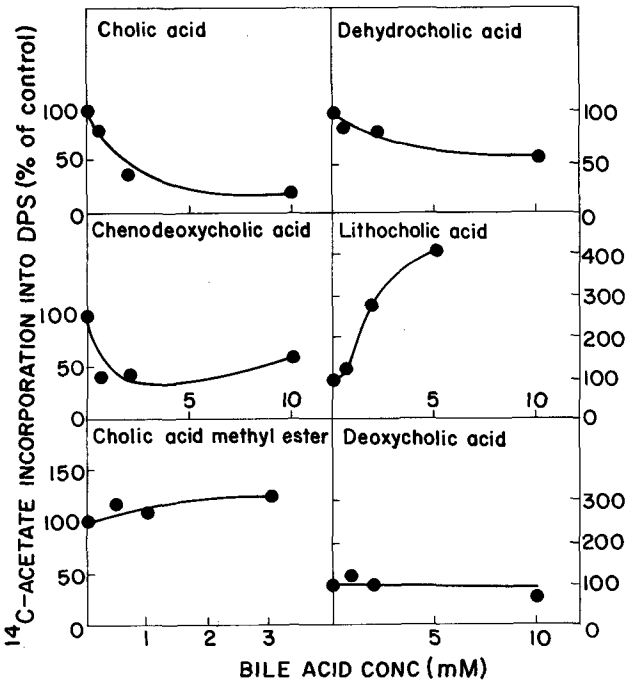

FIG. 3. Effect of nonconjugated bile acids on cholesterol synthesis in enterocytes. Cells (2×10^6) were incubated for 3 hr at 37 C in the presence of 12 μCi/ml [^{14}C]acetate and the indicated concentrations of bile acids. Preparation of bile acid solutions and determination of the amount of [^{14}C]acetate incorporated into DPS are described in Materials and Methods. Each point represents the mean data of triplicate determinations.

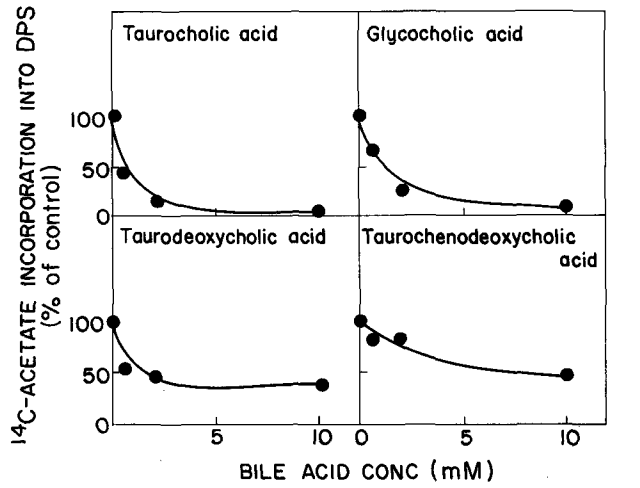

FIG. 4. Effect of conjugated bile acids on cholesterol synthesis in enterocytes. Experiments were carried out as described in the legend to Fig. 3.

Figure 4 shows the effect of conjugated bile acids on cholesterol synthesis in isolated enterocytes. Glycocholic, taurocholic, taurochenodeoxycholic and taurodeoxycholic acids inhibited cholesterol synthesis in enterocytes in a dose-dependent manner by a maximum of 93, 98, 52 and 65%, respectively. Loading of the cells with either free cholesterol (50–800 μM) or LDL (20–200 μM cholesterol) did not cause any statistically significant effect on de novo cholesterol synthesis (not shown).

Neither bile acids nor cholesterol taken at the maximal concentration changed the cell viability as measured by the trypan blue exclusion test. To rule out the possibility of a toxic effect not reflected in the trypan blue exclusion test, we assessed the impact of bile acids on the incorporation of [^{14}C]acetate into fatty acids. No bile acids, except taurodeoxycholic, affected fatty acid synthesis in enterocytes (Table 1).

To assess the specificity of the bile acid action, we determined their effect on cholesterol synthesis in cultured human skin fibroblasts (Table 2). Cholic and dehydrocholic acids did not affect cholesterol synthesis in fibroblasts. However, all conjugated bile acids and lithocholic acid caused a uniform decrease in the rate of cholesterol synthesis in cultured human skin fibroblasts. Deoxycholic, chenodeoxycholic and taurodeoxycholic acids caused a rapid detachment of fibroblasts, preventing evaluation of their effect on cholesterol synthesis in these cells.

DISCUSSION

In the present study, we investigated the effect of individual bile acids and cholesterol on cholesterol synthesis in isolated human enterocytes. It is already known that the liver synthesizes primary bile acids in conjugated form. The acids undergo deconjugation and chemical modifications by the intestinal flora of the gut, which results in the formation of a complex mixture of primary and secondary bile acids in conjugated and nonconjugated form. Both primary and secondary bile acids are absorbed by the intestine, conjugated and again secreted with bile (23). All classes of bile acids were tested in this study.

It was shown that both primary conjugated and nonconjugated bile acids, cholic and chenodeoxycholic acids, and two secondary bile acids, dehydrocholic and taurodeoxycholic acids, inhibit cholesterol synthesis in human enterocytes to a different extent. The effect is not due to their toxic properties since fatty acid synthesis was not impaired in response to incubation of the cells with these bile acids. Two other secondary bile acids, deoxycholic acid and methyl ester of cholic acid, did not affect cholesterol synthesis in enterocytes. Unexpectedly, lithocholic acid caused more than a fourfold enhancement of cholesterol synthesis in enterocytes. It appears that the more chemical transformations the primary conjugated bile acids undergo, the less inhibitory effect on cholesterol synthesis they exhibit in enterocytes. Bile acids originating from cholic acid have a stronger inhibitory effect than those originating from chenodeoxycholic acid. The mechanism of the bile acid action is not clear. Because the rate of action is very rapid, a direct or indirect effect on HMG-CoA reductase activity seems most probable. It may include enzyme modification (e.g., phosphorylation), competitive inhibition, or both. Further studies are needed to determine whether various effects of different bile acids reflect certain regulatory mechanisms. Alternatively, the variations may be due to the differences in the physicochemical properties of the bile acid (e.g., solubility) or may reflect different intercellular concentration. Bile acid composition may change in response to loading of hepatocytes with cholesterol (24).

Bile acids were used in a millimolar concentration, i.e., in the physiological concentration characteristic of the

TABLE 1

Effect of Bile Acids on Fatty Acid Synthesis in Isolated Human Enterocytes

Bile acid (2 mM)	[^{14}C]Acetate incorporation into fatty acids		
	pmol/mg P/3 hr[a]	% of control	p[b]
Experiment 1			
None	6.41 ± 0.80	100	
Cholic acid	8.35 ± 0.73	130	$p > 0.05$
Dehydrocholic acid	5.90 ± 0.59	92	$p > 0.05$
Chenodeoxycholic acid	7.82 ± 1.34	122	$p > 0.05$
Taurodeoxycholic acid	2.74 ± 0.67	43	$p < 0.01$
Experiment 2			
None	35.6 ± 4.70	100	
Glycocholic acid	40.4 ± 9.3	113	$p > 0.05$
Taurocholic acid	38.4 ± 4.20	108	$p > 0.05$
Taurochenodeoxycholic acid	25.8 ± 7.20	72	$p > 0.05$

[a]Mean ± SEM of triplicate determinations.

[b]According to student's t-test.

TABLE 2

Effect of Bile Acids on Cholesterol Synthesis in Cultured Human Skin Fibroblasts

Bile acid (2 mM)	[^{14}C]Acetate incorporation into DPS		
	nmol/mg P/24 hr[a]	% of control	p[b]
Experiment 1			
None	12.7 ± 2.0	100	
Cholic acid	9.9 ± 2.3	78	$p > 0.05$
Dehydrocholic acid	12.8 ± 3.0	100	$p > 0.05$
Lithocholic acid	4.6 ± 0.5	36	$p < 0.001$
Taurodeoxycholic acid	4.5 ± 0.1	35	$p < 0.001$
Experiment 2			
None	21.0 ± 1.0	100	
Glycocholic acid	6.9 ± 0.9	33	$p < 0.001$
Taurocholic acid	8.4 ± 0.2	40	$p < 0.001$

[a]Mean ± SEM of triplicate determinations.

[b]According to student's t-test.

intestine (19). Nonconjugated bile acids were used at concentrations at which they exist in monomeric and micellar form (19). The only exception was lithocholic acid, whose critical micellar temperature is above 37 C. It is possible that the unusual effect of lithocholic acid on cholesterol synthesis in enterocytes is related to its uncommon physicochemical properties.

Cholesterol does not affect its own de novo synthesis in enterocytes. These data are consistent with the results from other laboratories that indicate a low degree of down-regulation of intestinal cholesterol synthesis (2,4-9). On the other hand, it has been shown that under similar conditions cholesterol is easily taken up and can stimulate high density lipoprotein binding by enterocytes (25).

The effect of bile acids on cholesterol synthesis in cultured human skin fibroblasts differs from that in enterocytes. All but two bile acids decrease cholesterol synthesis in these cells by 60-70%. It should be noted that the bile acid concentrations used in the experiments with fibroblasts exceeded those in blood. Therefore, in spite of unchanged cell viability and morphology, the specificity of the effect is not clear.

The rates of cholesterol and fatty acid synthesis varied considerably between experiments. This may be ascribed to unknown peculiarities of individual patients or to differences in their diets or treatment. However, the effect of different substances on the incorporation of [^{14}C]-acetate into DPS was quantitatively reproducible.

The results obtained make it possible to postulate that bile acids may regulate cholesterol synthesis in isolated human enterocytes.

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Spectroscopic Investigations of the Water Pool in Lecithin Reverse Micelles

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The nature of the water pool formed in the reverse micellar system, lecithin/nonpolar solvent/water, has been investigated by means of near infrared, ultraviolet, fluorescence emission and visible spectroscopic techniques. The three nonpolar solvents chosen in this study were benzene, carbon tetrachloride and cyclohexane. Near infrared spectroscopic studies revealed that the amount of water present in the bulk organic phase is negligible at all water concentrations studied in all three solvents. The results of the polarity probe and 8-anilinonaphthalene sulfonic acid (ANSA) fluorescence emission maxima studies indicate that the polarity of the water pool is much lower than that of bulk water. The difference in polarity between the water pool and bulk water decreases with increasing water concentration in benzene and carbon tetrachloride systems. However, in the cyclohexane system, at a water content of 6 moles of water per mole of lecithin, where the system is known to change from isotropic reverse micelle to anisotropic liquid crystalline state, the polarity of the water pool is found to decrease.
***Lipids 21,* 764-768 (1986).**

Reversed micelles are the aggregates of amphipathic molecules such as synthetic surfactants and phospholipids in nonpolar media (1). These aggregates are capable of solubilizing a large amount of water in their polar interior, which is then called a water pool (2). These systems are of interest mainly because they are thought to resemble the bioaggregates containing water pockets like mitochondrial membranes (3). Also, reverse micelles are attracting increasing study of catalysis by water-soluble enzymes entrapped in the water pool (4,5), and very recently it has been shown that reverse micelles can be used in the separation and extraction of proteins (6). The size and polarity of the water pool and the aggregation number in surfactant reverse micelles has been very well characterized by a variety of spectroscopic and physical techniques (5,7-9). Similar studies on phospholipid reverse micelles were aimed at determining the aggregation number of phospholipid (10,11) or looking at phospholipid polar head group-water interaction (12-20). However, studies on the polarity of the water pool in phospholipid reverse micelles are few (21). In this paper, we present a combined spectroscopic characterization of the water pool polarity in the lecithin/nonpolar solvent/water system. The spectroscopic methods are based on the fact that the effective polarity, acidity and microscopic viscosity of the water pools are expected to be substantially different from those in bulk water (8).

EXPERIMENTAL PROCEDURES

The extraction of chromatographically homogeneous egg lecithin was carried out using the method of Singleton et al. (22). A single spot on a Silica Gel G (Merck) thin-layer chromatogram confirmed the purity of the extracted lipid. All solvents used were of AnalaR grade and were further dried and distilled. The water used was deionized and double-glass distilled.

Near infrared (IR) spectra were recorded using a Cary-17D spectrophotometer. A solution containing lecithin in nonpolar solvent of the same concentration as that of the sample solution was used as a reference to record the spectra of solubilized water in the reverse micellar system. Values of λ_{max} presented here were reproducible to within $\pm$ 0.5 nm. UV absorption spectra were also recorded on the same Cary-17D instrument. AnalaR-grade potassium nitrate was used to prepare the solutions for the polarity probe studies.

Fluorescence emission spectra were run on an Aminco-Bowmann spectrofluorimeter model SPF-125S. ANSA (Sigma Chemical Co., St. Louis, Missouri) was converted to its magnesium salt and recrystallized from water after charcoal treatment, and the resulting product was used as a fluorescent probe. Quantum yields have been calculated according to standard methods (23). The λ_{max} values presented in the fluorescence spectra were reproducible to $\pm$1 nm, and the quantum yields were accurate up to $\pm$0.01.

All the measurements were carried out at a number of R values, where R is defined as the molar concentration ratio (=$[H_2O]/[lecithin]$) and at a temperature of 25 $\pm$ 1 C. Methods of preparation of the samples and the addition of different amounts of water were described earlier (35).

RESULTS AND DISCUSSION

The near IR spectra of the lecithin/benzene/water system are presented in Figure 1 for small amounts of water added. A shoulder is seen around 1890 nm at very low concentrations of water apart from the dominant 1930-1960 nm band. This shoulder weakens and vanishes into the trailing portion of the 1930-1960 nm band at higher water concentrations.

In nonpolar solvents containing amphiphiles, some water is located in the bulk organic phase, where the water molecules are mostly freed from hydrogen bonding among themselves. Another group of water molecules is in contact with the polar head group of the amphiphile. In the near IR region, the former exhibits an absorption band around 1900 nm and the latter over a range of 1920-2020 nm (24,25).

The features of the near IR spectra presented in Figure 1 may be explained as follows: Two types of water exist in the lecithin reverse micellar solutions; one is water-dispersed in the organic phase, which shows a shoulder at about 1890 nm, and the other is water-solubilized in the reverse micellar interior, which shows a broad band at 1930-1960 nm.

The data given above are further utilized to make a quantitative estimate of the relative amount of water present in the bulk organic phase and in the reverse

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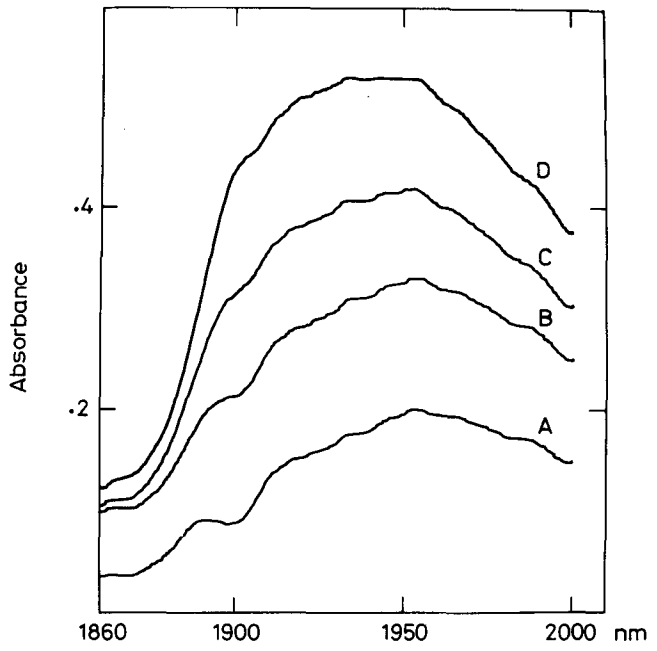

FIG. 1. Near IR spectra of lecithin/benzene/water system at different water concentrations. A, R = 0.17; B, R = 0.34; C, R = 0.66; D, R = 0.99.

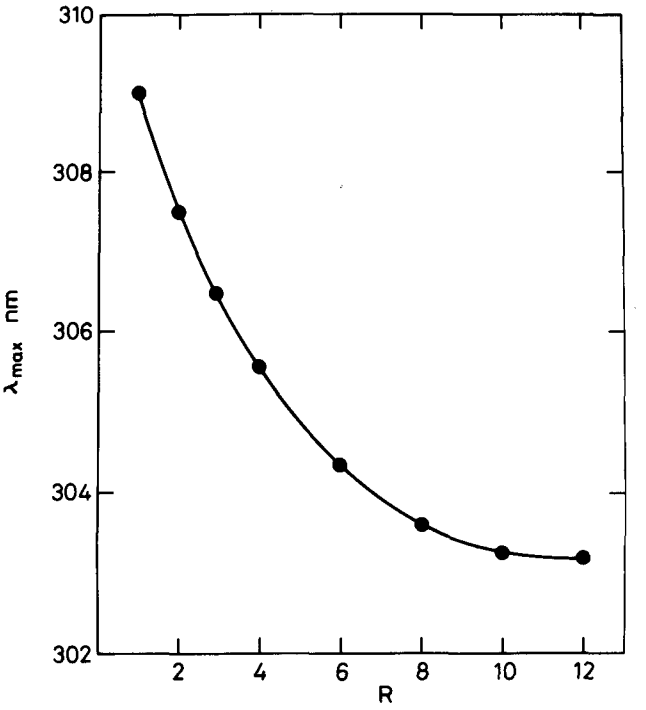

FIG. 2. Variation of λ_{max} of n —— π^* band of NO_3^- ion in lecithin/carbon tetrachloride/water system as a function of added water.

micellar interior (as pools). The relative intensities of the 1890 nm shoulder and 1930–1960 nm band assessed from expanded plots should correspond to the ratio of water in the water pools to water in the solvent phase. From this it is estimated that not more than 0.05% (v/v) of water enters the bulk solvent phase at any water concentration in these systems. This means that at water concentrations R > 1, the amount of water distributed in the bulk phase (organic) can be neglected and all the water can be treated as being present in the water pools. These low values for the concentration of water in the bulk phase, while reminiscent of those obtained for dodecylammonium propionate reverse micelles in benzene (25), are much lower than the 2.8% (v/v) obtained for the potassium oleate/hexanol/hexadecane system (26). The above results and arguments are for the benzene solvent system and are qualitatively valid for carbon tetrachloride and cyclohexane solvent systems also.

Polarity probe studies. The nitrate ion has an n-π^* band around 300 nm, which is quite sensitive to the dielectric constant of the solvent used (27). The solvent sensitivity of this band arises from the differences in the H-bonding ability of the ground and excited states of the nitrate oxygen electrons. The solvent shift is therefore a measure of the hydrogen bonding ability of the solvent. Keeping these facts in mind, we have utilized the n-π^* band of the nitrate ion as a probe of the polarity of water pools formed in the three solvent systems that we have studied.

Figure 2 shows the variation in λ_{max} of the n-π^* band of the nitrate ion in carbon tetrachloride system as a function of added water (R). At low R values, the n-π^* band appears around 309 nm, indicating a lower strength of interaction between the pool water and nitrate ion. At higher R values, the strength of the nitrate ion-water pool interaction increases, blue-shifting the band to its normal value of around 301.5 nm, as seen in bulk water (λ_{max} of NO_3^- ion, for comparison, is 309.0 nm in chloroform and 301.5 nm in water [27]). It is clear that the weakened solvent-ion interaction is indicative of a lower effective polarity of the water pool, in comparison with bulk water, since at all times the $(H_2O)/(NO_3^-)$ ratio is at least 50:1. The results of our polarity probe studies are further supported by our fluorescence emission studies presented in the next section. Qualitatively, similar behavior is observed for benzene and cyclohexane systems.

In similar studies, other solvent-sensitive polarity probes have also been utilized. Menger et al. (7) used pyridine 1-oxide, and Fendler and co-workers (8,28,29) utilized 1-ethyl-4-carbomethoxy-pyridinium iodide, vitamin B_{12} and hemin. These organic probes may not report the unperturbed solvent polarity of the entire water pool since these probes are large and are likely to be oriented at the interface. On the other hand, a small ionic inorganic probe such as the one used in our system is expected to dissolve completely in the water pool and report the average polarity of the entire water pool.

