Properties of Diacylglycerol Acyltransferase from Spinach Leaves

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ABSTRACT

Diacylglycerol-acyltransferase (EC 2.3.1.20) was partially purified and characterized from spinach leaves. The enzyme had a pH optimum of 8.0 and activity was stimulated 2-fold by the addition of 20 mM Mn^{2+} or Mg^{2+}. Diolein and dipalmitin were examined as *sn*-1,2-diacylglycerol substrates. Only diolein gave detectable triacylglycerol synthesis. In addition, the saturation kinetics of the enzyme with 16:0-CoA, 18:0-CoA and 18:1-CoA were examined. The highest apparent Km was observed with 18:1-CoA, the lowest with 16:0-CoA. Endogenous spinach leaf glycerolipids were extracted and analyzed. The leaves contained 70 nmol triacylglycerol (g fresh weight)$^{-1}$.

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INTRODUCTION

Triacylglycerol (TG) is an important storage lipid present in the tissues of many plant and animal species (1). The pathway for TG biosynthesis is assumed to be fundamentally similar in most animal and plant tissues. In that regard, the basic steps of TG synthesis initially involve the acylation of *sn*-glycerol-3-phosphate, catalyzed by the enzyme glycerophosphate acyl-transferase (EC 2.3.1.15) and the acylation of lysophosphatidic acid by 1-acylglycerol phosphate-acyltransferase (EC 2.3.1.51). The subsequent formation of *sn*-1,2-diacylglycerol (DG) is catalyzed by the enzyme phosphatidate phosphatase (EC 3.1.3.4.). The enzyme diacylglycerol acyltransferase (EC 2.3.1.20) catalyzes the synthesis of TG from *sn*-1,2-DG and an acyl-thioester donor (2).

Diacylglycerol acyltransferase (DGAT) is the only enzyme that is unique to the TG biosynthetic pathway. The DGAT reaction has been characterized extensively in various mammalian tissues. Activities recently have been demonstrated in the microsomal fractions of rat adipocytes (3), rat liver (4), bovine mammary gland (5), and pig perinephric adipose tissue (6). Partially purified DGAT from animal tissues generally exhibits rather broad specificity for acyl-thioester substrates. Discrimination, however, may be observed in the propensity of certain acyl-thioesters esterified at the *sn*-3 position of TG. In such cases, the apparent differential utilization of the acyl-thioester substrates by DGAT is not readily correlated to the critical micelle concentrations of the substrates (7). Although the endogenous concentration of specific acyl-thioester substrates utilized in TG biosynthesis may in part determine the acyl composition of TG at the *sn*-3-position, differential affinities for various substrates by DGAT could also be a factor in the mediation of acyl composition in TG. In that regard, the regulation of TG fatty acid composition may occur at the level of DGAT and has been proposed to be under specific genetic control in plant tissues (8-11).

Evidence for genetic control of TG composition in plants has been demonstrated most frequently through investigations of genetic variability for fatty acid composition in a number of agronomic crops (12). Although TG biosynthesis in plant tissues is well documented (13-16), the kinetic properties of DGAT in plant tissues have not been described. Hence, a direct establishment of the biochemical basis for genetic control of plant TG composition with respect to the role of DGAT cannot be made at this time. In order to assess the probability that TG fatty acid composition is regulated by, or that genetic variability for DGAT activity exists within a given plant species, the solubilization, purification, and characterization of DGAT from a plant source must be demonstrated. As a prelude to such studies, information concerning the nature of DGAT activity from spinach leaf tissue is presented in this report.

MATERIALS AND METHODS

Tissue Preparation

Spinach (*Spinacia oleracea* L., cv Long Standing Bloomsdale) plants were grown in a greenhouse under a 12-hr photoperiod. Day/night temperatures were 22/17 C. Fully expanded leaves were harvested and chilled to 4 C for all subsequent operations. Leaf tissue, 60 g fresh weight, was chopped with an electric razor knife in 100 ml of buffered grinding

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medium. The grinding medium contained 50 mM Bicine (N,N-*bis* [2-hydroxyethyl glycine)], pH 8.0, 20 mM $MgCl_2$, 0.4 M sucrose, 10 mM mercaptoethanol and 2% (w/v) polyvinyl polypyrrolidone. $MgCl_2$ was omitted and 1 mM Na EDTA was added to the grinding buffer to determine the effects of divalent ions on enzyme activity. The homogenate was filtered through 4 layers of cheesecloth and one layer of Miracloth. The filtrate was centrifuged at 20,000 × g for 20 min at 4 C. The supernatant obtained was centrifuged at 100,000 × g for 1 hr. The pellet was suspended in 1 ml 25 mM Bicine, pH 8.0, 10 mM mercaptoethanol and 20 mM $MgCl_2$ buffer. Pellets were gently dispersed with a Ten-Broeck tissue homogenizer. The suspensions were used immediately, or frozen with liquid N_2.

Solubilization of DGAT

The pellet obtained as described above, was suspended in buffer containing 25 mM Bicine, pH 8.0, 20 mM $MgCl_2$ and 10 mM mercaptoethanol saturated with KCl at 4 C. After stirring for 30 min, the suspension was centrifuged at 20,000 × g for 20 min. This procedure was repeated twice and the supernatant fractions were combined and dialyzed exhaustively against 25 mM Bicine, pH 8.0. After dialysis, the solution was centrifuged at 20,000 × g for 20 min. The pellet obtained was resuspended in a buffered solution containing 25 mM Bicine, pH 8.0, 20 mM $MgCl_2$ and 0.35 M KCl and was centrifuged at 100,000 × g for 1 hr. Soluble enzyme activities were assayed from the resulting supernatant fraction. This procedure resulted in a recovery of about 20% of the original enzyme activity. Protein concentrations were determined by a modification of the Lowry method (17).

Glycerolipid Analysis

The lipid composition of whole spinach leaves was determined by the following procedures. Tissue (10 g fresh weight) was homogenized consecutively with a Brinkman Polytron and Ten-Broeck tissue grinder in 40 ml chloroform/methanol (2:1, v/v). The homogenate was filtered with an additional 20 ml chloroform/methanol (2:1, v/v) and 30 ml methanol. After filtration, 50 ml deionized water was added to the filtrate. The mixture was shaken and centrifuged at 1000 × g for 20 min to form a biphasic solution. The phase containing lipids was dried in vacuo and stored in 2 ml chloroform/methanol (2:1, v/v). Polar lipids (TPL), DG, and TG were separated from total lipid extracts by thin layer chromatography (TLC) using petroleum ether/diethyl ether/glacial acetic acid (70:30:1, v/v/v) as the developing solvent. Glycerolipids were extracted from the TLC gels after transfer to 5-ml disposable pipette tips plugged with glass wool, and elution with 3 ml each of chloroform/methanol (2:1, v/v), chloroform/methanol (1:2, v/v), and methanol. All filtrate volumes were reduced under nitrogen. Fatty acid methyl esters were prepared from each glycerolipid fraction by the addition of 0.1 ml Meth-Prep II (Applied Science, Division of Milton Roy, Inc.) in 1 ml toluene. Gas chromatographic (GC) analysis of the methyl ester preparations and lipid quantification was conducted as previously described (11).

DGAT Assay

Assays were performed in culture tubes (13 × 100 mm) at 30 C in a final volume of 200 µl. The reaction mixture contained 50 mM Bicine, pH 8.0, 25 µM [1-^{14}C]acyl-thioester (16:0, 18:0 or 18:1; sp act 2.5 to 5.0 mCi.mMol^{-1}), 0.4 mM *sn*-1,2-diolein, 0.7 mM Zwittergent 2-08 (Calbiochem Inc.), 10 mM $MgCl_2$ and 0-30 µg protein. The reaction was initiated by addition of the radioactive acyl-thioester. Variations of these conditions were noted in the appropriate figure legends. BSA (1 mg/ml) gave slight stimulation of DGAT activity but was not used generally because of possible chemical interaction with various acyl-CoA thioesters. The reaction was terminated by addition of 2 ml chloroform/methanol (1:1, v/v). After 5 min, 0.7 ml deionized water was added and the phases were blended with a vortex mixer. The mixture was centrifuged at 1000 × g for 10 min. The upper phase (methanol/water) was discarded and the chloroform phase was dried under a stream of nitrogen. TG was isolated and recovered from the chloroform fraction by TLC as previously described. TLC gel containing radioactive TG was scraped into vials containing 10 ml of Aquagel scintillation cocktail (Packard). Radioactivity was determined by liquid scintillation with channel ratio quench correction.

RESULTS

Factors Affecting DGAT Activity

Freshly prepared enzyme suspensions from spinach leaf tissue lost ca. 50% of initial DGAT activity when stored for 24 hr at 4 C. The activity was stabilized, however, when frozen with liquid N_2. Representative DGAT activities could be maintained for as long as 6 months when handled in that manner (data not shown). The temperature optimum for spinach DGAT

activity assayed as described in Materials and Methods occurred between 25 and 30 C (Fig. 1), and the optimum pH was 8.0 (Fig. 2).

Enzyme preparations isolated in the absence of Mg^{2+} or Mn^{2+} ions demonstrated a stimulation of TG synthesis with the addition of 10 mM $MgCl_2$ or $MnSO_4$ (Fig. 3). No evidence for a divalent ion effect could be obtained without the deletion of Mg^{2+} or Mn^{2+} from the isolation procedure. Similar concentrations of Ca^{2+} or Cu^{2+} ions had no effect upon enzyme activity.

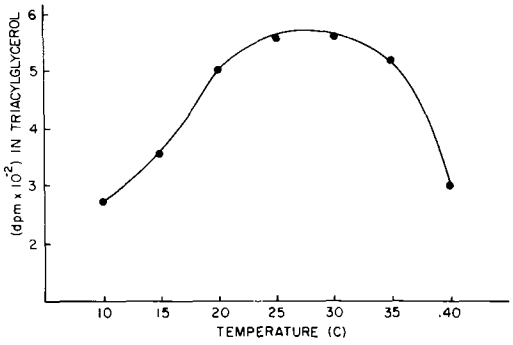

FIG. 1. DGAT activity in response to temperature. The assays were performed with enzyme from the 100,000 × g pellet as described, for 10 min with [1-^{14}C] 18:1 CoA. Samples were preincubated for 5 min at the appropriate temperature before initiation of the reaction.

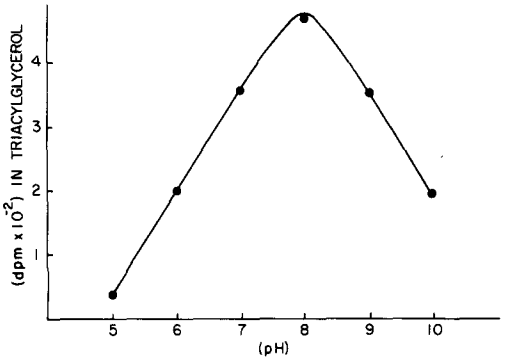

FIG. 2. Dependence of DGAT on pH. The assays were performed using [1-^{14}C] 18.1 CoA and enzyme from the 100,000 × g pellet as described, except MES buffer was used from pH 5.0–6.0, BES from pH 7.0–8.0 and AMPSO from pH 9.0–10.0

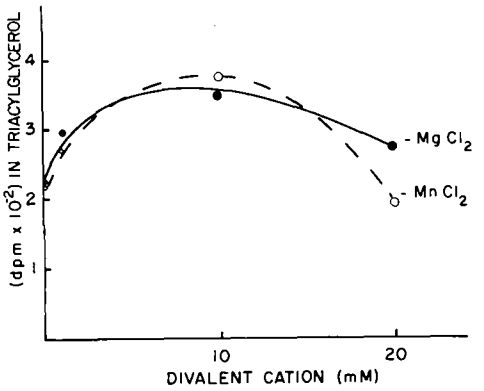

FIG. 3. Effect of Mg^{2+} and Mn^{2+} on DGAT. The assays were performed using [1-^{14}C] 18:1 CoA and enzyme from the 100,000 × g pellet as described. The enzyme was prepared in the absence of $MgCl_2$ or $MnCl_2$.