Fluorescence probe studies. Since fluorescence emission spectra are very sensitive to the polarity and polarizability of solvent molecules around the chromophore, the fluorescence emission spectra could be used in favorable circumstances to measure the polarity of the binding site (30). ANSA is one of the extensively used fluorescence probes. The emission maxima of ANSA and the quantum yield or efficiency are well correlated with the Kosower solvent polarity index, Z (31,32). The more polar a solvent, the more red-shifted (shift to longer wavelengths) fluorescence emission maxima of ANSA and lower the fluorescence quantum yield.

Viscosity, specifically microviscosity, plays an important role in the quantum yield and λ_{max} of the emission

spectrum (33). If the microviscosity of the monitored environment increases, it effectively freezes the solvent dipole and prevents the solvent dipole from reorienting itself about the excited state of ANSA molecules. This results in an increased overlap between absorption and emission with a concomitant increase in fluorescence quantum yield and a blue shift of the emission spectrum. Fluorescence quantum yields have been utilized in the past to measure the microviscosity of the binding state (34).

Figures 3 and 4 show the fluorescence emission spectra of ANSA at various R values in benzene and cyclohexane systems, respectively. Figure 5 shows the variation in the fluorescence emission maxima of ANSA-dissolved benzene and cyclohexane systems as a function of added water, R.

It can be seen from Figure 5 that, in the benzene system, the emission maxima, λ_{max}, red-shifted upon addition of water. This shift of λ_{max} upon addition of water suggests that polarity of the water pools in which the ANSA molecules are localized increases with increasing concentration of water. The large λ_{max} red-shifts suggest that, at low water concentrations, the polarity of the water pool as a whole is significantly less than that of bulk water. This result further supports our polarity probe studies presented in the previous section.

It can also be observed from Figure 5, however, that in the cyclohexane system, λ_{max} shows a blue shift around an R value of 6. This blue shift indicates that the effective polarity of the water pool decreases around an R value of 6. It is difficult to visualize any mechanism whereby the water pool becomes less polar with increasing water concentration in this region. However, this can be attributed to the formation of a liquid crystalline phase with an increase in microscopic as well as macroscopic viscosity in this region. Indeed, we have shown by a variety of physical as well as ^{1}H and ^{31}P NMR studies (35-37) that the lecithin/cyclohexane/water system exhibits anisotropy around an R value of 6, suggesting the

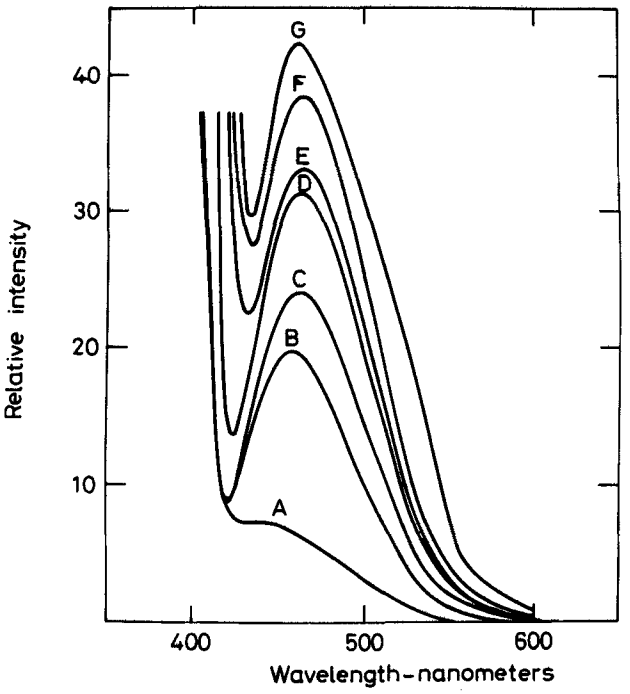

FIG. 4. Fluorescence emission spectra of lecithin/cyclohexane/water system at various concentrations of added water. A, R = 0; B, R = 1; C, R = 2; D, R = 3; E, R = 4; F, R = 5; G, R = 6.

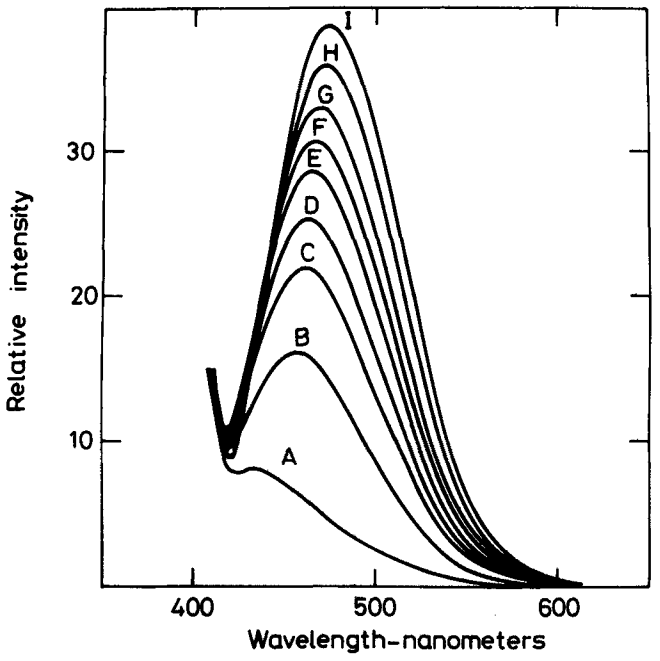

FIG. 3. Fluorescence emission spectra of lecithin/benzene/water system at various concentrations of added water. A, R = 0; B, R = 1; C, R = 2; D, R = 3; E, R = 4; F, R = 5; G, R = 6; H, R = 8; 8, R = 10.

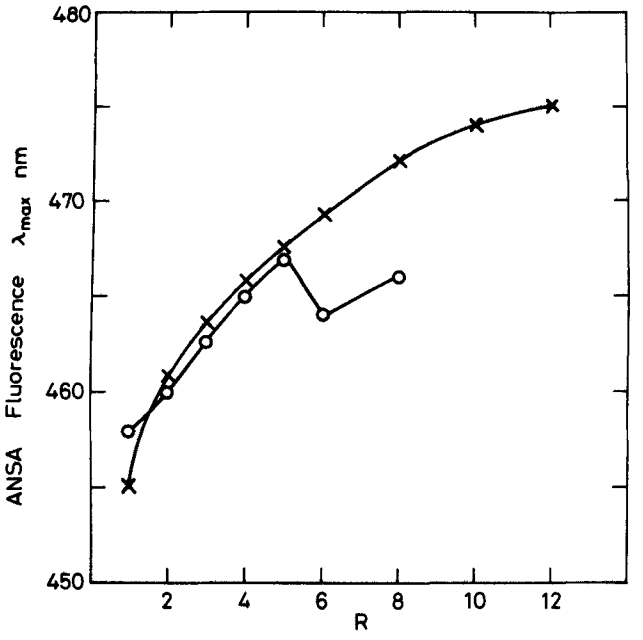

FIG. 5. Variation of fluorescence emission maximum of ANSA in lecithin/benzene/water and lecithin/cyclohexane/water systems as a function of added water. ×, Data obtained for benzene system. ○, Data obtained for cyclohexane system.

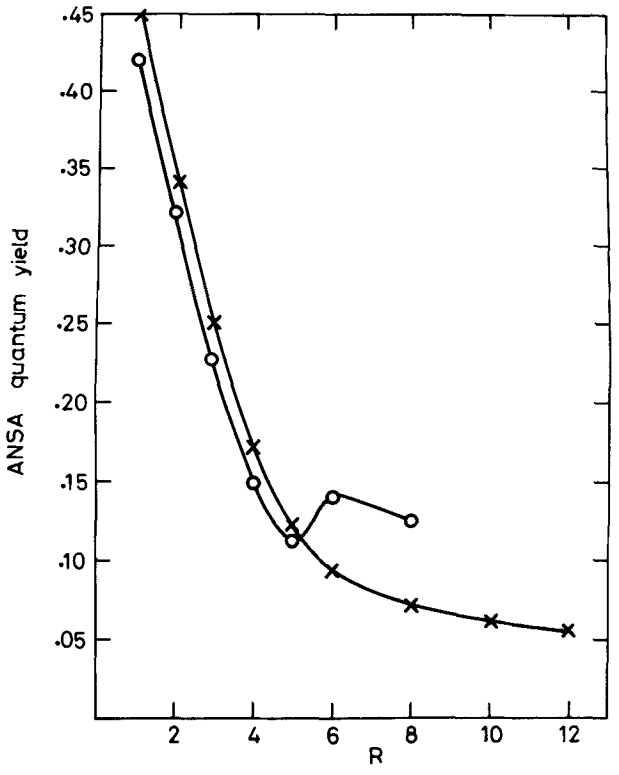

FIG. 6. Variation of quantum yield of ANSA in lecithin/benzene/water and lecithin/cyclohexane/water systems as a function of added water. ×, Data obtained for benzene system. ○, Data obtained for cyclohexane system.

formation of liquid crystalline phase. The relative viscosity of this system is found to increase enormously around an R value of 6 (35).

We have calculated the fluorescence quantum yield of ANSA in benzene and cyclohexane systems as a function of added water by standard methods (23). The results of the quantum yield as a function of R are presented in Figure 6.

It can be seen from Figure 6 that, in the case of the benzene system, the quantum yield continuously decreases with increasing R values. From this it is clear that the polarity of the water pool monitored by ANSA increases with increasing water concentration.

From Figure 6 it may also be noted that, in the case of cyclohexane system, the quantum yield of the fluorescence probe decreases up to an R value of 6. At higher R values, the quantum yield shows an increase. This may be due to the formation of the liquid crystalline phase, with a consequent increase in microviscosity and also macroviscosity (35). This increased microviscosity would indeed effectively freeze out the solvent dipoles and thereby prevent their orientation about the excited state of ANSA molecules (33). The behavior of the CCl_4 system is qualitatively similar to that of the benzene system.

The following can be concluded from our near IR, polarity probe and fluorescence emission studies: (i) our near IR results show that the amount of water present in the bulk organic phase is negligible at all water concentrations studied; (ii) our polarity probe and fluorescence emission studies not only complement each other, but reveal that the polarity of water pool in these systems is much lower than that of bulk water; (iii) our polarity probe studies indicate only a weak interaction between the water pool and the nitrate ion at low water concentrations.

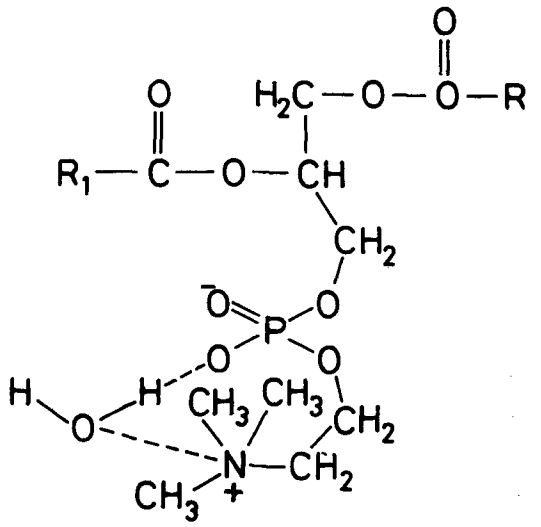

FIG. 7. Interaction of water with the polar head group of lecithin.

Our extensive proton NMR spin-lattice relaxation (T_1) studies (37) on these systems indicate that one water molecule is tightly bound to the polar head group at all water concentrations. Our conclusion is in excellent correspondence with the deuterium NMR (T_1) result of Fung and McAdams (17) as well as the proton NMR line width and infrared studies of Davenport and Fisher (16). Both these research groups have shown that one mole of water is tightly bound to one mole of lecithin head group. Most likely, the binding of one water molecule to the zwitterionic head group could be represented as shown in Figure 7 by a weak hydrogen bonding.

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Species Differences in Lipid Peroxide Levels in Lung Tissue and Investigation of Their Determining Factors

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Marked species differences in thiobarbituric acid reactant value (TBA value) in normal lung tissue of five species of animals were found. The order of the values was mouse > hamster > rat > guinea pig > rabbit, and the value for mice was 3.6 times higher than that for rabbit. The vitamin E (VE) and nonprotein sulfhydryls (NPSH) contents in lungs varied widely among the five animal species. Species differences were also observed on polyunsaturated fatty acid composition in lung phospholipids. The peroxidizability index (PI), which shows the relative rate of peroxidation reaction, was calculated from the composition ratio and the reactivity of each polyunsaturated fatty acid, and the PI was found to be significantly correlated to the TBA value in lungs ($r = 0.853$, $p < 0.001$). The PI value was normalized by the contents of VE and/or NPSH. Finally, the log-value of PI, normalized by the log values of the reciprocals of VE and NPSH, log(PI/VE·NPSH), showed the highest correlation coefficient ($r = 0.907$, $p < 0.001$). Normalization by the activities of antioxidative protective enzymes in lungs did not show any significant correlation against TBA value. These results suggest that TBA value as an index of lipid peroxides in the lungs of animals may be regulated mainly by the contents of VE and NPSH, the composition ratio and the reactivity of each polyunsaturated fatty acid in lung phospholipid fraction.
Lipids 21, 769–775 (1986).

The lung is a primary target organ for high concentrations of oxygen and air pollutants. It is well known that various species of animals (1,2) and strains of mouse (2–4) show different susceptibilities to oxidant gases such as nitrogen dioxide or ozone. One mechanism by which damage with these air pollutants is initiated may be lipid peroxidation (5–7). In vivo lipid peroxidation has been said to be an important parameter in damage to cells by oxygen (8,9), air pollutants (7,10–12), some phases of atherosclerosis (13), some forms of liver injury (14), damage to cells by drugs (15) and in aging (16,17).

Lipid peroxidation in lungs is of particular interest since the lung is the point of entry for oxidant gases contaminating the atmosphere. Kornbrust and Mavis (18) reported that rates of in vitro lipid peroxidation (as measured by thiobarbituric acid [TBA] reaction) of lung microsomes varied widely among species of animals, and the order of the lipid peroxidation rate in lungs was mouse > human > rat > rabbit. They also noted that the ratio of vitamin E (VE) to peroxidizable polyunsaturated fatty acids (PPUFA) in lung microsomes of four animals varied in the reverse order to in vitro lipid peroxidation, which accounted for the relative resistance of lung to lipid peroxidation (18). However, the expression "peroxidizable polyunsaturated fatty acids" cannot reflect the reactivity of PPUFA with different numbers of double bonds in their structure. In addition, little information is available regarding species differences of in vivo lipid peroxidation in lungs.

We found that in vivo lipid peroxidation, expressed as TBA value, and the related factors in lungs varied widely among many species of animals. Lipid peroxide determining factors, which reflect the composition and reactivity of each polyunsaturated fatty acid and the contents of the antioxidants, are discussed.

MATERIALS AND METHODS

Animals. Male animals from five species—ICR mouse, Golden hamster, Wistar rat, Hartley guinea pig and New Zealand White rabbit—were used in this experiment. The mice, hamsters and rats were purchased at the age of six weeks from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka City, Japan) and were fed a commercial stock diet (CE-2) obtained from Japan Clea Co. Ltd. (Tokyo, Japan) (JCL) for four weeks. After four weeks, body weights of mice, hamsters and rats were 36.4 ± 2.4 g, 107 ± 8 g and 346 ± 28 g, respectively. The stock diet for mouse, hamster and rat contained 3.5% crude lipids, 4.5% crude fiber, 24% crude protein, 56% sucrose and 6% water. The fatty acid composition in the crude lipids was 18.3, 21.0, 54.8 and 2.8% for palmitate, oleate, linoleate and linolenate, respectively. Vitamin E and A contents in the diet were 23 mg and 10,000 IU/kg diet, respectively.

Guinea pigs and rabbits were purchased from the same company. They were fed a commercial stock diet (RC4) from the Oriental Yeast Industry Co. Ltd. (Tokyo) for four weeks, at which time body weights of guinea pigs and rabbits were 517 ± 53 g and 3.47 ± 0.17 kg, respectively. The stock diet for guinea pig and rabbit contained 2.7% crude lipids, 13.3% crude fiber, 21.4% crude protein, 46.6% sugar and 7.4% water. Vitamin E and A contents in the diet were 8.8 mg and 2150 IU/kg diet, respectively.

All animals were subjected for four weeks to a daily cycle of 14 hr of light and 10 hr of darkness in a room kept at a constant temperature of 24–26 C with a relative humidity of 50–70%. They were killed by removing sufficient blood from the neck artery under anesthesia with diethyl ether. The left part of the lungs was removed, and the right part of the lungs was perfused via the right ventricle with deaerated and N_2-bubbled saline. The right part of the lungs was rinsed with saline and stored at −80 C until assay in vials purged with nitrogen. Half of the left part of lungs was used immediately after sacrifice for the assay of α-tocopherol and nonprotein sulfhydryls (NPSH). The sample number was six for each species. One sample consists of the pooled lungs from eight mice or two hamsters, respectively. The other sample of rat, guinea pig and rabbit consists of a part of lung obtained from one animal.

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Assays of α-tocopherol and NPSH. Half of the left lung lobe of each animal was homogenized in a glass-Teflon homogenizer under N_2, and a 10% homogenate was made with 50 mM Na, K-phosphate buffer (pH 7.5), which was deaerated with degasser (Erma Optical Works Ltd., (Tokyo, Japan) ERC-3310) and bubbled with N_2. The homogenate was centrifuged at 200 × g for 5 min, and the pellet fraction was washed with 1/4 volume of the same buffer. The supernatants were combined. Approximately 87% or more proteins were recovered into 200-×-g supernatant. The 200-×-g supernatant fraction was used for the assay of α-tocopherol and NPSH. The former was measured by the high performance liquid chromatography method of Abe et al. (19). α-Tocopherol was extracted from 1 ml of the supernatant with 5 ml of n-hexane after addition of 1 ml of ethanol. This extraction was repeated once again, and the combined n-hexane extracts were evaporated under N_2. The residue was dissolved in n-hexane, and the aliquot was injected into Finepak SIL-NH_2 column (Nihon Bunkô Co. Ltd., Tokyo, Japan). The solvent for separation was n-hexane/isopropyl ether/acetic acid (90:10:0.5, v/v/v). The eluted tocopherols were determined using a fluorometer (Ex 298 nm; Em 325 nm). NPSH in this 200-×-g supernatant and heparinized blood were determined by the method of DeLucia et al. (20). Proteins in the assay sample were removed by addition of 10% trichloroacetic acid solution. The deproteinized sample was assayed according to 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) assay.

Assays of lipid peroxides and antioxidative protective enzyme activities. The perfused right lung was used for measurements of TBA reactants and the activities of antioxidative protective enzymes. The lung was homogenized and the 200-×-g supernatant fraction was obtained by the method mentioned above. The supernatant was used for determination of TBA reactants as an index of lipid peroxides. The TBA reaction was performed by the method of Ohkawa et al. (21): The 200-×-g supernatant fraction (0.2 ml) was mixed with 8% sodium dodecylsulfate (0.2 ml), 20% acetate buffer, pH 3.5 (1.5 ml), and 5% aqueous solution of TBA reagent. After heating at 95 C for 60 min, the pink pigment produced was extracted with n-butanol (5 ml). The pink pigment was measured fluorometrically at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. An aliquot of 200-×-g supernatant was centrifuged at 10,000 × g for 10 min, and the supernatant was again centrifuged at 105,000 × g for 60 min. The supernatant fraction obtained was used for the measurements of activities of glutathione peroxidase (GPx), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) and glutathione S-transferase (GSH-tase). An aliquot of the supernatant was dialyzed against 50 mM Na, K-phosphate buffer (pH 7.5) containing 0.1 mM EDTA for 20 hr at 4 C; the dialysis buffer was changed twice. The dialyzed supernatant was used for assays of superoxide dismutase and disulfide reductase (DSR).