Because of the hydrophobic nature of diacylglycerol, selected treatments were necessary to increase substrate solubility. The effects of various agents upon DGAT activity in the presence of diolein was determined (Table 1). Maximal activity was obtained with Zwittergent 2-08.

Effect of DG upon TG Formation

The presence of endogenous DG in enzyme suspensions obtained by centrifugation was determined by TLC and GC analysis. Endogenous DG concentrations were less than 1.0 mM. The relative utilization of exogenous DG required solubilization of the DGAT protein

TABLE 1

Effect of Agents Used to Solubilize Diolein Upon DGAT Activity

Agent	Concentration	TG Synthesis
	%[a]	dpm[b]
Ethanol	5.0 (v/v)	47
Triton X-100	0.02 (w/v)	0
Na-cholate	0.02 (w/v)	0
Zwittergent 2-08	0.02 (w/v)	550
Zwittergent 3-10	0.02 (w/v)	300
Zwittergent 3-12	0.02 (w/v)	87
Zwittergent 3-14	0.02 (w/v)	104
Zwittergent 3-16	0.02 (w/v)	145

[a]Final concentration.

[b]Radioactivity in TG per 20 min reaction.

complex to remove endogenous DG. Enzyme obtained in this manner had a specific activity of 0.6 to 2.0 nmol min^{-1} [mg protein]$^{-1}$. DGAT activity in crude extracts was only 0.05 to 0.06 nmol min^{-1} [mg protein]$^{-1}$. Hence, enzyme obtained by salt solubilization as described in Methods represented a 10- to 40-fold purification and was used to determine diolein-dependent formation of TG (Fig. 4). Dipalmitin was also tested as a DG substrate for DGAT. Dipalmitin, however, was a poor substrate, in that no TG formation was observed with concentrations up to 500 μM.

DGAT Utilization of Acyl-Thioester Substrates

Kinetics for substrate saturation were determined for acyl-thioesters using radioactive 16:0-CoA, 18:0-CoA, or 18:1-CoA with *sn*-1,2-diolein (Fig. 5). With each of the respective acyl-thioester substrates, saturation occurred at about 20 μM. Slight inhibition was noted at acyl-thioester concentrations above 25 μM. A comparison of the relative affinities between the acyl-thioesters tested was determined from a Lineweaver-Burk plot (Fig. 6). The apparent Km values derived from these data were as follows: 16:0:CoA (3.0 μM), 18:0-CoA (7.0 μM), and 18:1-CoA (28 μM). Similar results were obtained from an Eadie-Hofstee plot (18).

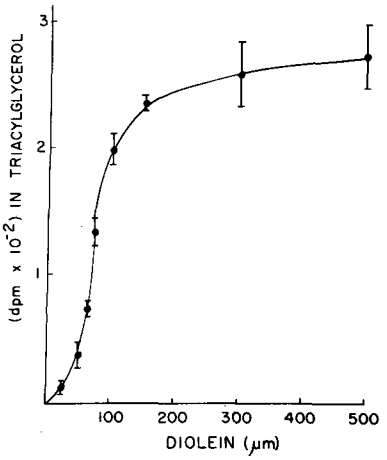

FIG. 4. Saturation kinetics of DGAT with diolein. The assays were performed using [1-^{14}C]18:1 CoA as an acyl donor as described for 20 min at 30 C. The enzyme was solubilized with 25 mM Bicine pH 8.0 saturated with KCl. Diolein in 0.2% (w/v) Zwittergent 02-8 was prepared by sonication, and centrifugation at 200,000 X g for 1 hr. The supernatant was used in the respective assays. The concentration of diolein in the supernatant was determined by GC as described in Methods.

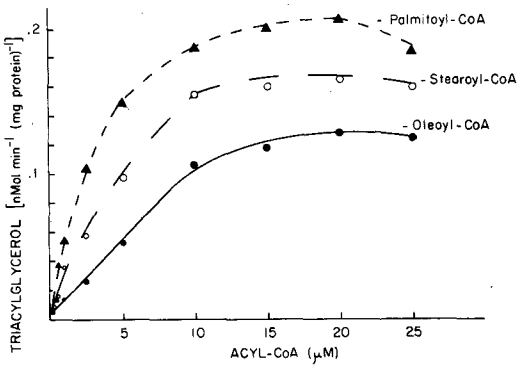

FIG. 5. Saturation kinetics of DGAT with acyl-CoA. The assays were performed as in Methods with boiled enzyme as a blank.

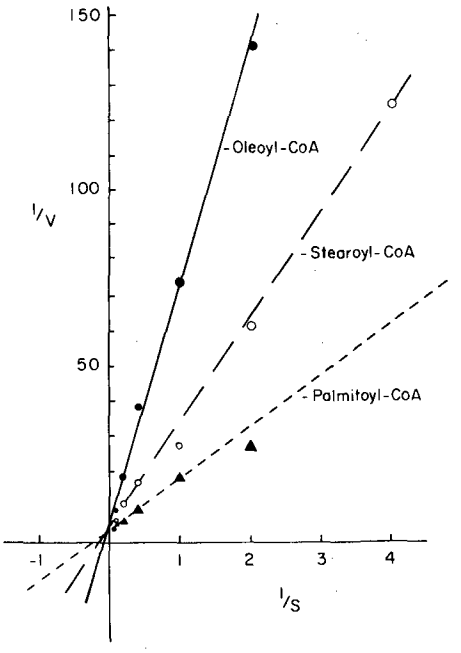

FIG. 6. Lineweaver-Burk plot of DGAT saturation kinetics with acyl-CoA. The data was plotted according to a linear regression analysis. The Km listed in Discussion are in agreement with those determined from an Eadie-Hofstee plot. Only data from 20 μM acyl-CoA and below was used for these calculations.

Glycerolipid Analysis

The fatty acid composition of the major glycerolipid classes present in spinach leaf tissue is shown in Table 2. The TPL (predominantly glycolipids), DG, and TG fractions accounted for 93.3, 5.1, and 1.6% of the spinach leaf glycerolipids, respectively.

TABLE 2

Endogenous Composition of Spinach Leaf Glycerolipids

Lipid[a]	Fatty acid							Total
	16:0	16:1	16:3	18:0	18:1	18:2	18:3	
	mol %							μmol / 10 g fresh wt
TPL	19.1	5.9	4.6	1.6	9.2	15.3	43.5	38.83
DG	9.9	1.8	0.0	6.4	18.5	8.1	55.3	2.13
TG	21.5	2.8	0.0	6.0	16.5	22.5	30.7	0.68
Total	19.4	5.6	4.3	1.9	9.8	15.0	44.0	41.64

[a]TPL, total polar lipid; DG, diacylglycerol; TG, triacylglycerol.

DISCUSSION

To our knowledge, DGAT has not been characterized previously from any plant tissue. A preliminary attempt to characterize DGAT from plants was conducted with developing soybean cotyledons, however, the nature of that tissue was not conducive to the establishment of reaction conditions. Although the metabolic function and origin of TG in leaf tissue has not been demonstrated and could be dissimilar to that in developing oilseeds, the observation of TG in spinach leaf tissue suggested the presence of DGAT. The DGAT purification and solubilization scheme employed with spinach leaf tissue was successful. Hence, the first attempt to develop an assay for DGAT activity in plant tissues was conducted with spinach leaves.

Differential centrifugation of spinach leaf homogenates yield a number of membrane fractions containing DGAT activity. Among those fractions, the 100,000 x g pellet contained the highest specific activity and thus was used in subsequent experiments. Salt solubilized DGAT from spinach leaf accepted diolein but not dipalmitin as a diacylglycerol substrate. The difference in utilization of the diacylglycerol compounds as substrates was similar to that reported for microsomal DGAT from rat adipose tissue (3). Because of the physical limitations of lipoidal substrates in an aqueous reaction, agents were employed to enhance the solubility of diolein. Assays for DGAT from animal sources (3) indicated that ethanol, Triton X-100, or Na-cholate were potential dispersal agents. With the preparation from spinach leaves, however, those agents did not give satisfactory results. Diolein dispersed in a 0.02% (w/v) solution of the detergent Zwittergent 2-08 enhanced the activity of DGAT about 12.0 times over that obtained with ethanol.

The utilization of the acyl-thioesters, 16:0-CoA, 18:0-CoA, and 18:1-CoA, by spinach DGAT was similar to the relative substrate affinities described for microsomal preparations from adipose tissue (3). In addition, the relative utilization of those substrates did not appear to be related to the respective critical micelle concentrations (19). The apparent inhibition of spinach DGAT by acyl-thioester concentrations above 20 μM was similar to that reported for partially purified DGAT from rat liver microsomes (20) and could be related to micelle formation. Recent data has suggested that 16:0-CoA formed micelles at concentrations between 30 to 60 μM (21). Crude microsomal preparations, however, did not exhibit that phenomenon (3).

Endogenous acyl-CoA analysis of spinach leaves (22) indicated that 16:0-CoA (25.9%) and 18:1-CoA (38.4%) accounted for a major portion (64.3%) of the total acyl-CoA pool. The levels of those acyl-thioesters coupled with the apparent Km values found in this report suggested that DGAT substrate specificity could be a factor in determining TG fatty acid composition. Additional work is being conducted to clarify that interaction and to localize DGAT activity in subcellular fractions from spinach leaves. Preliminary results have indicated that spinach DGAT activity is present in chloroplast envelopes and in the fat layer or oil bodies from spinach leaf homogenates (23). In that regard, the pH optimum of spinach DGAT activity was similar to that of other enzymes of the glycerolipid pathway in spinach chloroplasts (24). Hence, a possible function for this enzyme in leaves might be synthesis of intraplastidic oil bodies which have been observed in micrographs of chloroplasts from many tissues (25).

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Effect of Environmental Temperature Changes on Rat Liver Fatty Acid Desaturases

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ABSTRACT

Female rats warm-adapted at 30-32 C for 20-25 days and then shifted to 13-15 C for 12, 24, 48, 72 and 120 hr showed that Δ9 desaturase and fatty acid synthetase activity decay after 24 hr of cold exposure, while Δ6 and Δ5 desaturases were increased after this period of time. These results were confirmed by an increase of arachidonic acid of heart and liver microsomes phosphatidylcholine and a decrease of oleic acid. Neither NADH-cyt b_5 reductase nor NADH-cyt c reductase activity of liver microsomes were significantly affected. Male rats warm-adapted under the same conditions and then shifted to 13-15 C for 120 hr did not show significant changes in fatty acid synthetase, Δ9 and Δ6 desaturases and enzymes of the microsomal electron transport chain. Therefore, the desaturase response to environmental temperature changes could be plausibly linked to female hormones.
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INTRODUCTION

Good evidence has been gathered showing the effects of environmental temperature on unsaturated fatty acid biosynthesis in microorganisms (1,2). Martin et al. (3) and Skrivers and Thompson (4) have made contributions concerning temperature changes in *Tetrahymena piriformis*, which alter the fatty acid desaturation activity of endoplasmic reticulum membrane. In this organism, the endoplasmic reticulum would constitute a kind of self-regulated system for maintaining an optimal physical state by means of activation or deactivation of desaturation reactions. In more evolved poikilothermic organisms as fish, Torrengo and Brenner (5) have shown that the temperature acclimation of *Pimelodus maculatus* from 38 C to 18 C increases the specific activity of Δ6 desaturase.