Activities of the enzymes, except DSR, were measured by a JEMSAC (General Medical Science/Atomic Energy Commission) autoanalyzer. Enzyme activity was a linear function of time and protein concentration at 30 C. GPx was measured by the method of Little and O'Brien (22) using cumene hydroperoxide and hydrogen peroxide as the substrates. GR was assayed by the method of Bergmeyer (23). G6PD was measured by the method of Wilhelm and Waller (24). 6PGD was measured in the same way as G6PD, using 0.1 M Tris-HCl buffer (pH 8.5) containing 20 mM $MgCl_2$ and 6.5 mM cysteine, with 6-phosphogluconate as the substrate. GSH-tase was measured by the method of Habig et al. (25). 1-Chloro-2,4-dinitrobenzene was used as a substrate. Superoxide dismutase was measured in terms of its ability to prevent the reduction of cytochrome c by superoxide anion radicals produced by the xanthine-xanthine oxidase system according to the method of McCord and Fridovich (26). DSR was measured by the method of Tietze (27). Cystine was used as a substrate, and cysteine formed by the enzymatic reaction was determined by the method of DeLucia et al. (20).

Extraction and analysis of lung lipids. Three-hundred mg of nonperfused left lung was homogenized with a polytron tissue disrupter (Kriens Co. Ltd., Luzern, Switzerland, CH-6010 type) in 2 ml chloroform/methanol (2:1, v/v) containing 0.005% (w/v) butylhydroxytoluene (BHT) as antioxidant under an N_2 atmosphere. This homogenate was centrifuged at 3,500 rpm for 10 min at 4 C. The tissue residue was extracted once more with 4 ml chloroform/methanol (2:1, v/v), and the chloroform layer was separated. The two extracts were combined. Removal of nonlipid components contaminating the extracts was done according to the method of Folch et al. (28). This semipurified lipid was dissolved in 5 ml chloroform/methanol (2:1, v/v); 0.05 ml of this sample was used to determine total phospholipids by the method of Rouser (29). The remaining extracts were stored at −20 C until analysis.

The lipids were separated by thin layer chromatography on Kieselgel 60 (Merck Ltd., Darmstadt, Germany). *n*-Hexane/ethyl ether/acetic acid (80:40:1, v/v/v) and chloroform/methanol/acetic acid/H_2O (50:30:8:1, v/v/v/v) were used as developing solvents for the separation of total phospholipids and individual phospholipid classes, respectively. Concentrations of individual phospholipids were calculated from total lipid phosphorus and percentage distributions of phosphorus in the isolated phospholipid classes.

Total phospholipids were hydrolyzed to free fatty acids in 0.5 N NaOH in methanol, and free fatty acids (in the presence of Keiselgel) were esterified in 14% BF_3 in methanol. The fatty acid methyl esters were separated by gas liquid chromatography (Shimadzu GC-7A). Shinchrome E-71 (5% on 80/100 mesh Shimalite) was used as the stationary phase in a 3 m × 3 mm ID glass column with nitrogen as carrier gas. The temperature was set at 230 C. The detector linearity was checked using commercially available mixed standards of fatty acid methyl esters. Peak area measurements made with an electronic integrator (Shimadzu Chromatopack C-R1B) were used for quantitation.

Statistical method. The results of this study are presented as mean ±SD. Student's t-test at the 0.05 significance level was used to examine the statistical significance of the mean responses between mouse and the other animals.

RESULTS

TBA values, α-tocopherol and NPSH contents in lung tissue. TBA values, α-tocopherol and NPSH contents in the lungs of the five animal species are shown in Table 1.

TABLE 1

TBA Values as an Index of Lipid Peroxides, α-Tocopherol, Nonprotein Sulfhydryl and Protein Contents in Lungs of Mouse, Hamster, Rat, Guinea Pig and Rabbit

Animals	TBA value[a] (nmol/mg·prot)	α-Tocopherol[a] (μg/mg·prot)	Nonprotein sulfhydryl[a] (nmol/mg·prot)	Protein[b] (mg/g·lung)
Mouse	1.336 ± 0.123	0.116 ± 0.008	14.8 ± 2.6	51.7 ± 2.7
Hamster	1.056 ± 0.107	0.157 ± 0.014	20.9 ± 5.1	48.9 ± 2.0
Rat	0.643 ± 0.103	0.335 ± 0.029	18.1 ± 1.7	60.0 ± 4.5
Guinea pig	0.573 ± 0.076	0.181 ± 0.011	52.7 ± 7.6	47.6 ± 3.6
Rabbit	0.375 ± 0.074	0.267 ± 0.065	40.1 ± 6.7	48.2 ± 7.1

[a]All values significantly different from mouse values.
[b]Value of rat is different from all other animal values.

TABLE 2

Fatty Acid Compositions and Peroxidizability Index (PI) in Lung Phospholipids of Mouse, Hamster, Rat, Guinea Pig and Rabbit

	Mouse	Hamster	Rat	Guinea pig	Rabbit
14:0	1.86 ± 0.08	1.37 ± 0.11	2.66 ± 0.15	3.15 ± 0.21	2.41 ± 0.55
16:0	37.60 ± 0.44	34.14 ± 0.47[a]	34.83 ± 0.44[a]	34.60 ± 1.16[a]	34.39 ± 1.88[b]
16:1	6.04 ± 0.10	4.75 ± 0.08[a]	5.16 ± 0.17[a]	4.02 ± 0.45[a]	3.46 ± 0.45[a]
18:0	11.01 ± 0.09	11.46 ± 0.21[a]	11.79 ± 0.15[a]	11.52 ± 0.35[b]	10.56 ± 0.75
18:1	10.04 ± 0.18	15.33 ± 0.22[a]	11.67 ± 0.11[a]	17.71 ± 0.49[a]	18.01 ± 0.21[a]
18:2	7.01 ± 0.15	10.40 ± 0.18[a]	7.99 ± 0.19[a]	9.91 ± 0.87[a]	13.43 ± 1.32[a]
20:4	8.78 ± 0.07	8.99 ± 0.19[c]	11.59 ± 0.27[a]	8.37 ± 0.39[c]	9.06 ± 0.64
22:4	1.82 ± 0.06	1.72 ± 0.06[c]	2.41 ± 0.09[a]	1.49 ± 0.12[a]	0.90 ± 0.05[a]
22:5	2.84 ± 0.07	2.76 ± 0.11	2.85 ± 0.07	1.96 ± 0.15[a]	1.22 ± 0.12[a]
22:6	6.71 ± 0.06	2.97 ± 0.09[a]	2.42 ± 0.07[a]	N.D.	N.D.
PI	129.2 ± 1.9	104.6 ± 1.4	110.8 ± 2.8	72.2 ± 3.5	69.7 ± 3.3

[a]Significantly different from mouse values, $p < 0.001$.
[b]Significantly different from mouse values, $p < 0.01$.
[c]Significantly different from mouse values, $p < 0.05$.

Marked species differences were found in TBA values. The order of the value was mouse > hamster > rat > guinea pig > rabbit; this order varied inversely to the body size of each species. The value for mouse was 3.6 times that for rabbit, but the ratio of body size between rabbit and mouse is far higher. The order of α-tocopherol content was mouse < hamster < guinea pig < rabbit < rat. It is characteristic that the α-tocopherol content for rats is the highest. The content in rat lungs was 2.9 times that in mice. The order of NPSH content was mouse < rat < hamster < rabbit < guinea pig. The NPSH content for guinea pigs was 3.6 times that for mice. These two antioxidants showed an inverse relationship to TBA values with correlation coefficients of -0.549 ($p < 0.02$) and -0.726 ($p < 0.001$), respectively.

The lungs of various animals contained an increment of NPSH due to blood in the tissue, because they were determined using nonperfused lungs. NPSH contents in the lungs originating in blood were estimated by the assays of hemoglobin and NPSH contents in blood and in the 200-×-g supernatant fraction of lung homogenate, respectively, using mouse, hamster, rat and guinea pig. Percentages of 14.8 ± 0.9, 12.5 ± 1.7, 14.1 ± 1.0 and 13.0 ± 1.2 of NPSH in the lungs were originated from blood of mouse, hamster, rat and guinea pig, respectively. These percentages are not negligible, but there are not significant differences among the four species of animals. Furthermore, NPSH contents in blood of mouse, hamster, rat and guinea pig were 13.3 ± 0.4, 12.3 ± 0.2, 15.3 ± 0.2 and 17.7 ± 1.5 nmol/mg·hemoglobin. The differences among the values in blood of four animals are not very significant in view of the marked species differences observed in the lungs of four animals.

Fatty acid composition and peroxidizability index in lung phospholipids. The fatty acid composition and peroxidizability index (PI) in lung phospholipids of the five animal species are shown in Table 2. The composition of polyunsaturated fatty acids varied noticeably among the five animal species, although the variety of saturated fatty acid composition was not so marked. The compositions of PPUFA, such as docosatetraenoic ($C_{22:4}$), docosapentaenoic ($C_{22:5}$) and docosahexaenoic ($C_{22:6}$) acids, showed marked species differences (Fig. 1). Differences in the content of docosahexaenoic acid ($C_{22:6}$), which is most peroxidizable, were most notable and were related inversely with TBA values in the lungs of the five animal species.

TABLE 3

Total Phospholipid Contents and the Composition of Individual Phospholipid Classes in Lungs of Mouse, Hamster, Rat, Guinea Pig and Rabbit

	Mouse	Hamster	Rat	Guinea pig	Rabbit
Lysophosphatidylcholine	1.7 ± 0.2	1.3 ± 0.1[a]	1.9 ± 0.1	0.8 ± 0.1[a]	0.9 ± 0.4[b]
Sphingomyelin	9.5 ± 0.2	10.4 ± 0.3[a]	10.2 ± 0.1[a]	11.2 ± 0.3[a]	11.7 ± 0.8[a]
Phosphatidylcholine	46.9 ± 0.4	47.4 ± 0.4	47.1 ± 0.6	46.2 ± 0.3[b]	47.0 ± 3.0
	0.7 ± 0.3	0.5 ± 0.2	1.3 ± 0.1	0.3 ± 0.1	0.7 ± 0.2
Phosphatidylinositol + phosphatidylserine	12.7 ± 0.2	12.9 ± 0.2	12.5 ± 0.2	13.1 ± 0.5	13.0 ± 0.8
Phosphatidylethanolamine	21.9 ± 0.6	21.9 ± 0.3	21.6 ± 0.3	22.3 ± 0.4	21.3 ± 1.5
	4.5 ± 0.4	4.1 ± 0.4	3.7 ± 0.3	3.5 ± 0.3	2.8 ± 0.6
	1.8 ± 0.2	1.6 ± 0.1	1.6 ± 0.1	2.5 ± 0.4	2.3 ± 0.4
Solvent front	0.5 ± 0.2	0.1 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.5 ± 0.4
	(% ratio of total phosphorous of lung lipid)				
Total phosphorous (mmol/g·lung)	39.2 ± 1.2	35.8 ± 1.9	33.6 ± 0.6	34.6 ± 1.4	35.7 ± 4.5

[a]Significantly different from mouse values, $p < 0.001$.
[b]Significantly different from mouse values, $p < 0.01$.
[c]Significantly different from mouse values, $p < 0.05$.

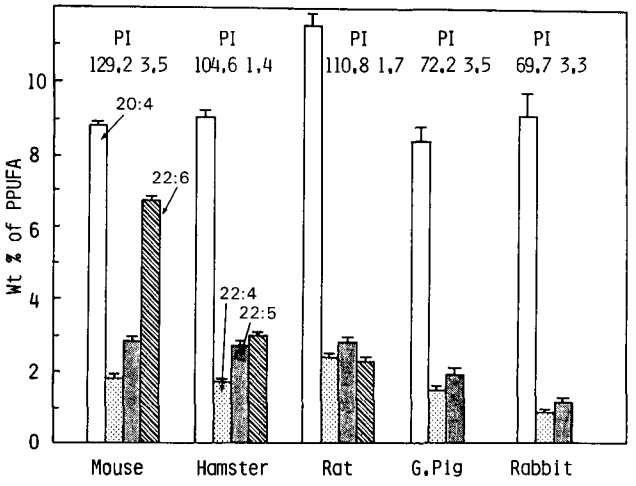

FIG. 1. Comparison of polyunsaturated fatty acid compositions in lung phospholipids of mouse, hamster, rat, guinea pig and rabbit. 20:4, Arachidonic acid; 22:4, docosatetraenoic acid; 22:5, docosapentaenoic acid; 22:6, docosahexaenoic acid.

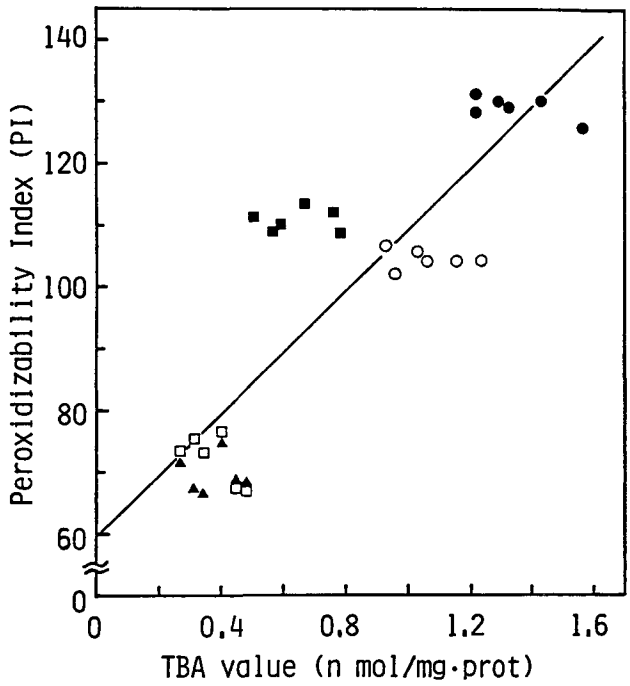

FIG. 2. Relationship between TBA values and peroxidizability index in lung phospholipids of five animal species. Correlation coefficient (r) was 0.853 ($p < 0.001$). ●, Mouse; ○, hamster; ■, rat; □, guinea pig; ▲, rabbit.

The change of arachidonic acid ($C_{20:4}$) was not so striking, although the value for rats was relatively higher than that for the other four animals. The PI was calculated from the following equation (30): PI = (% monoenoic × 0.025) + (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 4) + (% pentaenoic × 6) + (% hexaenoic × 8). This PI value reflects the relative rate of peroxidation, because the PI is calculated from the composition ratio and reactivity of each fatty acid. The PI value for mouse was roughly twice that for rabbit. The PI for the five animal species showed a significant relationship with the TBA values of each animal, as shown in Figure 2. The correlation coefficient (r) was 0.853 ($P < 0.001$).

Total phospholipid contents and the composition of individual phospholipid classes in lungs. The total phospholipid contents and composition of individual phospholipid classes are shown in Table 3. These parameters did not show any significant relationship with TBA values among the five animals.

Activities of antioxidative protective enzymes. Enzyme activities of GPx system in lungs are shown in Table 4, and the activities of GSH-tase, superoxide dismutase and DSR are shown in Table 5. The activities of antioxidative protective enzymes also varied widely among the five

TABLE 4

Enzyme Activities of Glutathione Peroxidase System in Lungs of Five Animal Species

Enzyme	Animal	Mean ± SD	%[a]
G6PD[b]	Mouse	213 ± 16	100
	Hamster	127 ± 9[c]	66
	Rat	108 ± 31[c]	51
	Guinea pig	208 ± 38	98
	Rabbit	115 ± 33	54
6PGD[b]	Mouse	123 ± 11	100
	Hamster	140 ± 8[e]	114
	Rat	128 ± 11	104
	Guinea pig	181 ± 28[d]	147
	Rabbit	44 ± 8[c]	36
GR[b]	Mouse	300 ± 13	100
	Hamster	252 ± 17[c]	84
	Rat	191 ± 14[c]	64
	Guinea pig	324 ± 39	108
	Rabbit	260 ± 8[c]	87
GPx-cumene·OOH[b]	Mouse	197 ± 13	100
	Hamster	64 ± 5[c]	32
	Rat	106 ± 11[c]	54
	Guinea pig	25 ± 4[c]	13
	Rabbit	149 ± 16[c]	76
GPx-H_2O_2[b]	Mouse	57.1 ± 1.9	100
	Hamster	14.1 ± 4.6[c]	25
	Rat	61.3 ± 9.0	107
	Guinea pig	N.D.	0
	Rabbit	128.9 ± 15.3[c]	226

G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; GR, glutathione reductase; GPx-cumene·OOH, glutathione peroxidase measured using cumene hydroperoxide as a substrate; GPx-H_2O_2, glutathione peroxidase measured using hydrogen peroxide as a substrate.

[a]Values show percent ratio against the value of mouse.

[b]Values are expressed as nmol of NADPH formed or reduced/mg·protein/min.

[c]Significantly different from mouse value, $p < 0.001$.

[d]Significantly different from mouse values, $p < 0.01$.

[e]Significantly different from mouse values, $p < 0.05$.

TABLE 5

Activities of Glutathione S-transferase (GSH-tase), Superoxide Dismutase (SOD) and Disulfide Reductase (DSR) in Lungs of Five Animal Species

Enzyme	Animal	Mean ± SD	%[a]
GSH-Tase[b]	Mouse	1.46 ± 0.05	100
	Hamster	0.41 ± 0.03[c]	28
	Rat	0.14 ± 0.01[c]	10
	Guinea pig	1.54 ± 0.23	105
	Rabbit	0.62 ± 0.04[c]	42
SOD[d]	Mouse	55.7 ± 6.5	100
	Hamster	121.2 ± 48.4[e]	218
	Rat	63.9 ± 5.2[e]	115
	Guinea pig	43.1 ± 13.5	77
	Rabbit	30.9 ± 2.8[c]	55
DSR[f]	Mouse	115 ± 13	100
	Hamster	236 ± 37[c]	205
	Rat	277 ± 29[c]	241
	Guinea pig	157 ± 18[g]	137
	Rabbit	63 ± 15[c]	55

[a]Values show percent ratio against the value of mouse.

[b]Values are expressed as μmol of metabolites formed/mg·protein/min. Metabolite shows the reactant from 1-chloro-2,4 dinitrobenzene.

[c]Significantly different from mouse value, $p < 0.001$.

[d]Values are expressed as unit/mg·protein. One unit is defined from the calculation by equation $(V-v)/v$. V shows the reaction rate (OD/min) without SOD sample in reaction mixture, and v shows the reaction rate with SOD sample in reaction mixture.

[e]Significantly different from mouse value, $p < 0.05$.

[f]Values are expressed as nmol of cysteine formed/mg·prot/hr.

[g]Significantly different from mouse value, $p < 0.01$.

animals, and species differences of GPx and GSH-tase were especially notable.

If the activity of glutathione peroxidase was measured using cumene hydroperoxide as a substrate (GPx-cumene·OOH), the value for mouse was 8.6 times that for guinea pig. If the activity was measured using hydrogen peroxide as a substrate (GPx-H_2O_2), the value for rabbits was nine times that for hamsters. It is characteristic that the activity of this enzyme is deficient in guinea pig. Furthermore, the activity of GSH-tase in mouse was 11 times that in rat. Although many enzyme activities in mouse were generally higher than those in other animals, the TBA value in mouse was the highest among the five animal species. On the other hand, none of the antioxidative protective enzymes showed significant relationships to TBA values.

DISCUSSION

In the present study, marked species differences for TBA values in the lung tissue of five normal animals were found, and it is proposed that four factors—the composition ratio and reactivity of each polyunsaturated fatty acid in phospholipids and the contents of VE and NPSH—were important determiners for TBA values.

Within the data cited in Results, the highest correlation coefficient was observed between TBA value and PI value ($r = 0.853$, $P < 0.001$). This evidence shows that lipid peroxide levels in the lungs of the five animal species are regulated mainly by the composition ratio and the reactivity of each polyunsaturated fatty acid. However, it is well known that VE is concerned in the determination of lipid peroxide levels in many tissues, and numerous studies have indicated that lipid peroxidation is greatly influenced by VE levels in tissues (31).