Although the effect of temperature on membrane fluidity is not obvious in homeothermic animals as it is in poikilotherms, other reactions may be triggered by a change of environmental temperature altering the unsaturated fatty acid biosynthesis. In 1974, Peluffo and Brenner (6) showed in the rat that Δ6 and Δ9 desaturases are not only diet-dependent enzymes but also change their activity according to seasons. Other authors have demonstrated that cold-exposed homeotherms show an increase on catecholamine levels (7,8), oxygen consumption (9,10), oxidations (11,12), thermal generation (13,14), glucagon and free fatty acids (15, 16). Cold exposure also affects prostaglandins (17), corticosteroids (18), cyclic AMP (19) and enzyme levels in mitochondria, peroxisomes and lysosomes (20).

Taking into account the above information, we are interested to study the effect of environmental temperature on the saturated and polyunsaturated fatty acid biosynthesis in the female rat.

MATERIALS AND METHODS

[1-^{14}C]Palmitic acid (56 mCi/mmol), [1-^{14}C]linoleic acid (55 mCi/mmol), [1-^{14}C]-eicosa-8,11,14-trienoic acid (61 mCi/mmol) were provided by New England Nuclear, Boston, MA. Cytochrome c was provided by Sigma Chemical Company Inc., St. Louis, MO. Cofactors for enzyme reactions were purchased from Boehringer Argentina, Buenos Aires, Argentina.

Animal Treatment

Thirty female Wistar rats, 130-150 g weight, were divided into groups of 5 animals each, and were placed in a warm room at 30-32 C for 20-25 days under purina chow diet and water, ad libitum. After that period of time, 5 groups were placed in a temperature-controlled chamber at 13-15 C for 12, 24, 48, 72 and 120 hr, respectively, under the same diet conditions.

Ten male Wistar rats were divided in 2 groups of 5 animals each and were warm-adapted at 30-32 C for the same period of time and then one of them was placed in the cold chamber for

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120 hr. After these periods of time, all the animals were killed at 7 A.M. to avoid circadian effects. Livers were homogenized in a solution containing 0.25 M sucrose, 0.15 M KCl, 62 mM phosphate buffer (pH 7) and 1.5 mM glutathione. The homogenate was centrifuged at 10,000 x g for 20 min, the pellet was discarded and the supernatant was centrifuged again at 110,000 x g during 60 min to obtain the microsomes and cytosol.

Enzyme Assays

Fatty acid desaturase assay was performed using 50 nmol of labeled palmitic, linoleic or eicosa-8,11,14-trienoic acids. Each acid was incubated with 3 mg of microsomal protein at 35 C for 10 min. In these conditions, the enzymes were saturated by the substrate. The incubation solution contained: 0.25 M sucrose, 0.15 M KCl, 0.04 M phosphate buffer (pH 7.0), 1.5 mM glutathione, 0.04 M KF, 1.3 mM ATP, 0.06 mM CoA, 0.87 mM NADH, 5 mM $MgCl_2$ and 0.33 mM nicotinamide in final volume of 1.6 ml. Fatty acids were saponified, esterified and the conversion was measured by gas liquid radiochromatography in a Packard apparatus with a proportional counter (21). NADH-cyt b_5 reductase activity was measured by NADH oxidation at 340 nm, using potassium ferricyanide as terminal electron acceptor. The reaction mixture contained ferricyanide (70 nmol), microsomal protein (2-10 μg) and NADH (30 nmol) in a final volume of 0.27 ml of 0.05 M Tris acetate (pH 8.1), 1 mM EDTA. An extinction coefficient of 6.22 $mM^{-1} \times cm^{-1}$ was used. NADH cyt c reductase activity was measured at 550 nm using cytochrome c as a terminal electron acceptor. The reaction mixture contained 20 nmol of cyt c, 30 nmol of NADH and 2-10 μg of microsomal protein in a final volume of 0.27 ml of 0.05 M Tris acetate (pH 8.1), 1 mM EDTA. The absorption increase at 550 nm was followed as a function of time. An extinction coefficient of 18.5 $mM^{-1} \times cm^{-1}$ was used.

The fatty acid synthetase activity was assayed by the method of Bruckdorfer et al. (22) measuring the NADPH oxidation at 340 nm.

Phosphatidylcholine Fatty Acid Analyses

Liver, heart and liver microsomal total lipids were extracted, phosphatidylcholine isolated and methyl esters were prepared and analyzed by gas liquid chromatography (GLC) in a Hewlett-Packard 5840-A gas chromatograph equipped with the 5840-A GC terminal and using a 6-ft column filled with 10% Sp 2330 on 100-200 Chromosorb WAW (23).

RESULTS AND DISCUSSION

Liver fatty acid desaturase activity is modified by several factors such as diets, hormones (24), cytosolic soluble proteins (25), ethanol (26) and clofibrate (27). In this experiment, the effect of environmental temperature variations is described. Figure 1 shows that Δ9 desaturase conversion increases in a nonstatistically significant way during the first 12 hr of temperature, shifting from 30-32 C to 13-15 C, being followed by a marked decrease. At 48 hr, the Δ9 desaturase reaches the lowest value that remains constant after this time. Similar behavior was shown by the fatty acid synthetase that also decreases but reaching the lowest value with a slight delay of 24 hr. The similarity of Δ9 desaturase and fatty acid synthetase behavior promoted by diet modification has already been pointed out by Jeffcoat and James (28) and other authors (26,27), and the present results are new evidence that the activity of both enzymes may be intimately related. This decrease of Δ9 desaturation is confirmed by the decrease of oleic acid shown on the fatty acid composition of both heart and liver microsomes phosphatidylcholine (Table 1).

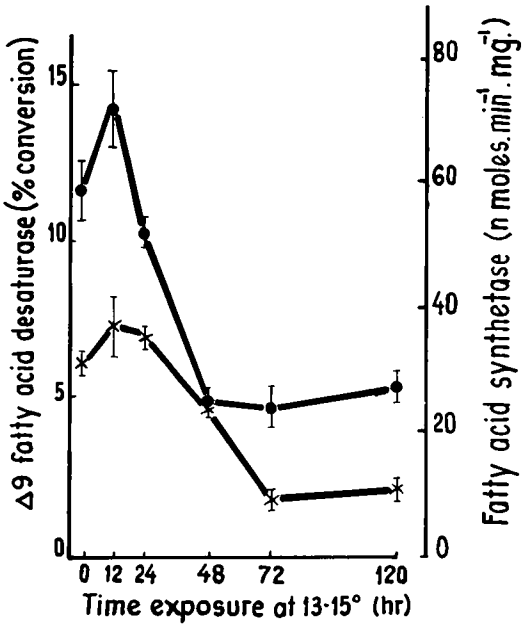

FIG. 1. Fatty acid synthetase (X—X) and Δ9 desaturase (•—•) variations in female rat liver previously warm-adapted (20-25 days at 30-32 C) and then shifted to 13-15 C for 12, 24, 48, 72 and 120 hr. Results are the mean of 5 samples ± SE.

TABLE 1

Liver Microsomes and Heart Phosphatidylcholine Fatty Acid Composition of Female Rats Exposed at 30-32 C for 20 Days and Then Shifted to 13-15 C for 120 hr

Fatty acids	Liver microsomes		Heart	
	30-32 C	13-15 C	30-32 C	13-15 C
16:0	25.6 ± 0.5	19.0 ± 0.3[b]	19.3 ± 0.4	18.0 ± 0.8
16:1	2.6 ± 0.1	2.3 ± 0.5	1.5 ± 0.2	1.2 ± 0.1
18:0	28.8 ± 0.6	31.4 ± 0.2[a]	26.0 ± 0.6	25.5 ± 1.0
18:1	12.9 ± 0.4	10.1 ± 0.1[b]	13.0 ± 0.3	10.4 ± 0.3[b]
18:2(ω6)	7.9 ± 0.2	8.5 ± 0.3	14.1 ± 0.8	9.1 ± 0.7[a]
20:3(ω6)	1.9 ± 0.1	1.2 ± 0.1[a]	–	–
20:4(ω6)	12.6 ± 0.9	20.7 ± 0.7[b]	20.9 ± 0.7	27.6 ± 0.9[b]
22:5(ω3)	1.9 ± 0.5	0.9 ± 0.2	1.6 ± 0.1	2.8 ± 0.4
22:6(ω3)	5.8 ± 0.6	5.9 ± 0.2	3.6 ± 0.3	5.4 ± 1.0

Minor components were not considered. Results are the mean of 5 samples ± 1 SE. 30-32 C vs 13-15 C: [a]$p < 0.01$, [b]$p < 0.001$.

The decreased environmental temperature effect on the conversion of 18:2 to 18:3 by Δ6 desaturase was completely different since the conversion increased progressively according to the time of cold exposure after a lag period of 24 hr. A similar effect was shown on Δ5 desaturase, since the conversion of 20:3 to 20:4 was also increased after a lag period of 24 hr. However, a plateau of maximal conversion was reached at 48 hr (Fig. 2).

The activity of enzymes of the microsomal electron transport chain such as NADH-cyt b_5 reductase and NADH-cyt c reductase, which are involved in the fatty acid desaturation reaction, were not modified significantly (Fig. 3) by the environmental temperature shift. Therefore, it may be admitted that the effect would be exerted on the terminal fatty acid desaturase and, whatever the mechanism of desaturase temperature-dependence might be, the results mentioned above show that saturated and monounsaturated fatty acid synthesis is diminished, while that of polyunsaturated fatty acids is increased, when warm-adapted female rats are shifted from 30-32 C to 13-15 C.

Since the analysis of the fatty acid composition of phospholipids by GLC may be used to show the status of a desaturase more precisely than does its enzymatic assay (29), the fatty acid composition of the phosphatidylcholine of liver microsomes and heart was also investigated. In our experiments, the increase of Δ6 and Δ5 fatty acid desaturation suggested an increase of arachidonic acid. The total lipid fatty acid composition of microsomal membrane (not reported here) showed only a small increase of arachidonic acid that was not statistically significant. However, the increase of

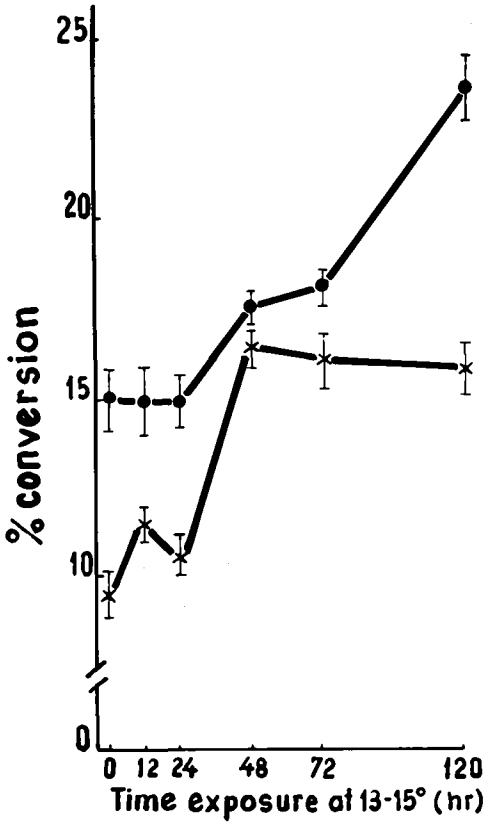

FIG. 2. Δ6 (• –•) and Δ5 (X --X) desaturase variations in female rat liver microsomes previously warm-adapted (20-25 days at 30-32 C) and then shifted to 13-15 C for 12, 24, 48, 72 and 120 hr. Results are the mean of 5 samples ± SE.