Bieri and Anderson (32) demonstrated that the ability of tissue homogenates to undergo in vitro lipid peroxidation was inversely related to the dietary VE status of the animal. Tappel's group (33) also demonstrated that breath pentane levels, as an index of lipid peroxides in the whole body, were inversely proportional to the log of dietary VE concentration. Furthermore, it is well known that VE levels in tissue vary with the log of dietary VE levels (34). Kornbrust and Mavis (18) reported that the ratio of VE per PPUFA showed an inverse relationship with TBA value in in vitro experiments with lung microsomes. Therefore, investigation of the VE content in tissue as a determining factor of TBA value as an index of lipid peroxidation is very important. In the present study, the

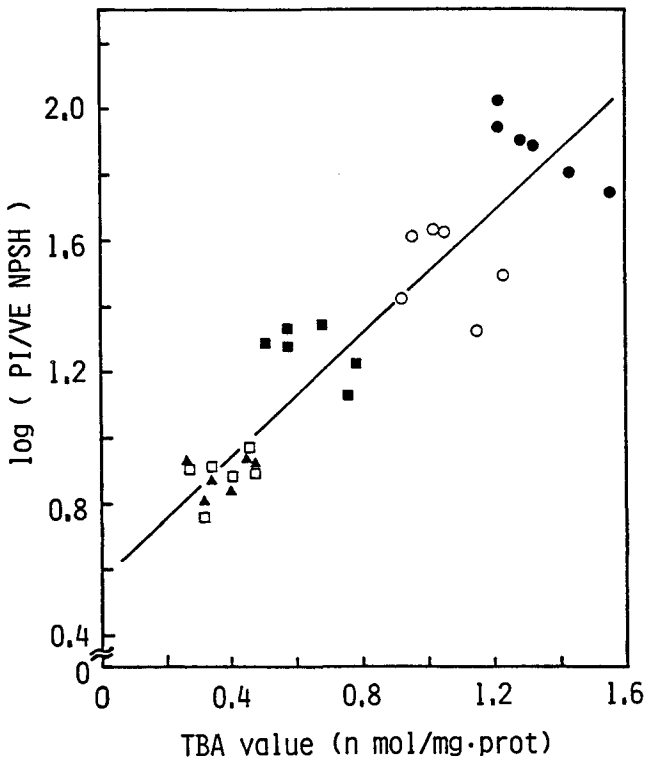

FIG. 3. Relationship between TBA values and log (PI/VE·NPSH) of five animal species. Correlation coefficient (r) was 0.907 ($p < 0.001$). ●, Mouse; ○, hamster; ■, rat; □, guinea pig; ▲, rabbit.

correlation coefficient (r) of PI/VE with TBA value raised to 0.876 ($p < 0.001$).

To obtain a higher correlation coefficient, we tried a normalization of PI/VE by other factors, including the activities of the antioxidative protective enzymes. As a result, the log value of PI normalized by the reciprocals of VE and NPSH, log (PI × 1/VE × 1/NPSH), showed a higher correlation coefficient ($r = 0.907$, $P < 0.001$) than that of PI/VE, as shown in Figure 3. At least 90% of the NPSH in lung is reduced glutathione (GSH). Therefore, the normalization by NPSH might be reasonable, for the following reasons: First, GSH is itself a very important antioxidant and prevents the formation of lipid peroxides (35,36). Second, GSH is an important coenzyme for many glutathione-related enzymes, such as GPx and GSH-tase, which can metabolize lipid peroxides or detoxify foreign compounds (37,38). Third, GSH functions in the maintenance of thiol groups of proteins (39). Fourth, GSH regulates the hexose monophosphate pathway that can produce NADPH, which is important as a reducing agent (40–42). These evidences suggest that GSH plays many important roles in protecting cells from oxidative damages. Therefore, it is reasonable that the normalization of PI/VE by 1/NPSH has the highest correlation coefficient against the TBA value.

The antioxidative protective enzymes examined in the present study are also very important to protect cells from oxidative damages. The GPx system can metabolize lipid peroxides (37). GSH-tase also catalyzes the same net reaction as GPx (25,43). GR, G6PD and 6PGD act to maintain reducing substances such as glutathione and NADPH that are essential for normal cell function and for protection of cells against oxidative damages. Superoxide dismutase provides the basic defense against the potential cytotoxic reactivities of superoxide anion radicals (26). An increase of superoxide dismutase activity in lungs may represent adaptive changes that reduce oxidative damage, one of which may be formation of mixed disulfide. The disulfide formed by oxidant gases is reduced to each free sulfhydryl by DSR. Therefore, DSR also plays an important role in the reduction of the mixed disulfide produced by oxidative stress (27).

In spite of the important roles of these protective enzymes, TBA values in the lungs of five animals were not related to the protective enzymes. Though many enzyme activities in mouse were generally higher than those in other animals, the TBA value in mouse was the highest among the five animal species. These results suggest that the antioxidative protective enzymes are not related directly with TBA values in the lungs of various animals.

On the other hand, the formation of lipid peroxides is also influenced by the enzymatic systems in biological membranes such as lysosomes (44), microsomes (45,46) and mitochondria (47). Future investigation of these enzymatic systems on lipid peroxidation might lead to more detailed clarification of the lipid peroxide determining factors. Furthermore, it is well known that oxygen consumption per g tissue in small animals is greater than that in large animals (48). Therefore, the TBA value observed in the lungs may relate to the metabolic oxygen consumption of each animal. The relationship is a subject for future investigation.

It is well known that fatty acid composition in each tissue varies with diet and VE content. In the present study, we used two diets in which the VE content and fatty acid composition were different. One diet was for mice, hamsters and rats; the other was for guinea pigs and rabbits. We believe that the same diet should be used for all animals in such an experiment. However, in an experiment to examine the species difference of susceptibility to many drugs or toxic agents under usual breeding conditions (except for the examination of nutritional effects), it is very difficult to use the same diet for all animals. This is a common problem for experiments aimed at extrapolating the effects on experimental animals to humans, because it is impossible to control human diet under usual human life styles. The VE content in lung tissue did not exactly reflect the VE content in diet among the different animal species; the VE content in the lungs of mice and hamsters was lower than that of guinea pigs and rabbits (Table 1), although the VE content in the diet for mice and hamsters was 2.6 times that for guinea pigs and rabbits. There were also large differences in fatty acid composition, especially polyunsaturated fatty acid, among mice, hamsters and rats, as well as between guinea pigs and rabbits (Fig. 1). Therefore, we emphasize that there are genetic species differences in lipid peroxide levels, and that their determining factors can be accounted for by genetic as well as nutritional factors.

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Specific Induction of Lauric Acid ω-Hydroxylase by Clofibrate,diethylhexyl-phthalate and 2,4-Dichlorophenoxyacetic Acid in Higher Plants

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Recently, we have found in plant microsomes two laurate hydroxylases that catalyze the terminal hydroxylation or the in-chain hydroxylation of the fatty acid. These two hydroxylases, which are both cytochrome P-450 enzymes, are never found in the same plant. This study shows that the hypolipidemic drug clofibrate induces the lauric acid ω-hydroxylase activity in *Vicia* and soybean seedlings. The marked increase in activity (>20-fold) produced by clofibrate was dose-dependent but was not paralleled by an enhancement of bulk cytochrome P-450 or cinnamic acid 4-hydroxylase. Compounds related to clofibrate by structure (2,4-dichlorophenoxyacetic acid) or effects (diethylhexyl-phthalate) also stimulated the ω-hydroxylating system in these plants. In contrast, the lauric acid in-chain hydroxylase from Jerusalem artichoke tubers was induced less than cinnamic acid 4-hydroxylase activity and bulk cytochrome P-450 in tissues incubated in clofibrate solution. This suggests that clofibrate induces preferentially the laurate ω-hydroxylating isozyme in plants.
Lipids 21, 776–779 (1986).

Lauric acid is hydroxylated in Jerusalem artichoke tubers, tulip bulbs, maize seedlings and several other plants (1) by an in-chain hydroxylase producing a mixture of ω-2, ω-3 and ω-4 monohydroxylaurates (1,2). We have recently reported the presence in other plants, mainly leguminosae, of a laurate ω-hydroxylase hydroxylating exclusively the methyl terminus of the molecule (3). The two activities never coexist in the 12 plant species previously analyzed (1) and are supported by different cytochrome P-450 species.

The generally low level of cytochrome P-450 in most plants is significantly increased by exposure to various xenobiotics (4), endobiotic substrates (5), fungi infections (6), light irradiation (7,8), wounding of the tissues and subsequent aging (6,9). We have shown that 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic plant hormone, increased the bulk of cytochrome P-450 (spectrophotometrically detectable) in Jerusalem artichoke tuber tissues (10). Similarly, cytochrome P-450 content and more specifically the activities of the lauric acid in-chain hydroxylase and ω-hydroxylase were substantially induced by phenobarbital in various plants (1,11).

Clofibrate (ethyl 2-[4-chlorophenoxy]-2-methylpropanoate) is a hypolipidemic drug causing a proliferation of mitochondria, smooth endoplasmic reticulum and peroxisomes in mammalian liver (12). Induction of cytochrome P-450 (13) and, more specifically, of lauric acid ω-hydroxylase activity (14) was observed as well as several other metabolic modifications (15–18). Similar symptoms are elicited by di-(2-ethylhexyl)-phthalate (DEHP), a widely used industrial plasticizer (19,20).

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We here report some effects of clofibrate, DEHP and 2,4-D on cytochrome P-450 content and laurate hydroxylase activities in plant systems.

MATERIAL AND METHODS

Plant material and incubation conditions. Jerusalem artichoke tubers (*Helianthus tuberosus* L. cv. Blanc commun), grown locally, were stored in polyethylene bags at 4 C in darkness. Tubers were sliced (1-mm thick) and washed several times with deionized water.

Vicia sativa L. cv. Septimane, *Vicia faba* L. cv. Partim and *Glycine max* (L.) Merr. cv. Map. arrow seeds were germinated on moist filter paper in the dark at 25 C for 4–5 days.

Ca. 100 g fresh weight of tuber slices or etiolated seedlings (after removal of roots and teguments) were incubated for 48 hr, in the dark at room temperature, in 2-l Erlenmeyer flasks containing 1.5 l distilled water or distilled water plus the compounds to be tested. Clofibrate and DEHP, which are insoluble in water, were emulsified by vigorous shaking. The solutions were continuously bubbled with a filtered and hydrated air stream (4.5 l/min).

Enzyme sources and assays. Plant microsomes were prepared as previously described (9), resuspended in 0.1 M sodium phosphate buffer, pH 7.4, containing 10 mM 2-mercaptoethanol and 30% glycerol (v/v), and stored at −20 C without appreciable loss of activity for several weeks.

Cinnamic acid 4-hydroxylase was measured using the radiochemical method described previously (9). The lauric acid in-chain hydroxylase from Jerusalem artichoke tubers yields three isomers monohydroxylated at the C8 (15%), C9 (60%) and C10 (25%) positions. The lauric acid ω-hydroxylase from *Vicia* and soybean yields 12-hydroxylauric acid (85%) and an unidentified metabolite (15%).

The rate of hydroxylation of lauric acid by in-chain and ω-hydroxylases was measured using the radiochemical method described previously (11), with minor modifications that make it less time-consuming.

Assay for laurate ω-hydroxylase. ω-Hydroxylase was assayed in a final volume of 0.2 ml. The reaction mixture contained 1 mM NADPH, 6.7 mM glucose 6-P, 0.4 unit glucose 6-P dehydrogenase, [1-^{14}C]lauric acid (about 700 × 10^3 CPM) and sodium laurate to a final concentration of 50 μM, 0.1 to 1.0 mg microsomal protein and 0.1 M sodium-phosphate buffer (pH 7.4). Incubations were run for 20 min at 25 C and terminated by the addition of 0.2 ml pure methanol or acetonitrile. After 15 min in ice and a rapid centrifugation at 3000 RPM, an aliquot of the supernatant (0.1–0.2 ml) was spotted directly with authentic 12-hydroxylauric acid (100 μg) on a Silica Gel F 254 plate. The developing solvent was diethyl ether/light petroleum (b.p. 40–60 C)/formic acid (70:30:1,

v/v/v). Radioactive peaks were detected and 12-hydroxylauric acid was revealed by exposure to iodine vapor. Lauric acid (Rf = 0.75) and 12-hydroxylauric acid (Rf = 0.38) were well separated. Radioactive metabolite peaks were scraped into scintillation vials, and radioactivity was measured in a liquid scintillation counter.

Assay for laurate in-chain hydroxylase. The same incubation and product isolation procedures were used to measure in-chain hydroxylase activity. The three hydroxylaurate isomers formed migrate as a single peak (Rf = 0.54), which was scraped off the plate; radioactivity was counted as a whole or individually evaluated by high performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry analysis. The three isomers may be separated by the isocratic HPLC procedure described below.

Instrumentation. High-pressure liquid chromatographic analyses were carried out on a 15-cm × 4.6-mm Beckman Ultrasphere ODS 5-μm C18 reverse-phase column with acetonitrile/water/acetic acid (25:75:0.2, v/v/v) as the elution solvent (2 ml/min). The eluent was monitored with an Isomess Ramona-D radioactivity detector equipped with a solid scintillation cell.

Figure 1 shows a typical HPLC separation of 10-hydroxylauric (**a**), 9-hydroxylauric (**b**) and 8-hydroxylauric (**c**) acids obtained after incubation of phenobarbital-induced Jerusalem artichoke microsomes with [1-^{14}C]-lauric acid. When clofibrate-induced *Vicia* or soybean microsomes were incubated with the fatty acid, only 12-hydroxylauric acid (arrow **d**) was eluted by the solvent. An unknown radioactive compound that is neither (ω-1)-hydroxylauric nor dodecanedioic acid, considering their retention times, was eluted with pure acetonitrile (not shown).

Radioactive hydroxylated fatty acids (Fig. 1) were collected, concentrated and subjected to mass spectra analysis after methylation with diazomethane and silylation with N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA). Electron-impact (70 eV) mass spectra were obtained on a gas chromatograph (column packed with 1% Dexil on chromosorb W-AW DMCS) programmed to rise from 100 to 200 C at 4 C/min and coupled to a LKB 9000S mass spectrometer with an LKB 2130 computer one-line system.

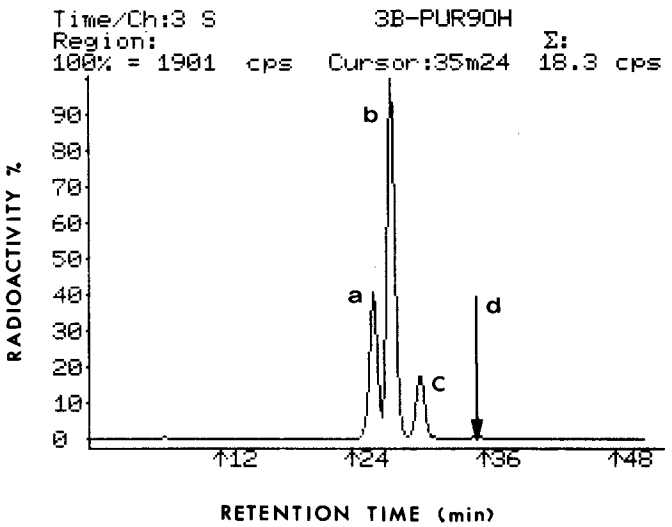

FIG. 1. Reverse phase chromatography of radioactive hydroxy laurates formed by microsomal incubations from Jerusalem artichoke (peaks a, b, c) or from *Vicia* (arrow d) with [1-^{14}C]lauric acid. The assigned structures of peaks a (10-hydroxylauric acid), b (9-hydroxylauric acid), c (8-hydroxylauric acid) and peak represented by the arrow d (ω-hydroxylauric acid) were obtained by mass spectra analysis. The conditions of incubation, HPLC and mass spectra analysis are described in Materials and Methods.

The mass spectra (not shown) are those expected for the methyl hydroxylaurate trimethylsilyl derivatives with characteristic fragments at m/e 131 and 273 corresponding to 10-hydroxylauric acid (peak **a**), m/e 145 and 259 to 9-hydroxylauric acid (peak **b**), m/e 159 and 245 to 8-hydroxylauric acid (peak **c**) and m/e 89,103 corresponding to ω-hydroxylauric acid (represented by the arrow **d**) with ions commonly present (21) in all these compounds at m/e 287 (M-15), m/e 271 (M-31), m/e 255 (M-47) and the base peak at m/e 73 (trimethylsilyl).

Cytochrome P-450 concentrations were measured on a Shimadzu MPS 2000 UV-visible spectrophotometer by the method of Omura and Sato (22) assuming an absorption coefficient of 91 mM-1.cm-1 for A 450-490 nm.

Results expressed in tables and figures are means of duplicates or triplicates with S.E. <5% in all cases. All experiments were repeated several times. However, significant differences were noted in seed germination, which may be associated with seed quality, storage conditions and seed age. Furthermore, enzyme induction in aging plant tissues is a physiological process strongly affected by seasonal endogenous rhythms. Thus, length and intensity of enzyme induction waves vary greatly during the year from one experiment to another. This may explain why the intensity of induction of laurate ω-hydroxylase from *Vicia* seedlings by clofibrate varied from 20 to 100 times in 10 separate experiments.

No attempt was made to assess the uptake of clofibrate, DEHP or 2,4-D by the tissues. The more lipophilic and neutral clofibrate and DEHP are probably better absorbed than 2,4-D. Therefore, the effects of these compounds are not to be compared on a quantitative level.

RESULTS

Clofibrate effects on lauric acid hydroxylase activities. The induction of lauric acid ω-hydroxylase in 5-day-old etiolated *Vicia* seedlings incubated for 48 hr on different clofibrate solutions is shown in Figure 2. It is seen that whereas cytochrome P-450 and cinnamic acid 4-hydroxylase activity were only weakly increased, ω-hydroxylase activity was stimulated by 700%. The response to clofibrate was virtually saturated at 0.5 mM.

Cytochrome P-450, cinnamic acid 4-hydroxylase and laurate hydroxylase activities were measured in Jerusalem artichoke tuber slices and in *Vicia* seedlings incubated for 48 hr on clofibrate (Table 1). In tuber tissues, in-chain hydroxylase is enhanced two times, which is in proportion to the stimulation of bulk cytochrome P-450, but sensibly less than the sixfold enhancement of cinnamate 4-hydroxylase activity. In sharp contrast, in *Vicia* seedlings, cinnamate 4-hydroxylase and cytochrome P-450 are enhanced 1.5 times and the laurate ω-hydroxylase is stimulated 30 times.

Effects of 2,4-D and DEHP on laurate ω-hydroxylase activity. Clofibrate and 2,4-D share a halogen-substituted

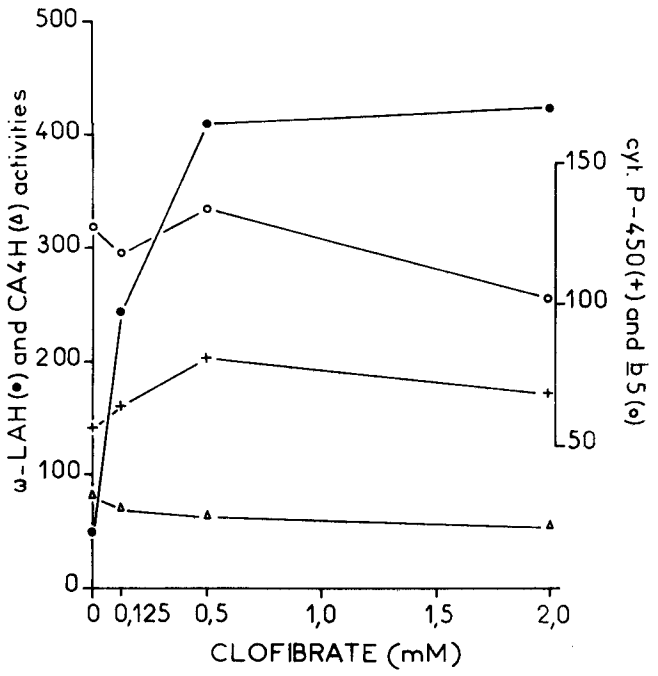

FIG. 2. Effects of increasing clofibrate concentrations on lauric acid ω-hydroxylase (ω-LAH) (●) and cinnamic acid 4-hydroxylase (CA4H) (Δ) activities in pmol $min^{-1}mg^{-1}$ protein or cytochrome P-450 (+) and cytochrome b_5 (○) contents in pmol mg^{-1} protein of *Vicia faba* etiolated seedlings incubated in aerated clofibrate solutions for 48 hr.

TABLE 1

Comparative Effects of Clofibrate on Lauric Acid ω- and In-chain Hydroxylase Activities

Incubation conditions of plant tissues	*H. tuberosus*[a]		*V. faba*[a]	
	Water	Clofibrate	Water	Clofibrate
Lauric acid ω-hydroxylase[b]	0	0	15	429
Lauric acid in-chain hydroxylase[b]	7	16	0	0
Cytochrome P-450[c]	53	119	58	89
Cinnamic acid 4-hydroxylase[b]	580	3510	172	281

[a]Microsomes from *Vicia faba* seedlings and *Helianthus tuberosus* slices incubated in water or 2 mM clofibrate solution.

[b]Enzyme activities are in pmol min^{-1} mg^{-1} protein.

[c]Cytochrome P-450 content in pmol mg^{-1} protein.

phenoxy ring. This and the fact that 2,4-D stimulated both cinnamic acid 4-hydroxylase and cytochrome P-450 content but not lauric acid in-chain hydroxylase in tuber tissues (10) prompted us to compare the effects of 2,4-D and clofibrate on the lauric acid ω-hydroxylase from *Vicia faba* seedlings. Laurate hydroxylase was stimulated five times in seedlings treated for 48 hr with 0.5 mM of either clofibrate or 2,4-D. This effect was not paralleled by an equal increase of total cytochrome P-450 and cinnamic acid hydroxylase, which were only weakly affected.

The plasticizer DEHP is one of the major organic atmospheric pollutants. It has been reported recently that DEHP induces similar form(s) of cytochrome P-450 as clofibrate in hepatic microsomes (19,20). DEHP also enhances the fatty acid ω-hydroxylase specifically, but to a lesser extent than clofibrate in rats.

Four-day etiolated soybean or *Vicia* seedlings were incubated on clofibrate or DEHP solutions. The results in Table 2 show that the laurate ω-hydroxylase is enhanced by DEHP over 3 times in soybean and 1.3 times in *Vicia*, whereas the cinnamic acid 4-hydroxylase was decreased 1.4 times. In vivo studies have shown that soybean and wheat cell suspension cultures are able to metabolize DEHP to polar conjugates (23).

In these first studies of DEHP effects on the microsomal hydroxylating system, we have not defined the optimal concentration for induction of lauric acid ω-hydroxylase activity. Clofibrate produced a strong stimulation of laurate ω-hydroxylase in soybean seedlings as well as in *Vicia* seedlings.

DISCUSSION

Our results provide the first evidence that cytochrome P-450 isozymes with identical substrate specificity and regioselective hydroxylating capacities are induced in plants and animals by a common inducer. It appears clear that the effects of clofibrate on the plant monooxygenase system are both species and isozyme-specific. Laurate in-chain hydroxylase of tuber tissues is less induced than the cinnamic acid 4-hydroxylase. The reverse is observed in *Vicia* seedlings, where the laurate ω-hydroxylase is enhanced over 30 times whereas cinnamate 4-hydroxylase is barely affected. In mammalian tissues containing two distinct laurate hydroxylases, an ω- and an (ω-1)-hydroxylase, clofibrate induces specifically the ω-hydroxylating isoenzyme (14). The very strong stimulation of laurate ω-hydroxylase activity in plants is not paralleled by an

TABLE 2

Effects of Clofibrate or DEHP on the Microsomal Hydroxylating System

Incubation conditions of etiolated seedlings	*Glycine max*[a]			*Vicia sativa*[a]		
	Water	Clofibrate	DEHP	Water	Clofibrate	DEHP
Lauric acid ω-hydroxylase[b]	5	81	17	40	901	53
Cytochrome P-450[c]	—	—	—	50	88	51
Cinnamic acid 4-hydroxylase[b]	99	68	97	190	228	136

[a]Microsomes from *Glycine max* and *Vicia sativa* seedlings incubated for 48 hr in water or in 1 mM clofibrate or DEHP solutions.

[b]Enzyme activities are in pmol min^{-1} mg^{-1} protein.

[c]Cytochrome P-450 content in pmol mg^{-1} protein.

equal increase of cytochrome P-450 content. This suggests the induction of a specific, but quantitatively minor, form of cytochrome P-450. It is also possible that physiological regulation mechanisms offset the increase of one form of cytochrome P-450 by a decrease of another.

DEHP, another "peroxisomal proliferator" that elicits responses in animals similar to clofibrate, but bears no structural resemblance to it, was also tested. This compound induced the ω-hydroxylase in soybean, and to a smaller extent in *Vicia*, while cinnamate 4-hydroxylase was not enhanced.

The effects of 2,4-D are very similar with respect to microsomal hydroxylating systems to those of clofibrate in the tuber as well as in the leguminosae system. Since 2,4-D, like several other halogenated phenoxy compounds, is a potent hormone of the auxin family, the laurate ω-hydroxylase induction in plants by 2,4-D may be accounted for by an auxin-like effect of this compound. The question remains open whether lauric acid ω-hydroxylase induction by clofibrate in plants and animals share a common mechanism. But the high induction of laurate ω-hydroxylase activity, elicited by clofibrate in leguminosae, provides a new tool for the study of selective isoenzyme induction in plants and opens perspectives for the search of structural relationships between laurate hydroxylase enzymes from animal, yeast and plant origins.

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A Phospholipase C with a High Specificity for Platelet-Activating Factor in Rabbit Liver Light Mitochondria

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The light mitochondrial fraction from rabbit liver was found to catalyze the hydrolysis of platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) by the phospholipase C reaction to form 1-O-alkyl-2-acetyl-glycerol and phosphocholine. The highest specific phospholipase C activity occurred in the liver and kidney. A subcellular survey showed that the enzyme was of lysosomal origin. The enzyme was solubilized with 2% Triton X-100 from rabbit liver light mitochondria and purified ca. 600- to 700-fold with a 17% yield using procedures that included hydroxyapatite, Sepharose 4B and isoelectric focusing column chromatography followed by fast protein liquid chromatography. The enzyme consists of two forms having a pI of 4.7 and 5.8. Each form was purified to a homogeneous state as judged by sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis. The enzyme migrated to positions corresponding to apparent molecular weights of 33,000 and 75,000, respectively. The purified enzymes of pI 4.7 and 5.8 had pH optima of 8.2 and 8.5 and apparent Km values of 55.6 and 45.5 μM for PAF, respectively. Furthermore, their phospholipase C activity was significantly inhibited by the addition of 1 mM EDTA. EDTA-inactivated enzyme, however, recovered completely upon addition of Ca^{2+} to the original level. p-Chloromercuribenzoate markedly inhibited enzyme activity, suggesting that phospholipase C is a −SH enzyme. The physiological role of the enzyme should be evaluated, considering its specificity for a highly potent, biologically active ether-phospholipid.
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PAF is a highly potent mediator that stimulates platelets and some other cells (1). PAF expresses potent biological activities (2–4), including platelet aggregation, neutrophile activation, increase of vascular permeability, bronchoconstriction and hypotensive effects (1,5). Specific enzymatic reactions involved in the biosynthesis of PAF have been documented by recent reports. These reactions include an acetyltransferase (6–8) and a cholinephosphotransferase (9). Both of these enzymes are of microsomal origin in rat spleen. On the other hand, acetylhydrolase, which is responsible for the hydrolysis of the acetate at the *sn*-2-position, was found in the cytosol fraction of rat liver (10).

This article presents results to reveal the presence in various rabbit tissues of a phospholipase C that would be relatively specific to PAF. Previously, the main if not the only pathway of PAF degradation has been thought to be via an acetylhydrolase. Furthermore, the enzyme differs from another lysosomal phospholipase in that it is inhibited by EDTA.

EXPERIMENTAL PROCEDURES

Materials. PAF and lyso-PAF were prepared semisynthetically from ratfish *(Hydrolagus colliei)* liver oil as described previously (11). The alkyl moiety consisted of 16.7% hexadecyl and 78.2% octadecyl. 1-O-Oleyl-2-acetyl-*rac*-glycerol was synthesized starting from 1-O-oleyl-*rac*-glycerol (selachyl alcohol) (12,13). [^{3}H]PAF (1-O-alkyl-[1′2′-^{3}H]-) (mixture of hexadecyl and octadecyl alkyl ethers) (115 Ci/mmol) was from Amersham (Arlington Heights, Illinois). The specific activity was diluted to 0.5 Ci/mol with unlabeled PAF, and the radiochemical purity was 98% by thin layer chromatography (TLC). 1-O-Hexadecyl-*rac*-glycerol (chimyl alcohol) and phospholipase C *(Bacillus cereus)* were from Sigma (St. Louis, Missouri). Precoated Kieselgel 60 plates were from E. Merck (Darmstadt, Federal Republic of Germany). Triton X-100 and Triton WR-1339 were from Nakarai (Tokyo, Japan). Spheroidal hydroxyapatite was from BDH (Poole, England). Hydroxyapatite, Bio-Beads SM-2 and dye reagent for protein determination were from Bio-Rad (Richmond, Virginia). Sepharose 4B was from Pharmacia (Uppsala, Sweden). Carrier Ampholite (pH 4–6) was from LKB (Uppsala, Sweden). Marker proteins for molecular weight determination were from Boehringer (Mannheim, Federal Republic of Germany). Various natural phospholipids were purchased from Serdary (London, England). The purity of phospholipids (3-*sn*-phosphatides) was assessed by TLC in the solvent system chloroform/methanol/acetic acid/H_2O (75:11:11:3, v/v/v/v). The phospholipids were found to migrate as a single spot on the thin layer plate. All other reagents were of analytical grade.

Subcellular fractionation. Subcellular fractionation of liver and other tissue homogenates was carried out according to the procedure of de Duve et al. (14) with the following modification: Rabbit (male New Zealand white) tissues were perfused with ice-cold 0.25 M sucrose, excised and homogenized with a Potter-Elvehjem homogenizer in 4 vol of 20 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. The homogenate was centrifuged at 600 × g for 10 min (nuclear fraction), and the supernatant was centrifuged at 3,300 × g for 10 min (mitochondrial fraction). The resulting supernatant was centrifuged at 25,000 × g for 10 min (light mitochondrial fraction), and the supernatant was further centrifuged at 105,000 × g for 1 hr to yield a precipitate (microsomes) and a supernatant (cytosol).

Further separation of lysosomes and peroxisomes, which are contained in the light mitochondrial fraction, was performed by centrifugation in a sucrose density gradient using rabbit liver injected with Triton WR-1339 (15). As marker enzymes, acid phosphatase (16) and ureate oxidase (17) were used for lysosomes and peroxisomes, respectively.

Purification of PAF-selective phospholipase C. All the experiments were conducted at 0–4 C unless otherwise indicated.

• Step 1: solubilization. The liver light mitochondrial fraction prepared as described above was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl (20 mg of protein/ml) (fraction 1). Triton X-100

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was added to the suspension to the final concentration of 2%. After gentle stirring for 3 hr at 4 C, the suspension was centrifuged at 105,000 × g for 1 hr. The supernatant was used as solubilized enzyme (fraction 2).

- Step 2: batchwise spheroidal hydroxyapatite. The solubilized enzyme was stirred with 20 g of spheroidal hydroxyapatite equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.1% Triton X-100 for 30 min and was filtrated. The unbound fraction was pooled and concentrated using an Amicon macrosolute concentrator (B15) (fraction 3).
- Step 3: sepharose 4B column chromatography. Fraction 3 was applied to a Sepharose 4B column (3.4 × 85 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.1% Triton X-100 and was eluted with the same buffer. In this column chromatography, phospholipase C was eluted between catalase and human immunoglobulin G and pooled as fraction 4.
- Step 4: hydroxyapatite column chromatography. Fraction 4 was applied to a hydroxyapatite column (4 × 10 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.1% Triton X-100. After extensive washing with the equilibration buffer, the concentration of phosphate buffer was increased linearly from 20 to 350 mM. The enzyme activity was eluted at ca. 100 mM of phosphate, which was collected and concentrated (fraction 5).
- Step 5: isoelectric focusing. Fraction 5 was subjected to isoelectric focusing in a column with a pH gradient of 4–6. A solution of 1% carrier Ampholite was used as a support in a density gradient of 0–40% sucrose (18). There were two enzyme active peaks at pH 4.7 (termed pl 4.7 enzyme) and 5.8 (termed pl 5.8 enzyme), and both fractions were dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 0.1% Triton X-100.
- Step 6: fast protein liquid chromatography (FPLC). Each dialyzate was applied on FPLC using prepacked anion exchange column Mono-Q™ (Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 0.1% Triton X-100. After extensive washing with the equilibration buffer, the concentration of NaCl in the buffer was increased linearly to 500 mM. The enzyme active peak was pooled, concentrated and used as the final enzyme preparation (fraction 7).

Results of a typical purification procedure are summarized in Table 1. The final enzyme preparation showed ca. 600- to 700-fold purification, with a recovery of 17%.

Enzyme assays. Incubation mixtures consisted of the following: 50 μM [^{3}H]PAF dissolved in 1% methanol, 0.1 M Tris-HCl buffer (pH 8.0) and enzyme protein in a final volume of 1 ml. After incubation with shaking at 37 C for 3 hr, reactions were stopped by adding 4 ml of chloroform/methanol (2:1). Following vigorous mixing and centrifugation, the lower solvent phase was evaporated under nitrogen, dissolved in a minimum of chloroform and applied to thin layer plates which were developed with hexane/ethyl ether/acetic acid (60:40:1, v/v/v). The area corresponding to authentic 1-alkyl-2-acetyl-glycerol was scraped and counted in a liquid scintillation counter. Values given in Table 1 are means of triplicate determinations.

When 1 mM EDTA was added, the enzyme was completely inhibited as described in Table 2, and the EDTA-inactivated enzyme was recovered to the original level by an addition of 0.5 mM $CaCl_2$ (Table 3). However, the enzyme assay was performed in the absence of $CaCl_2$, because the addition of $CaCl_2$ did not affect significantly the enzyme not treated with EDTA.

Analytical methods. Polyacrylamide (7.5%) disc gel electrophoresis was carried out by the method of Weber and Osborn (19) in the presence of 0.1% sodium dodecyl sulfate. Protein was determined by the procedures of Lowry et al. (20) or using the Bio-Rad protein assay kit; bovine serum albumin was used as standard. Phospholipid liposomes were prepared as described previously using a Branson sonifier (21). Phospholipid phosphorus was determined by the method of Bartlett (22).

RESULTS

Subcellular distribution of liver phospholipase C with a high specificity for PAF. With conventional centrifugation, the light mitochondrial fraction from liver showed the highest specific activity, but microsomes and cytosol showed little activity (Table 4). Further separation of the light mitochondrial fraction by sucrose density gradient centrifugation demonstrated that phospholipase C is exclusively localized in lysosomes.

TABLE 1

Purification of Phospholipase C with a High Specificity for PAF from Rabbit Liver Light Mitochondria

Fraction		Volume (ml)	Total protein (mg)	Specific activity (nmol/mg)	Total activity (nmol)	Yield (%)	Purification (-fold)
1. Light mitochondria		130	3220	1.2	3864	100	1
2. Triton X-100		200	800	3.6	2880	74.5	3.0
3. Hydroxyapatite		230	644	4.2	2705	70.0	3.5
4. Sepharose 4B		30	36.0	62.4	2246	58.1	52.0
5. Hydroxyapatite		20	7.6	213.9	1626	42.1	178.3
6. Isolectric focusing	pl 4.7	2.0	2.4	305.1	732	18.9	254.3
	pl 5.8	2.0	0.9	744.0	670	17.3	620.0
7. Fast protein liquid chromatography	pl 4.7	1.5	0.9	718.5	647	16.7	598.8
	pl 5.8	3.0	0.8	844.2	675	17.5	703.5

TABLE 2

Effect of Various Agents on Phospholipase C

Chemicals added	Concentrations (mM)	Relative activity (%)[a] pl 4.7	pl 5.8
None	—	100	100
Reduced glutathione	2	111.0	98.4
NaF	2	90.2	82.6
Dithiothreitol	2	74.1	61.9
2-Mercaptoethanol	2	92.6	88.9
p-Chloromercuribenzoate	2	3.2	8.3
EDTA	1	0	0

[a]Protein concentrations of the purified pl 4.7 and 5.8 enzymes were 20 and 10 μg, respectively. The 100% activity of pl 4.7 and 5.8 enzymes corresponds to 363 and 675 nmol/mg, respectively.

TABLE 3

Effects of Various Divalent Cations on Phospholipase C

Cations added[a]	Relative activity (%)[b] pl 4.7	pl 5.8
None	100	100
Ca^{++}	140	115
Mg^{++}	43.2	20.0
Mn^{++}	11.7	2.1
Zn^{++}	6.7	1.0
Ni^{++}	26.7	81.7
Cu^{++}	18.3	53.3
Cd^{++}	0	3.5
Fe^{++}	98	68.3

[a]Concentrations of all cations were 0.5 mM, and the protein concentrations of the purified pl 4.7 and 5.8 enzymes were 20 and 10 μg, respectively.

[b]The standard incubation mixture described in "Experimental Procedures" was used, except that enzyme protein was preincubated with 1 mM EDTA for 2 min at 25 C, and then divalent cations were added as indicated. The 100% activity of pl 4.7 and 5.8 enzymes corresponds to 363 and 675 nmol/mg, respectively.

TABLE 4

Subcellular Distribution of Phospholipase C with a High Specificity for PAF in Rabbit Liver

Fraction	Enzyme activity (nmol/mg)
Nuclear and cell debris	0.33
Heavy mitochondria	0.57
Light mitochondria	1.20
Peroxisome	0.96
Lysosome	4.95
Microsome	0.06
Cytosol	0.12

TABLE 5

Phospholipase C Activity with a High Specificity for PAF in Light Mitochondrial Fraction from Various Rabbit Tissues

Tissues	Enzyme activity (nmol/mg)
Brain	0.75
Lung	0.51
Liver	1.20
Kidney	1.38
Testis	0.81

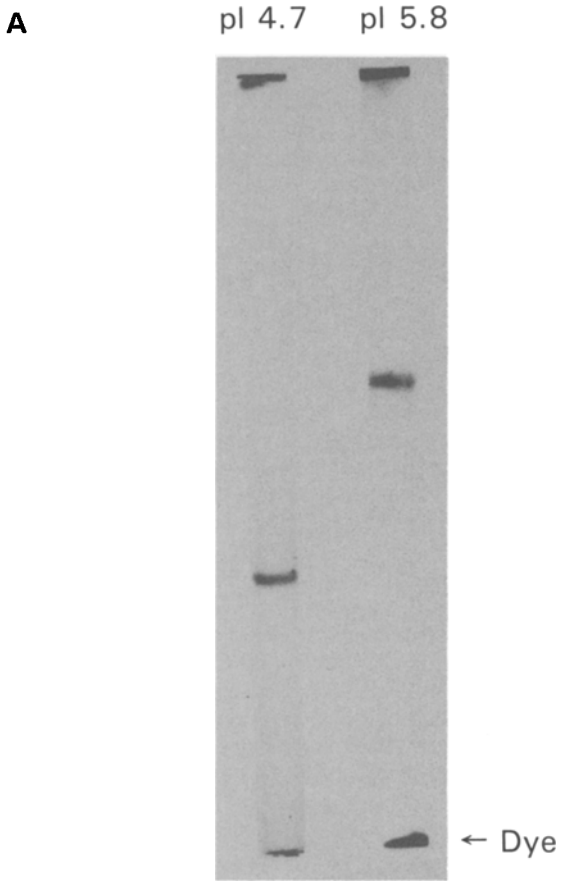
Distribution of phospholipase C activity in different rabbit tissues. The enzymatic activity in various rabbit tissues was examined using the light mitochondrial fraction. Kidney and liver had higher specific activities than other tissues, such as brain, lung and testis (Table 5). The enzymatic reaction product was also 1-alkyl-2-acetyl-glycerol in these assays. Liver light mitochondrial fraction was used for further characterization of the enzyme, in spite of the higher activity found in the kidney preparations, because of difficulties in obtaining sufficient amounts of the tissues.