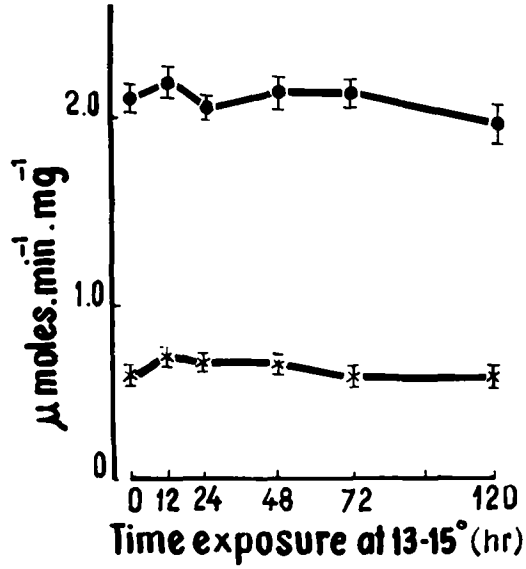

FIG. 3. NADH-cyt b_5 reductase (•—•) and NADH-cyt c reductase (×—×) activity variations in female rat liver microsomes warm-adapted (20-25 days at 30-32 C) and then shifted to 13-15 C; for 12, 24, 48, 72 and 120 hr. Results are the mean of 5 samples ± SE.

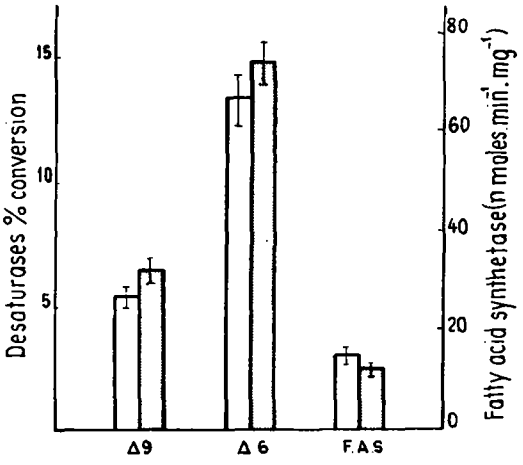

FIG. 4. Fatty acid synthetase, Δ9 and Δ6 desaturase variations in male rat liver warm-adapted (20-25 days at 30-32 C) (light bars) and then shifted to 13-15 C for 120 hr (heavy-strippled bars). Results are the mean of 5 samples ± SE.

arachidonic acid was statistically significant when the fatty acid composition of phosphatidylcholine was investigated in liver microsomes and heart (Table 1).

It is interesting that the changes of Δ9 and Δ6 fatty acid desaturase activity found in female rats were not present in males (Fig. 4). This result would suggest that female sex hormones could be involved in the temperature effect, and although it is too early to predict a mechanism for the process, it is important to consider Holloway's contribution showing that estradiol may alter microsomal Δ9 fatty acid desaturase in the rooster (30). This research is in progress.

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Lipid and Fatty Acid Characterization and Metabolism in the Sea Anemone *Phymactis clematis* (Dana)

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ABSTRACT

Neutral lipid, phospholipids and fatty acids of the sea anemone *Phymactis clematis* from the southwest Atlantic were characterized and quantified in spring and autumn. Neutral lipids predominated over phospholipids in both seasons. Triacylglycerol and diacylglycerol ethers were the major lipids. In spring, an increase of esterified sterols was noted. The major fatty acids found were 22:5ω3, 20:5ω3 and 16:0. The sea anemones were also incubated in vivo with either [1-^{14}C]linoleate or [1-^{14}C]α-linolenate for 2 hr. Isotope incorporation into lipids and their transformations into higher fatty acids were examined. Both precursors were incorporated into the lipids, mainly in triacylglycerols and monoacylglycerols, while α-linolenate was also incorporated into phospholipids. The radioactive linoleate was elongated to 20:2, 22:2 and 24:2 fatty acids, but not desaturated to 18:3ω6. α-Linolenate was desaturated by Δ6 desaturase to 18:4ω3. The specificity of Δ6-desaturase is discussed.
Lipids 18:12-17, 1983.

INTRODUCTION

The sea anemone *Phymactis clematis* (Dana) has a large geographical distribution along the Pacific coast of America from Baja California to Tierra del Fuego. In the southwest Atlantic, this species is an important member of the anemono-fauna in the coast of Buenos Aires province, with specimens showing a mean size of 2.7 cm wide and 3.7 cm long and generally containing symbiont zooxanthellae (1).

In the present work, neutral lipids, phospholipids and fatty acids of *P. clematis* from the southwest Atlantic coast were studied and characterized. The biotransformation of linoleic and α-linolenic acids as well as their incorporation into lipids by the combined algae host symbiotic system are reported.

MATERIALS AND METHODS

Organisms

Specimens of *P. clematis* were collected from rocks after tide retreat in the Atlantic coast near Mar del Plata, province of Buenos Aires, Argentina. All had endosymbiotic zooxanthellae. A group of 8 anemones was collected in each of autumn and spring, and were divided into two lots of 4. Each lot was processed independently to obtain total lipids.

Other groups were transported to the laboratory in vessels containing sea water. They were kept in an aquarium with aerated and filtered sea water for 1 week before the experiments were performed and were continuously illuminated with a 25-watt tungsten lamp at 30 cm from the surface.

Lipid Extraction and Analysis

Tissues were homogenized and total lipids were extracted according to the method described by Folch et al. (2) and weighed.

Total lipids were separated by silicic acid absorption into polar and neutral lipids (3). Each fraction was analyzed by thin layer chromatography (TLC) on plates covered with Silica Gel G. Neutral lipids were separated with hexane/ether/acetic acid (80:20:1, v/v) and with benzene/hexane (30:70, v/v). Polar lipids were fractionated with chloroform/methanol/acetic acid/water (65:25:4:4, v/v). The spots were localized by exposing the plates to iodine vapors. Lipids were identified by comparison of the R_f with standards or by spraying the plates with specific reagents. The quantitative determination of the lipids was performed by densitometry after charring with sulfochromic acid solution (4), suing a known amount of stearic acid as standard.