Purity and molecular weight. The final preparation of pl 4.7 and 5.8 enzymes was demonstrated to be nearly homogenous upon polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 1A). The molecular weight was estimated to be ca. 33,000 and 75,000, respectively (Fig. 1B).

Identification of the reaction product. Identification of the reaction product was carried out as follows: (i) The radioactive product was chromatographed on a thin layer plate and developed with hexane/ethyl ether/acetic acid (60:40:1, v/v/v) or benzene/ethyl ether/ethyl acetate/acetic acid (80:10:10:0.2, v/v/v/v). The enzyme reaction product had completely the same mobility as that of authentic 1-alkyl-2-acetyl-glycerol, although a part of the product was spontaneously isomerized (23) to 1-alkyl-3-acetyl-glycerol. (ii) Mild saponification of the reaction product completely converted the product to alkylglycerol. Furthermore, the ether bond of 1-O-[^{3}H]alkylglycerol produced by mild alkaline hydrolysis was cleaved to yield [^{3}H]fatty aldehyde by the action of alkylglycerol monooxygenase (24,25).

Effect of protein concentration and reaction time. The enzyme activity that catalyzes the hydrolysis of PAF was proportional to the protein concentrations over the ranges of 50–200 μg per incubation mixture. At a higher concentration of the enzyme protein, the enzyme reaction did not increase but tended to saturate. Although the reaction velocity showed approximate linearity until 30 min of incubation, it increased for a much longer period of time (Fig. 2). Lowering temperatures of incubation to 22 C remarkably decreased the enzyme activity but maintained the linearity of the reaction.

Kinetics. The results of varying the concentration of [^{3}H]PAF on the activity of phospholipase C and the linearity of the double reciprocal plots are shown in Figure 3. Phospholipase C activity was saturated with 260 μM of PAF in either pl 4.7 or 5.8 enzyme, and the Km value was determined to be 55.6 and 45.5 μM, respectively. On



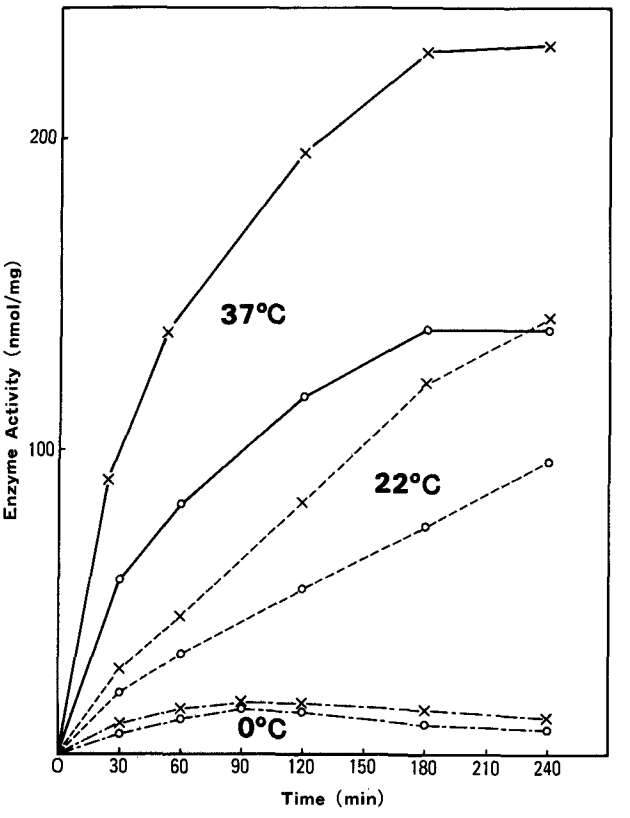


FIG. 2. Effect of incubation time. Enzymatic activity was determined as described in "Experimental Procedures," except that the incubation time was changed as indicated with 10 μg of enzyme protein. (○), pl 4.7 enzyme; (×), pl 5.8 enzyme.

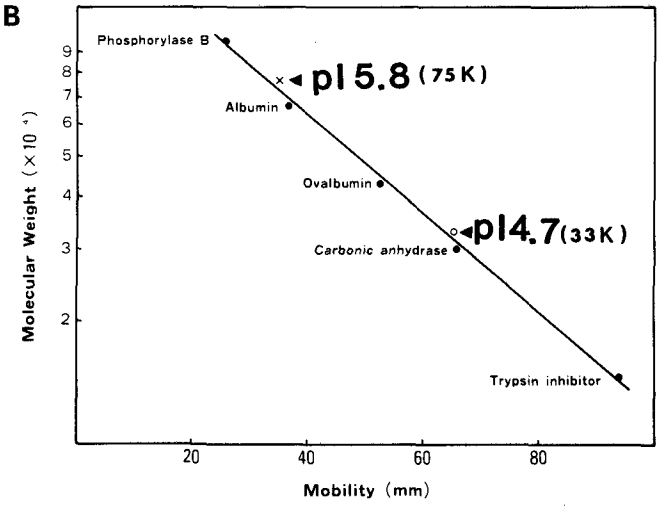


FIG. 1. Electrophoresis of purified phospholipase C (A) and determination of the molecular weight (B) on polyacrylamide gel (7.5%) in the presence of 0.1% sodium dodecyl sulfate. Seven μg protein of pl 4.7 and 5.8 enzymes was applied to the gel. Arrow indicates the position of the tracking dye, bromophenol blue. Proteins of known subunit molecular weight used to calibrate in the gel system include phosphorylase (94,000), bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000) and trypsin inhibitor (21,000).

the other hand, the Vmax values were 384 and 714 nmol/mg protein, respectively, which are considerably different.

Optimal pH. Both pl 4.7 and 5.8 enzymes showed a relatively sharp pH profile with optimums at 8.2 and 8.5, respectively, when Tris-HCl buffer was used.

Substrate specificity. Hydrolysis of various phospholipids was examined by measuring the inorganic phosphate of water-soluble products. As shown in Table 6, either pl 4.7 or 5.8 enzyme showed the highest activity for PAF. The other phospholipids, including lyso-PAF, were not hydrolyzed at significant rates in comparison with PAF under the conditions used.

Effect of various chemicals. These were summarized in Table 2. p-Chloromercuribenzoate strikingly inhibited the enzyme activity, suggesting that phospholipase C is a −SH enzyme. Addition of NaF and 2-mercaptoethanol had little effect, while EDTA completely inactivated the enzyme activity at a concentration of 1 mM.

Effect of divalent cations. When 1 mM EDTA was added to the reaction mixture, both pl 4.7 and 5.8 enzymes were completely inactivated, as already shown in Table 2. The EDTA-inactivated enzyme was recovered to 140 or 115% of the original activity by 0.5 mM Ca^{2+}, while Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu_{2+} and Cd^{2+} were much less effective or ineffective (Table 3). Fe^{2+} and Ni^{2+}, however, were effective for pl 4.7 and 5.8 enzymes, respectively.

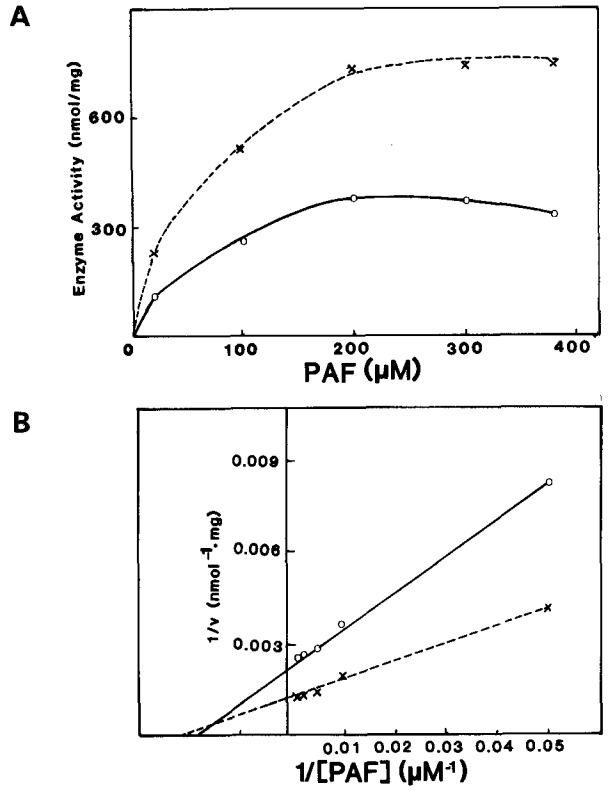


FIG. 3. Effect of substrate concentration (A) and the double reciprocal plot (B). Assay conditions were the same as those described in "Experimental Procedures," except that various concentrations of [^{3}H]PAF were added with 10 μg of enzyme protein. (○), pI 4.7 enzyme; (×), pI 5.8 enzyme.

TABLE 6

Substrate Specificity of the Purified Phospholipase C

Substrate[a]	Relative activity (%)[b] pI 4.7	pI 5.8
PAF	100	100
Phosphatidylcholine	34.0	34.5
Phosphatidylethanolamine	37.3	31.2
Sphingomyelin	64.7	20.8
Lyso-PAF	29.9	23.1
Phosphatidylinositol	27.8	14.1
Phosphatidylserine	24.5	16.5
Phosphatidic acid	10.5	8.5

[a]Concentrations of all substrates were 50 μM, and the protein concentrations of the purified pI 4.7 and 5.8 enzymes were 20 and 10 μg, respectively.

[b]The 100% activity of pI 4.7 and 5.8 enzymes corresponds to 363 and 675 nmol/mg, respectively.

DISCUSSION

Phospholipase C (EC 3.1.4.3) is a phosphodiesterase that acts on phosphoglycerides to hydrolyze the ester bond between diacylglycerol and the respective phosphoric acid-substituted polar head group. Phospholipase C was first purified from the growth media of several types of bacteria (26-29), but also is widely distributed in mammalian tissues (30). Although this enzyme is predominantly found in the cytosol of mammalian tissues (31-33), lysosomal phospholipase C was also found in brain (34) and liver (35). The membrane-bound form was distinctly different from the soluble enzyme. In contrast to cytosolic phospholipase C, lysosomal enzyme does not require divalent cations and is not inhibited by EDTA. It has an acidic pH optimum, while the pH optimum of the cytosolic enzyme is either neutral or slightly alkaline.

Certain types of phospholipase C show highest activity with PI as substrate and have been termed PI-specific phospholipases C (EC 3.1.4.10). They have been purified from rat liver and sheep vesicular glands (31,32). PI-specific phospholipases C are recognized as the major enzymes involved in signal transmission across membranes (36). Sphingomyelinase, which catalyzes a similar reaction, has also been described (37). However, it is not clear whether the various types of phospholipases C are closely related enzymes with different specificities due to minor changes in structure or represent distinctly different classes of enzymes. Furthermore, the precise specificities of the enzymes have not always been thoroughly defined.

In the present studies, novel types of phospholipase C with a high specificity for PAF were purified ca. 600- to 700-fold from rabbit liver light mitochondria. The recovery of the enzyme was about 17% with respect to activity. The phospholipase C described here appears to be the first ester hydrolase that is relatively specific for ether-linked lipids and is therefore important in the catabolism of alkyl phospholipids. Wykle et al. demonstrated that the derivatives containing acetate at the *sn*-2-position are very poor substrates for ether-linked lysophospholipase-D (38,39). Therefore, the phospholipase C activity for PAF is unlikely to be responsible for the combined action of lysophospholipase-D and phosphohydrolase. On the other hand, the two forms of enzymes purified in the present study appear to be functionally very similar; that is, they have similar substrate specificity and optimum pH and Km for PAF. Although the rate of phospholipase C activity is the greatest with PAF, substantial rates are also found with other phospholipids, which showed ca. 20-30% of the rate of PAF hydrolysis. Lysosomal phospholipase C of rat liver reported by Matsuzawa and Hostetler has a pH optimum of 4.4 (36), while the pH optimum for the present lysosomal phospholipase C is 8.2 and 8.5. In addition, rat liver lysosomal phospholipase C has no substrate specificity, while rabbit liver enzyme prefers PAF. Lysosomal phospholipase C with these properties has not been reported previously. Finally, the similarity between 1-alkyl-2-acetylglycerol and the diacylglycerol that is a well-known activator of protein kinase C (40,41) is noted. It is tempting to hypothesize that a breakdown product of PAF has some role in protein kinase C regulation (42). Further studies are necessary to define the quantitative importance and the physiological role of phospholipase C with a high specificity for PAF in mammalian tissues.

ACKNOWLEDGMENTS

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Influence of Dietary Vitamin E, Selenium and Age on Regional Distribution of α-Tocopherol in the Rat Brain

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Concentrations of α-tocopherol (α-T) in plasma, cerebrum, cerebellum, midbrain and brain stem and activity of selenium (Se)-dependent glutathione peroxidase (GSH-Px) in plasma were measured in 1- and 15-month-old male F344 rats fed diets containing vitamin E (E, IU/kg) and Se (ppm) in the following combinations: 30 E, 0.1 Se (control diet, minimum requirements); 200 E, 0.2 Se; 0.0 E, 0.2 Se; 200 E, 0.0 Se; 0.0 E, 0.0 Se for 8 or 20 weeks. α-T and GSH-Px levels in plasma were reflective of dietary treatment in young rats in which an interaction of the two nutrients was noted. A longer period of dietary vitamin E deficiency was necessary to deplete plasma α-T and depress GSH-Px activity significantly in the old rats. Among the brain regions of all ages, cerebrum and midbrain had the highest concentrations of α-T while cerebellum showed the lowest. However, cerebellum of young rats and cerebellum and brain stem of old rats had a greater α-T accumulation with doubly supplemented diets, whereas only cerebellum of young and old rats showed a marked increase of α-T with vitamin E supplementation. In old rats, vitamin E deficiency resulted in greater depletion of α-T in cerebellum and brain stem than cerebrum and midbrain regions. Se deficiency in brain stem of young and old rats significantly decreased α-T accumulation by vitamin E supplementation. Se supplementation marginally alleviates vitamin E depletion in brain. Cerebellum and brain stem of old rats fed the minimum requirement of vitamin E and Se for 20 weeks showed a significant decline in α-T. Therefore, cerebellum and brain stem appear to have a higher turnover of α-T than cerebrum and midbrain, and older rats may require a higher level of vitamin E in the diet to maintain steady state levels of α-T in these regions.
Lipids 21, 786–791 (1986).

Vitamin E is known to scavenge free radicals and protect cellular membranes from lipid peroxidation damage (1). Neuropathological evidence linking vitamin E deficiency with neurological abnormalities in abetalipoproteinemia, fat malabsorption disorders and experimentally induced dystrophy indicates that dietary vitamin E may play an important role in the central nervous system (CNS) with regard to membrane stability and physiological function (2–4). Lipofuscin, an aging pigment, is a by-product of membrane peroxidation and accumulates in neurons and other postmitotic cells during aging (5–8). Reduction of these fluorescent pigments has been demonstrated in animals fed vitamin E-supplemented diets (8). Several age related CNS changes, such as decrements in neuronal receptor sites, senile dementia, depression and sleep disorders have been suggested to result from free radical reactions and lipid peroxidation. This and other evidence (9–14) indicate that vitamin E may play an important role in the neurobiology of aging and that the CNS requirement for this nutrient may change during aging.

There is a biochemical interrelationship between vitamin E and selenium (Se) with regard to their protective antioxidant functions (15). The reciprocal influence of one on the other has been demonstrated in several tissues (16). Areas of the CNS are differently affected by disease, drugs and physiological processes; thus, the state of membrane physiochemistry may be partially dependent upon the antioxidant capacity of the neurons. The influence of dietary vitamin E and Se on the requirement of brain regions for α-tocopherol (α-T) in old rats has not been explored. This study examines the influence of dietary vitamin E and/or Se on the regional distribution of α-T in the brains of young and old rats.

MATERIALS AND METHODS

Forty-eight 1-month-old and seventy-eight 15-month-old male Fischer 344 rats were randomly assigned to six dietary treatment groups: group A (control) received the basal diet (containing no vitamin E or Se; Table 1) supplemented with the minimum requirement of vitamin E (30 IU α-tocopheryl acetate/kg diet) and Se (0.1 ppm Se as sodium selenite). Group B (+E+Se) received the basal diet supplemented with 200 IU α-tocopheryl acetate and 0.2 ppm Se. Group C (−E+Se) received the basal diet supplemented with 0.2 ppm Se. Group D (+E−Se) received the basal diet supplemented

TABLE 1

Composition of Basal Diet

Ingredient	Percentage
Torula yeast[a]	30.0
Sucrose[a]	56.7
α-Tocopherol stripped lard[a]	5.0
α-Tocopherol stripped corn oil[a]	2.0
DL-methionine[a]	0.3
Mineral mix (selenium free)[a,b]	5.0
Vitamin mix (vitamin E free)[c]	1.0
Total	100.0

[a]Teklad (Madison, Wisconsin).
[b]Hubbell-Mendel-Wakeman (3.08 g of $MnSO_4 \cdot H_2O$ per kg of mineral mix was added). Mineral contribution from torula yeast gave final percentage concentration in diet as follows: Ca, 1.16; P, 0.77; K, 1.2; Na, 0.16; Mg, 0.10; Fe, 0.017; Cu, 0.0023; Mn, 0.0055; Zn, 0.0037.
[c]Vitamin content in 1 kg of diet: thiamine HCl, 4.0 mg; riboflavin, 4.8 mg; pyridoxine HCl, 2.0 mg; calcium pantothenate, 20.0 mg; niacin, 100.0 mg; menadione, 1.0 mg; folic acid, 2.0 mg; biotin, 1.0 mg; vitamin B_{12} (0.1% trituration), 10.0 mg; retinyl palmitate, 100 mg; cholecalciferol, 4 mg; sucrose was added to make total vitamin mix 10 g.

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with 200 IU α-tocopheryl acetate/kg diet. Group E (−E−Se) received only the basal diet. Group F was pair-fed with the control diet to the double-deficient group E.

Rats were individually caged in stainless steel wire mesh cages and maintained at 22 C with 12-hr light-dark cycles. All the animals were fed ad libitum except for the pair-fed rats (group F).

After eight weeks of dietary treatment, all young animals and five 17-month-old (17M) rats from each group were sacrificed by decapitation. The remaining old animals were sacrificed at 20 months of age (20M) after 20 weeks of dietary treatment. Blood was collected in heparinized tubes, and plasma was separated and stored at −70 C. Brain was removed from the skull, placed on an ice-cold platform and immersed with ice-cold buffer. The cerebrum, cerebellum, midbrain (including hypothalamus, striatum and hippocampus) and brain stem (including medulla oblongata and pons) were dissected with precooled dissecting tools within 1-2 min of decapitation. Ten-percent homogenate of the left half of brain regions was prepared using ice-cold 1.15% KCl. After addition of α-T acetate containing 0.1% butylated hydroxy toluene in ethanol into the homogenate or plasma, α-T was extracted with hexane and dried under a stream of nitrogen and reconstituted in methanol. α-T was measured by the high performance liquid chromatography method described by Bieri et al. (17) using 100% methanol as the mobile phase and fluorescence detection (Exc, 292; Emm, 340 nm) for enhanced sensitivity. Measurement sensitivities for α-T in plasma and brain samples were 0.2 μg/ml and 10 μg/mg, respectively. Glutathione peroxidase (GSH-Px) activity in plasma was measured by the method of Paglia and Valentine (18). Data were tested by analysis of variance. Differences of means were evaluated for significance at 5% probability by Newman-Keul's multiple range test.

RESULTS

Young rats fed the vitamin E-supplemented diets (groups B and D) showed the highest percentage weight gain (431 and 451%, respectively), while double deficient group E and the matched pair-fed control (group F) had the lowest percentage weight gain (345 and 337%, respectively). The differences in weight changes between groups E and F and groups B and D were marginally significant. Group F, which was pair-fed to group E with the control diet, had no significant differences in plasma α-T and GSH-Px activity from those rats fed the control diet ad libitum (group A). None of the weight changes in young dietary groups were significantly different from group A (429% weight gain). Neither 17M nor 20M rats in the various dietary groups had significant weight changes at the end of the study.

Concentrations of α-T in plasma are shown in Table 2. Plasma α-T levels were markedly affected by the type of diet. The concentration of α-T in plasma of young rats supplemented with vitamin E (groups B and D) increased, while those which received vitamin E-deficient diets (groups C and E) showed decreased α-T compared with those fed the control diet (group A). Se deficiency had no effect on the α-T level of plasma in the young rats.

Feeding old rats the +E+Se diet (group B) for eight weeks (17M rats) significantly increased plasma α-T relative to control. The rise of plasma α-T in 17M rats fed the +E−Se diet (group D) was less than in those fed the +E+Se diet (group B). Feeding old rats for eight weeks with either vitamin E-deficient diet (−E+Se or −E−Se) did not decrease plasma α-T significantly from controls (groups A and F). However, continued feeding of old rats with vitamin E-deficient diets (−E+Se or −E−Se) for 12 more weeks (i.e., 20M rats) decreased plasma α-T significantly. Conversely, plasma α-T level increased significantly in the old rats fed vitamin E-supplemented diet. Interestingly, 20M rats fed +E+Se diet (group B) had significantly lower plasma α-T than those fed +E−Se diets (group D).

The activities of plasma GSH-Px in relation to dietary treatments and age are shown in Table 3. The activity of plasma GSH-Px reflected dietary Se treatment. The response of the young rats to either Se supplementation or deficiency was greater than that of old rats. Feeding Se-deficient diets (+E−Se and −E−Se) severely decreased the activity of plasma GSH-Px in the young rats. As old rats were fed Se-deficient diets for a longer period of time, the decline of enzyme activity was more pronounced.

Dietary vitamin E supplementation appeared to influence the activity of GSH-PX in young growing rats

TABLE 2

Concentration of α-Tocopherol in Plasma

Dietary group	Age of rats (months)		
	3	17	20
A (control)	4.57 ± 0.45 (5)^{a,*}	3.51 ± 0.31 (5)^{a,*}	—
B (+E+Se)	7.22 ± 0.44 (8)^{b,*}	8.05 ± 0.43 (6)^{b,*}	11.08 ± 0.61 (4)
C (−E+Se)	0.24 ± 0.08 (8)^{c,*}	4.20 ± 1.13 (6)^{ab}	1.70 ± 0.13 (4)^{a,*}
D (+E−Se)	7.35 ± 0.50 (8)^{b,*}	6.87 ± 0.69 (6)^{ab,*}	14.74 ± 1.80 (2)
E (−E−Se)	0.82 ± 0.21(8)^{c,*}	5.85 ± 1.83 (5)^{ab,†}	3.07 ± 0.17 (3)^{a,*,†}
F (Pair-fed)	4.54 ± 0.33 (5)^{a,*}	4.45 ± 0.27 (5)^{ab,*}	—

Each value represents mean ± SEM of α-T (μg/ml). Number of rats tested is indicated in parentheses. Means with a common letter superscript within an age group are not significantly different (P > 0.05). Means with a common * or † superscript within a dietary group are not significantly different (P > 0.05).

TABLE 3

Activity of Glutathione Peroxidase in Plasma

Dietary group	Age of rats (months)		
	3	17	20
A (control)	12.04 ± 0.71 (5)^{ab}	9.07 ± 0.51 (5)^{a,*}	8.69 ± 0.15 (6)^{a,*}
B (+E+Se)	13.04 ± 0.24 (5)^{a}	10.29 ± 0.69 (5)^{a,*}	9.46 ± 0.43 (9)^{a,*}
C (−E+Se)	10.80 ± 0.37 (5)^{b,*}	9.05 ± 0.86 (5)^{a,*}	9.85 ± 0.16 (5)^{a}
D (+E−Se)	0.15 ± 0.24 (5)^{c}	5.86 ± 0.37 (5)^{b,*}	3.47 ± 0.22 (7)^{b,†}
E (−E−Se)	0.24 ± 0.09 (5)^{c}	6.70 ± 0.48 (5)^{b,*}	3.55 ± 0.21 (6)^{b,†}
F (Pair-fed)	10.73 ± 0.75 (5)^{b,*}	9.64 ± 0.71 (5)^{a,*}	9.21 ± 0.33 (6)^{a,*}

Each value represents mean ± SEM of GSH-Px activity (U/ml). Number of rats tested is indicated in parentheses. Means with a common letter superscript within an age group are not significantly different ($P > 0.05$). Means with a common * of † superscript within a dietary group are not significantly different ($P > 0.05$).

but not in old rats fed experimental diets for eight weeks. The effect of vitamin E supplementation on GSH-Px activity in the young rats was not present in the groups fed Se-deficient diets (group D vs group E), but the effect was apparent when diet was supplemented with Se (group B vs group C).

Feeding old rats the −E+Se diet for 20 weeks (20M rats) produced a significant change in the activity of plasma GSH-Px compared to 17M rats, whereas +E+Se diet did not. In the 20M rats, Se-deficient diets (+E−Se and −E−Se) decreased the enzyme activity significantly relative to 17M rats that were treated with the same diets for eight weeks. Pair-fed groups of 17M and 20M rats did not show a significant difference in plasma GSH-Px activity when compared to control group A or Se-supplemented groups B and C.

The concentrations of α-T in the different brain regions are shown in Figure 1. Across all age groups, cerebrum and midbrain had higher concentrations of α-T than brain stem and cerebellum. Feeding young rats for 8 weeks and old rats for 8 or 20 weeks with control diet (containing the standard minimum requirements of vitamin E and Se) resulted in concentrations of α-T in a rank order of cerebrum > midbrain > brain stem > cerebellum. While there was no statistically significant difference between cerebrum and midbrain in α-T concentrations, the levels in brain stem and cerebellum were significantly different from each other and from cerebrum and midbrain (Fig. 1A). A similar pattern of α-T concentrations was found in brain regions of pair-fed rats (Fig. 1F). The cerebellum and brain stem of 20M pair-fed rats (group F), like control-fed rats (group A), had a significantly lower α-T concentration compared to 17M rats in the same dietary group.

Relative to control-fed rats, vitamin E-supplemented groups (+E+Se and +E−Se) had greater levels of α-T. Feeding the +E+Se diet significantly increased α-T concentration in the brain regions of all ages except midbrain of young rats and cerebrum of 17M rats (Fig. 1B). The cerebellum of young rats fed the +E+Se diet (group B) relative to control-fed rats (group A) had a significantly higher α-T increment than did cerebrum, brain stem and midbrain regions. Cerebellum and brain stem of 17M rats given the same diet had a higher α-T increment relative to cerebrum and midbrain. Similarly, higher increments relative to control-fed rats were found for 20M rats. The +E+Se diet in 20M rats significantly increased α-T concentration in cerebrum and midbrain compared to those of 17M rats.

In comparing α-T levels of brain regions to the respective regions of control-fed rats within an age group, cerebellum of rats fed the +E−Se diet had a significantly higher α-T increment than cerebrum, midbrain or brain stem. Comparing 20M rats to 17M rats, feeding the +E−Se diet for 12 more weeks (20M rats) increased α-T concentration in cerebrum and midbrain.

Comparing the effects of the doubly supplemented diet (+E+Se) to the single vitamin E-supplemented diet (+E−Se) on α-T concentration of brain regions showed that Se deficiency slightly lowers the α-T concentration in all brain regions for all ages (except midbrain in young rats). The difference was significant for cerebrum of 20M rats, cerebellum of 17M rats and brain stem of all ages.

Concentration of α-T in the brain regions (except cerebellum) for all ages was significantly lower in the −E−Se-fed rats compared to control-fed rats (Fig. 1E). Among the brain regions of young rats, the α-T decrements with −E−Se feeding were not significant. Old rats fed the −E−Se diet had a smaller α-T decrement than young in the all-brain regions. Old rats fed the same diet for 20 weeks (20M rats) showed a greater α-T level decrement relative to controls in cerebellum and brain stem regions than cerebrum and midbrain regions. A similar effect was detected when the α-T levels between 17M and 20M rats were contrasted. The magnitude of decline of α-T concentration as noted between group E (−E−Se) and group A (control) of 20M rats was not present between group F (pair-fed) and group A (control) of 20M rats.

The effect of the −E+Se diet compared to the control diet on α-T concentration of brain regions in the young rats was similar to the −E−Se diet. Feeding the −E+Se diet for an additional 12 weeks (20M rats) significantly decreased α-T concentration in cerebellum and brain stem compared to 17M rats. This diet was less effective in decreasing α-T concentration in cerebrum and midbrain. The effect of −E+Se diet on decreasing α-T concentration in brain regions of 20M rats relative to 17M rats was less than the effect of the −E−Se diet.

Comparing the effect on brain α-T concentration of the doubly deficient diet (−E−Se) with the single vitamin E-deficient diet (−E+Se) showed that Se supplementation marginally alleviated the decrement of α-T in all brain regions for all ages (except cerebellum of young rats).

DISCUSSION

The changes in α-T level and GSH-Px activity in plasma indicated that the dietary treatment was effective in modulating both micronutrients. In young rats, changes in dietary vitamin E and/or Se were reflected through changes in α-T levels in brain regions, although the magnitude of these alterations in some brain regions was not as great as in plasma α-T levels or GSH-Px activity. Old rats responded differently than young rats to the various dietary treatments, due to their prior 15-month chow diet as well as their age and postgrowth development stage. Perhaps due to redistribution of the vitamin from other tissues, e.g. adipose and liver, old rats fed vitamin E-deficient diets for eight weeks (17M rats) did not show a significant decline in plasma α-T. In contrast, old rats fed vitamin E-supplemented diets demonstrated a rise of plasma α-T relative to control groups. Moreover, plasma α-T increased as old rats were maintained on vitamin E supplementation for 12 additional weeks (20M rats). The decline of plasma α-T levels in 20M rats fed vitamin E-deficient diets was of much smaller magnitude than the rise seen in supplemented groups. Therefore, there is a relative resistance to vitamin E depletion in old animals but no barrier to increasing accumulation of this micronutrient for the period tested. Se supplementation in the young rats was not effective in increasing the activity of Se-dependent GSH-Px in the plasma, whereas the Se-deficient diet markedly depressed the activity of this enzyme.

Vitamin E is in a dynamic relationship with the activity of the Se-dependent GSH-Px enzyme (15). This relationship was apparent in young but not old rats, in which the rate of nutrient depletion is relatively low (19). The presence or absence of vitamin E in the Se-supplemented diets of young growing rats was reflected in the activity of plasma GHS-Px and demonstrated an interaction of these two nutrients through increased enzyme activity in group B (+E+Se) vs group C (−E+Se) (Table 3). The interaction of these two nutrients as reflected by GSH-Px activity was not detected in old rats (raised on chow diet) after 8 or 20 weeks of vitamin E and/or Se supplementation. However, depression of GSH-Px activity was significant with dietary Se deficiency with or without vitamin E supplementation. A greater decline of enzyme activity might have occurred if the old rats were fed an Se-deficient diet for more than 20 weeks.

Vatassery et al. (19) have found that three-month-old F344 rats had the highest α-T concentrations in frontal cortex and the lowest in the gray matter of cerebellum. Comparable results have been reported for adult Sprague-Dawley rat and guinea pig (20). Our findings are consistent with these studies. Across all ages and dietary groups, cerebrum and midbrain were found to contain the highest concentration of α-T while cerebellum contained the lowest. Grinna (21) has reported that the need for α-T in growing rats was higher than in old rats and decreases as they reach one year of age. However, an early report showing α-T concentration in whole brain of Wistar rats decreasing with increasing age (22) suggests that the α-T requirement of brain tissue is different than those of other tissues, such as liver in which the concentration of α-T increases with age. In contrast to our findings with control-fed animals, Vatassery et al. (23) reported that α-T concentration increases significantly with age in medulla and spinal cord. However, that study used rats raised on diets with unspecified vitamin E content. In this study, old rats fed control diet (containing 30 IU vitamin E and 0.1 ppm Se) for 20 weeks showed a significant decline of α-T concentration in cerebellum and brain stem. Thirty IU of vitamin E per kg of diet is the level recommended for growing and adult rats by the National Research Council (24) when diets contain up to 5% linoleic acid and adequate sulfur-containing amino acids and Se. The basal diet (Table 1) contained only 2% unsaturated fat and was adequate in sulfur-containing amino acids. Therefore, it appears that 30 IU/kg vitamin E is not adequate for maintenance of a steady state level of α-T in brain stem and cerebellum in old rats. The plasma α-T level in young, growing rats fed the control diet was not different from that of old rats fed the same diet for the same period of time (Table 2). Thus, with regard to plasma α-T levels, 30 IU α-tocopheryl acetate meets requirements for young rats. However, brain stem and cerebellum, but not cerebrum or midbrain, of young rats fed control diet did have lower levels of α-T than 17M rats. These findings, consistent with the observations of others (19,20), indicate that brain stem and cerebellum regions of rat brain may have higher requirements for α-T. Dopaminergic regions of brain such as midbrain possess a relatively high activity of GSH-Px in caudate-putamen and substantia nigra and have a low capacity for lipid peroxidation with a potentially lower requirement for α-T (25). Nondopaminergic regions, such as the cerebellum with its low GSH-Px activity, are more susceptible to free radical attack and lipid peroxidation; thus it appears to have a high turnover of the vitamin and a greater requirement for α-T protection (25,26).

The rank order for the percent increment of regional α-T concentration in young rats fed doubly supplemented diet (+E+Se) relative to controls was cerebellum > cerebrum > brain stem > midbrain, whereas in 17M and 20M rats it was cerebellum > brain stem > cerebrum > midbrain. These results are in agreement with Vatassery et al. (23), who showed an increased level of α-T in medulla and spinal cord of rats. The increased α-T concentration in cerebellum and cerebrum of young rats could be attributed to the developmental stage of these brain regions, whereas the high increase in the cerebellum and brain stem of old rats might be related to high demand and turnover of α-T.

The differential distribution and requirement of α-T in brain regions may depend on types of neurotransmitters, phospholipids and fatty acid components involved in the physiological function of brain regions. It may also be regulated by the presence of other antioxidant defense mechanisms and by free or complex

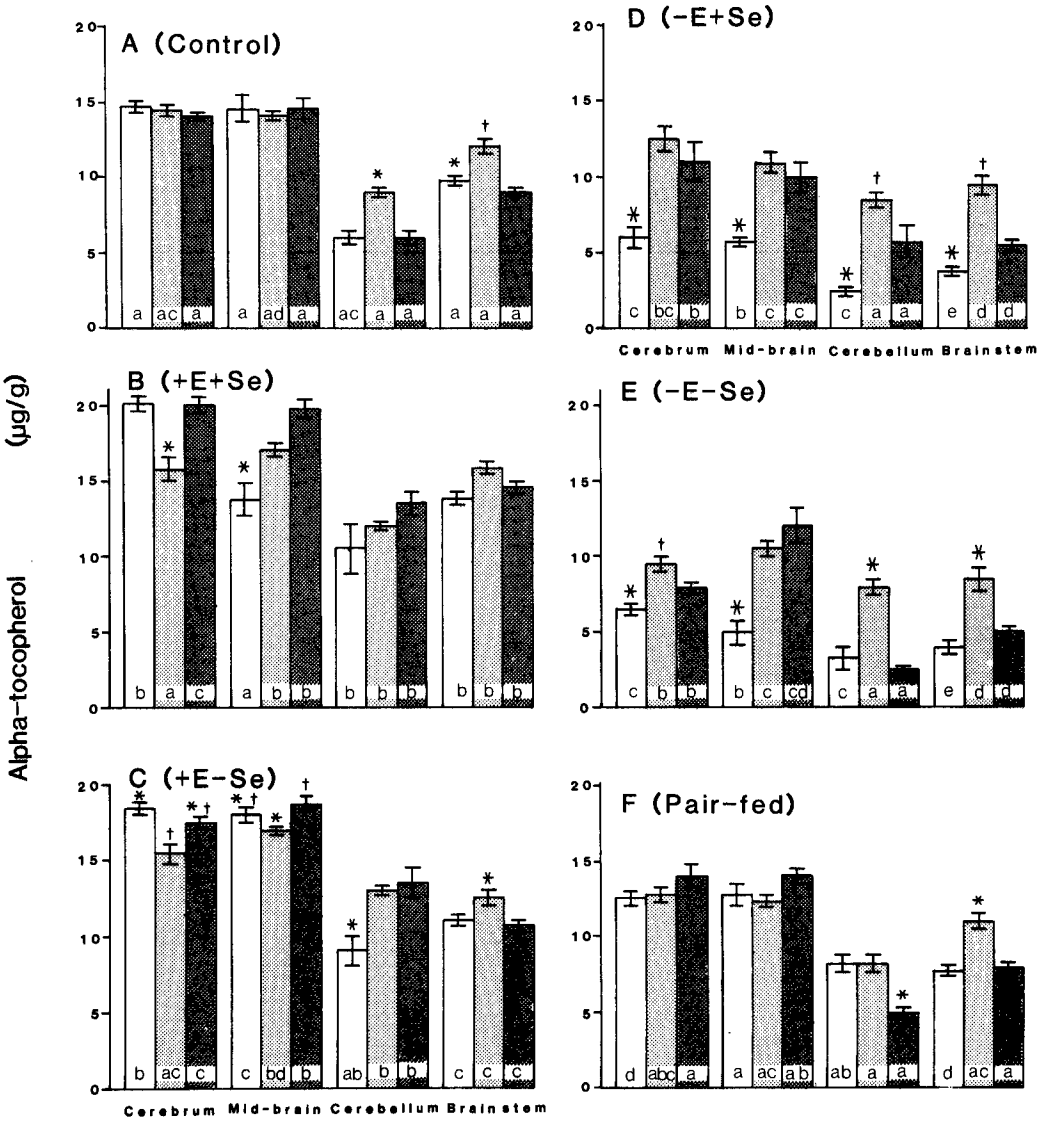

FIG. 1. Comparisons of α-T concentration in brain regions among dietary and age groups. □: Young (1-month-old, fed diet for 8 weeks); ▨: 17M (15-months-old, fed diet for 8 weeks); ▩: 20M (15-months-old, fed diet for 20 weeks). Means with different letters within a brain region and age group from different dietary treatments are significantly different ($P < 0.05$). Means with different * or † superscripts within a brain region and dietary treatment from different age groups are significantly different ($P < 0.05$).

iron with prooxidant capacity. Diet and aging may have a substantial influence on the composition and components in the CNS. Deficiency of vitamin E in the 20M rats had a more pronounced effect in cerebellum and brain stem than cerebrum and midbrain, whereas the brain of young rats did not show differences among the regions with vitamin E deficiency. These results further indicate that cerebellum and brain stem regions may have a higher turnover of α-T than cerebrum and midbrain regions and suggest older rats require more dietary vitamin E to meet the needs of these two brain regions.

Se nutriture does not appear to affect brain α-T as greatly as vitamin nutriture. However, Se deficiency slightly decreases α-T accumulation in brain regions by vitamin E supplementation (significant in brain stem of all ages). Se supplementation marginally alleviates α-T depletion from brain in dietary vitamin E deficiency.

Several reports indicate that spinocerebellar disorders such as areflexia and cerebellar ataxia are commonly associated with vitamin E deficiency in man (2,27-29). These brain regions may be more prone to vitamin E deficiency due to their higher metabolic demand for the vitamin. Our data (Figs. 1A and F) further indicate that the requirement for α-T in brain stem and cerebellum may increase with age and that recommended dietary vitamin E levels for the rat (24) may not be adequate to maintain a steady state concentration in these brain regions.

ACKNOWLEDGMENT

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METHODS

Limitations of the Method Using Peroxidase Activity of Hemoglobin for Detecting Lipid Hydroperoxides

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The method using peroxidase activity of hemoglobin (Hb) for the determination of lipid peroxides was examined by using pure methyl linoleate hydroperoxides, trilinoleoylglycerol hydroperoxides and egg yolk phosphatidylcholine hydroperoxides as substrates and tetramethyl benzidine as electron donor for the peroxidase reaction of Hb. The reactivities of these substrates were quite varied. Furthermore, some electron donors were tested for peroxidase activity of Hb, but none showed a complete reduction of methyl linoleate hydroperoxides. From these results, it seems the Hb method needs to be carefully applied to biological materials that contain mixtures of different types of lipid classes.
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Lipid hydroperoxides have attracted much attention regarding food and biological damage. Lipid peroxidation products are believed to be involved in the process of aging, mutagenicity and some diseases. Therefore, a simple microanalysis of lipid peroxides that can be applied to biological materials should be developed. In recent years, several methods have been proposed to measure lipid peroxide level in biological materials, such as the thiobarbituric acid (TBA) method (1-3), fluorometric method (4), chemiluminescence measurement (5,6) and enzymatic methods using horseradish peroxidase (7,8), hemoglobin (9-11), glutathione peroxidase (12) and activation of cyclooxygenase (13). However, these methods still have certain limitations when applied to biological materials. To clarify these limitations, it is necessary to evaluate and discuss the data obtained from biological systems.

We have been interested in the method that uses the peroxidase activity of hemoglobin (Hb) and have reported previously on some of the electron-donors which are used (10). Other papers concerning this Hb method have also appeared (9,11). When we examined this method in detail, we found some limitations in its application to biological materials. As a quantitative method for determining lipid peroxides, this method should be used only in the analysis of simple systems in which only one lipid class is to be measured. The method does not appear useful for systems containing a mixture of different lipid species.

MATERIALS AND METHODS

Materials. Hb from bovine blood-type I, methyl linoleate (99%), phosphorylcholine and methional were purchased from Sigma Chemical Co. (St. Louis, Missouri). Trilinoleoylglycerol (99%), Triton-X 100, ascorbic acid, uric acid, cysteine, DL-α-tocopherol, BHA, BHT and choline (50% in water) were purchased from Nakarai Chemical Co. (Kyoto, Japan). Tetramethyl benzidine (TMB; 4,4-di[N-dimethylamino]biphenyl) and hydroquinone were obtained from Wako Chemical Co. (Osaka, Japan). All reagents were of analytical grade and used without purification. Egg yolk phosphatidylcholine was prepared by the method described previously (14).

Preparation of lipid hydroperoxides. Methyl linoleate monohydroperoxides (15), trilinoleoylglycerol monohydroperoxides (16) and egg yolk phosphatidylcholine monohydroperoxides (14) were obtained by autoxidation or photosensitized oxidation and purified according to the method of Terao et al. (14-16).

Reduction of lipid hydroperoxides by the peroxidase activity of hemoglobin. Methyl linoleate monohydroperoxides, trilinoleoylglycerol monohydroperoxides and phosphatidylcholine monohydroperoxides were used as the substrates of peroxidase activity of Hb. TMB was used as the electron donor.

The method of Suzuki et al. (17) for the determination of peroxidase activity was used with slight modification. The reaction mixture consisted of 0.32 μmol TMB dissolved in N,N'-dimethylformamide (0.2 ml), 80 mM sodium phosphate buffer (pH 5.4) with 0.1% Triton-X 100, certain amounts of lipid hydroperoxides dissolved in 0.1 ml ethanol and 100 μg of Hb in a total volume of 3.4 ml.

The mixture was incubated for 10 min at 37 C. The colored reaction product, oxidized TMB (λ_{max}, 655 nm; ε, 5400) (18), was measured in a Shimadzu spectrophotometer (UV-240) at a wavelength of 655 nm. The amount of the colored product corresponds to the reduced amount of lipid peroxides.

Electron donors available for peroxidase reaction of hemoglobin. The reaction was carried out following the method described above except for 0.3 μmol of methyl linoleate hydroperoxides dissolved in 0.1 ml ethanol, 3 μmol of an electron donor in phosphate buffer (pH 5.4) and 200 μg of hemoglobin in a total volume of 3.4 ml.

After incubation for 10 min at 37 C, the lipid fraction was extracted three times by the method of Bligh and Dyer (19) and spotted on a Chromarod-SII apparatus. The mobile phase was composed of hexane/ethyl ether/acetic acid (8:4:0.2, v/v/v). The components separated on the Chromarod-SII were analyzed by an Iatroscan TH-10 (TLC/FID analyzer).

RESULTS

Reactivity of hemoglobin on peroxides. Purified methyl linoleate monohydroperoxides, trilinoleoylglycerol monohydroperoxides and phosphatidylcholine monohydroperoxides were used as substrates for the peroxidase reaction of Hb. The results are shown in Figure 1. The slope of the regression line for each compound showed extremely different values in a range from 1.32 to 36.15. This means that these three hydroperoxides did not react

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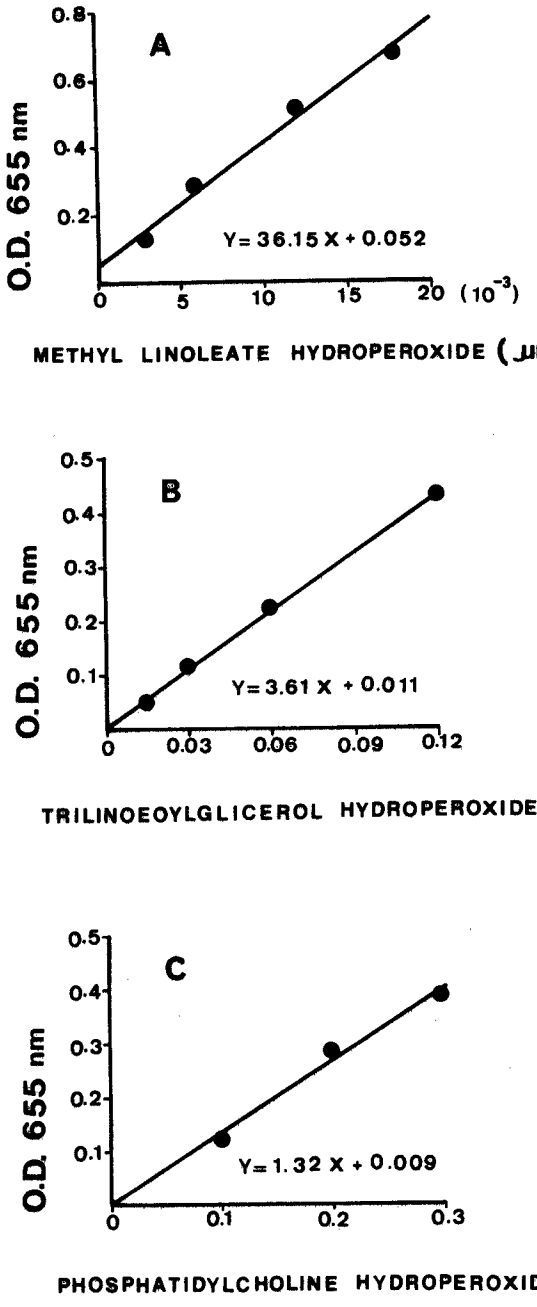

FIG. 1. Relationship between the amount of hydroperoxides and absorbance at 655 nm. Reaction conditions were the same as those described in the text. A, methyl linoleate monohydroperoxides; B, trilinoleoylglycerol monohydroperoxides; C, phosphatidylcholine monohydroperoxides. Regression line y = mx + b, where y = optical density and x = concentration of lipid peroxides.

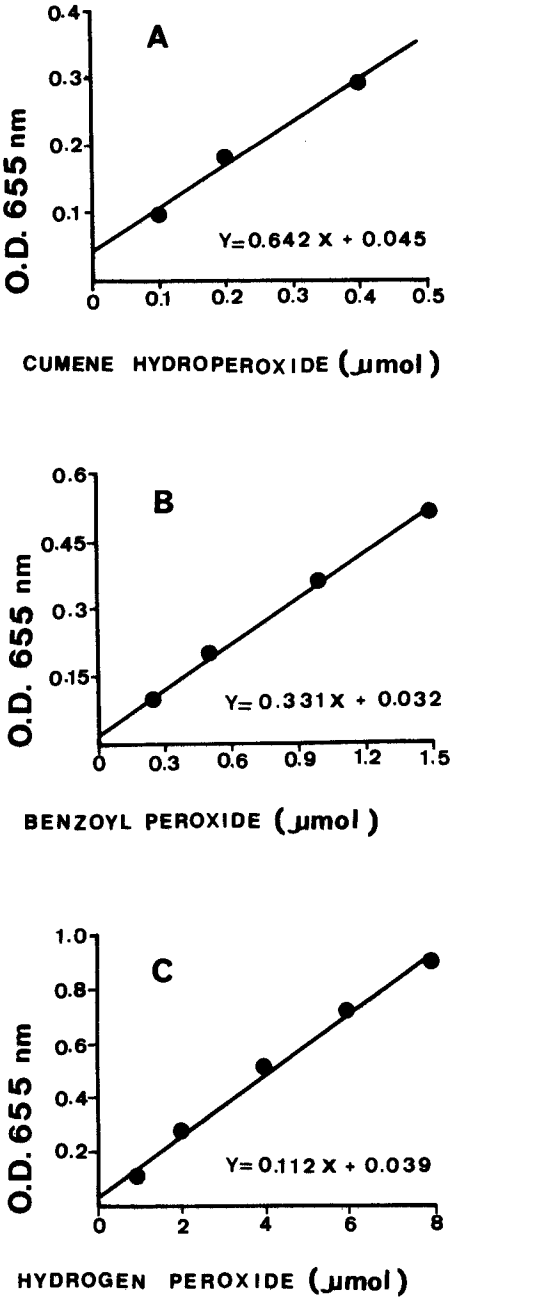

FIG. 2. Relationship between the amount of hydroperoxides and absorbance at 655 nm. Reaction conditions were the same as those described in the text. A, cumene hydroperoxide; B, benzoyl peroxide; C, hydrogen peroxide. Regression line y = mx + b, where y = optical density and x= concentration of peroxides.

similarly in this system. In order to get the same OD value (0.5) in the reaction, the amounts of methyl linoleate monohydroperoxides, trilinoleoylglycerol monohydroperoxides and phosphatidylcholine monohydroperoxides necessary were ca. 12, 150 and 500 nmol, respectively.

Other types of peroxides (cumene hydroperoxide, hydrogen peroxide, and benzoyl peroxide) also reacted differently from each other, as is shown in Figure 2.

Comparison of hydrogen donors on the peroxidase activity of hemoglobin. The electron-donating ability of some compounds was tested for the peroxidase activity of Hb. After the reaction, the reduced products, methyl hydroxy linoleate (ML-OH) from methyl linoleate hydroperoxides (ML-OOH), were determined. ML-OOH and ML-OH were separated on the Chromarod. Some of the chromatograms are shown in Figure 3 as examples. In the case of hydroquinone and ascorbic acid (Figs. 3C and 3D), ML-OH was formed, but a more polar product also was produced in the reaction. With the formation of ML-OH, therefore, a side reaction occurred during the

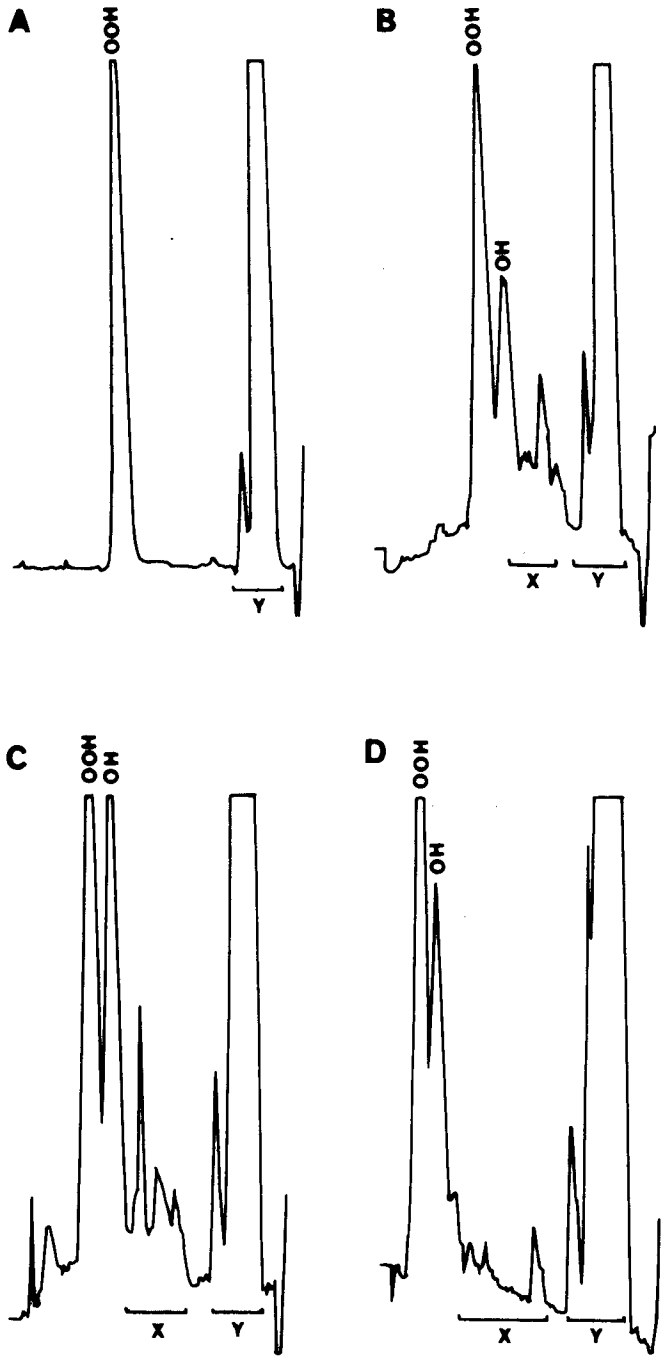

FIG. 3. Reaction products of the Hb method with methyl linoleate monohydroperoxides as the substrate and with different electron donors. Reaction conditions were the same as those described in the text. A, methyl linoleate monohydroperoxides only; B, Hb was added; C, Hb and hydroquinone were added; D, Hb and ascorbic acid were added; X, side reaction products; Y, solvent.

peroxidase reaction. The different electron donors tested and the ratio of the peak area of ML-OH to that of ML-OOH are listed in Table 1. Hb converted 36% of the ML-OOH to ML-OH without the addition of any electron donor. BHT and TMB showed higher ratios among electron donors used in this experiment. Methional showed a high ratio even in the system without Hb.

TABLE 1

Effect of Electron Donors for Hemoglobin Peroxidase Activity[a]

Added hydrogen donors	+ Hb −OH formed (%)	Without Hb −OH formed (%)
—	36	0
BHT	57	
TMB	54	
Methional	51	50
Hydroquinone	46	
Pyrogallol	46	
Ascorbic acid	36	
Uric acid	34	
BHA	32	
DL-α-tocopherol	30	18
Phosphorylcholine	26	
Iodide	21	
Choline	16	
Glutathione	12	
L-cysteine	11	

[a]The values show the relative percentage of methyl hydroxy linoleate formed and are the average of three experiments.

DISCUSSION

The TBA method is widely used to measure lipid peroxide levels in biological materials. However, the reliability of this method is questionable because of its nonspecificity for malondialdehyde and the occurrence of artifacts during test reaction. Instead of this method, a new method using the peroxidase activity of Hb has been proposed (9-11) recently to determine lipid peroxides. Hb has a broad substrate specificity for peroxidase activity and is more sensitive to such substrates than horseradish peroxidase. Lipid hydroperoxides are reduced to their hydroxy derivatives in the presence of an electron donor and Hb. Leuco-form dyes can function as hydrogen donors in this reaction and, therefore, the presence of peroxides can be determined by measuring the color produced by the oxidation reaction. Leuco-form dyes, such as a leuco-methyleneblue derivative (9), TMB (10) or the sesamol dimer (11), were used in the assay with Hb. The sensitivity of the Hb method may depend on the molecular extinction coefficient of each dye. However, the accuracy of the enzyme method depends on the substrate specificity and thus their affinity to the reaction site of Hb. As can be seen in Figures 1 and 2, the reactivities of the peroxides to Hb are different. Hb can react with fatty acid hydroperoxides at a level of several nmol. Linoleate hydroperoxides and arachidonate hydroperoxides react to a similar extent with Hb (11). However, Hb reacts with triacylglycerol hydroperoxides and phosphatidylcholine hydroperoxides to a lesser extent than fatty acid hydroperoxides. That is, a larger amount of triacylglycerol hydroperoxides and phospholipid hydroperoxides is needed to obtain the same color in the Hb reaction. Therefore, the Hb method seems more applicable to a pure lipid system rather than a biological system that contains different lipid classes. Besides, Hb itself induced decomposition of hydroperoxides when there was no electron donor, as is shown in Table 1. Although the extent of the reaction may be increased to some extent by improving the emulsification of the substrate with Hb and

the leuco-dye, the problem of steric hindrance as it affects the contact between substrates and the reaction site of Hb appears to remain.

These results indicate a need for caution in evaluating data obtained from biological systems using the Hb method.

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EDITORIAL

Lipids is celebrating its twentieth birthday. This is a time to reflect and project. *Lipids* was born in the mid-1960s amidst an unprecedented expansion in the lipid field. Since then, our journal has grown and matured, and it is facing adulthood with confidence and optimism.

Lipids was conceived through the foresight of its founding Editor, A. Richard Baldwin. The journal, from its beginning, was intended to include "many facets of basic lipid research" and was "devoted primarily to the more fundamental research on lipids." And so it all began.

Lipids is entering its third decennium with a larger format and a bright new face. The initial response to this change has been overwhelmingly positive. We trust that the new layout and page size will be welcomed by authors and readers alike and will further enhance the appeal of our journal.

A journal represents the collective efforts of many. As incoming Editor, I am most fortunate to be entrusted with the helm of a vessel of proven seaworthiness. I owe my special thanks to Dr. Ralph T. Holman, who kept our ship on course for the past eleven years, and to our Editorial Assistant, Donna Patten, for making the transition so smooth. It is comforting to know that I will be able to rely on the guidance of a distinguished Editorial Advisory Board and on the advice and experience of our Associate Editors. George Willhite, our Managing Editor, and his superb editorial staff have been instrumental in bringing about computerized typesetting and the facelift of both AOCS journals. *Lipids* enjoys the backing of a strong scientific organization. Thanks are due, in particular, to the members of the AOCS Governing Board and the Publications Committee. Dr. Baldwin's inspired and enthusiastic leadership in directing the Society's publishing endeavors deserves our special admiration and gratitude. Last, but not least, I wish to thank Dr. Robert T. Holt, Dean of the Graduate School of the University of Minnesota, for his encouragement and support.

Scientific publications are a most effective means of communicating scientific discoveries. They also serve as a record of scientific accomplishment and progress. We are called upon to disseminate scientific information that is reliable and of high quality. We are expected to do this as expeditiously as a fair and thorough peer review process will permit. A journal's success is measured by its appeal to contributors and readers. Scientists abhor trivia in their search for information. Our editorial philosophy will be guided by the desire to reach beyond the boundaries of past accomplishments. I invite your comments, criticisms and contributions.

Wolfgang J. Baumann
Editor