Fatty Acid Analysis

An aliquot of total lipids was saponified with 10% KOH in ethanol for 45 min at 80 C and, after extraction of the unsaponifiables with petroleum ether, the fatty acids were esterified with 3 N HCl in methanol.

The methyl esters were analyzed by gas liquid chromatography (GLC) in a Packard apparatus at 180 C. Two liquid phases were used: 15% EGSS-X on Chromosorb WPH 80/100 mesh and 15% EGSS-Y on Chromosorb WAW 80/100

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mesh.

The chromatographic peaks were tentatively identified using both columns by comparison of the retention times relative to methyl stearate with those of standards. This identification was checked by the graphical methods of equivalent chain length and separation factors reported by Ackman (5,6). Carbon chain length of the acids was checked by hydrogenation followed by GLC of hydrogenated products (7).

The number of double bonds in the fatty acids was confirmed by TLC on silica gel H-$AgNO_3$, followed by GLC of the separated fractions (8).

Incubation with Labeled Substrates

A total of 4 anemones was incubated with each radioactive tracer. Five μCi (1 μmol) of [1-^{14}C] ammonium linoleate or [1-^{14}C] ammonium-α-linolenate were injected into each animal, at the side of the mouth, and incubated for 2 hr at 20 C in the light conditions previously described. After that time, the anemones were briefly washed with sea water and the lipids extracted and analyzed. The lipids were separated by TLC and the radioactivity was measured on the plates with a scanner (Berthold, Germany).

Radioactive fatty acid methyl esters were analyzed by gas liquid radiochromatography in a Hewlett-Packard 402 apparatus equipped with a flame detector and coupled to a Panax Radiogas detector. A column packed with 10% SP-2330 on Chromosorb WAW liquid phase was used. Temperature was programmed for a linear increase of 2 C/min from 140 C to 220 C. Mass and radioactivity measures were simultaneously registered using a double-channel recorder.

RESULTS AND DISCUSSION

Lipid Composition

The lipid content of the whole tissue of *P. clematis* increased from 1.90 to 2.35% (wet weight basis) between autumn and spring. The neutral and polar lipids were separated by TLC into fractions that were quantitatively measured (Table 1).

Free and esterified sterols were recognized by color reactions with sulphuric acid/acetic acid (50:50, v/v). To identify the least polar component of the chromatogram, it was recovered from the plate and saponified as described above. The unsaponifiable fraction was chromatographed on silica gel plate with benzene/hexane (30:70, v/v) (9). Two spots appeared. A spot near the origin gave a color reaction characteristic of sterols and originated from the esterified sterols. The second spot with $R_f \cong 0.5$ was recognized as unhydrolyzed wax esters.

Spring specimens show higher contents of neutral lipids than those of autumn. This increase is largely found in the esterified sterols. Wax esters have been reported to be energy stores and important components of many marine animals (10-12). In *P. clematis*, only small amounts

TABLE 1

Seasonal Lipid Composition of *Phymactis clematis*

Lipid	Distribution (%) Autumn	Spring
Waxes	1.5[a] (0.5)[b]	2.4 (0.2)
Sterol esters	10.8 (1.5)	30.5 (1.7)
Diacylglycerol ethers	13.1 (0.5)	14.5 (0.5)
Triacylglycerols	25.7 (1.0)	26.4 (1.0)
Free fatty acids	traces	0.7 (0.7)
Fatty alcohols	1.1 (0.1)	1.6 (0.8)
Free sterols	12.5 (0.5)	11.0 (0.5)
Diacylglycerols		
Monoacylglycerols	3.8 (0.3)	2.1 (0.1)
Phosphatidylethanolamine	10.6 (0.2)	3.3 (0.3)
Ceramide aminoethyl phosphonate	14.9 (2.0)	5.1 (0.4)
Phosphatidylcholine		
Unidentified	traces	traces
Lysophosphatides	0.0 (0.5)	2.4 (1.0)
Total neutral lipids	68.5 (2.3)	89.2 (1.7)
Total polar lipids	31.5 (2.3)	10.8 (1.7)
Total lipids	1.90[c] (0.10)	2.35(0.15)

[a]Average of 2 samples of 4 animals each.

[b]Extreme deviation of the mean.

[c]% wt/wt wet tissue.

were found in the periods studied, suggesting that these lipids could not be significant energy stores.

Lipids running above triacylglycerols were identified as diacylglycerol ethers. Large amounts of these appear in some marine vertebrates (13) and in lesser proportion in invertebrates (14). They are usually considered to be related to wax esters since there is a similarity as regards composition and biosynthesis. The levels found in this case are half the porportion of triacylglycerols, an unusually high ratio.

To complete the picture of these related lipids, a spot with R_f 0.20 and coincident with a standard of linoleylol, maintained its position after saponification. It was identified as aliphatic alcohols.

In addition to triacylglycerols and diacylglycerols, monoacylglycerols were present. Pigments might be present in the monoacylglycerol spot, and therefore the values in Table 1 are uncertain.

The presence of several phospholipids was detected in the anemone (Table 1). Three spots gave a positive reaction with ninhydrin. The least polar was recognized as phosphatidylethanolamine and the most polar remained uncharacterized. The spot of intermediate polarity ran a little higher than phosphatidylcholine. It was attributed to a ceramide-aminoethyl phosphonate, since it gave positive reactions with specific reagents for sphingolipids and amino groups. This compound has been recognized in other marine (15,16) and freshwater invertebrates (17, 18). Large quantities of this compound were reported by Rouser et al. (19) and Mason (20) in sea anemones.

Phosphatides of serine and inositol, as well as the cerebrosides present in many aquatic organisms, including sea anemones (19,20), could not be detected.

Fatty Acid Composition

The fatty acid composition of total lipids of *P. clematis*, determined by GLC, is presented in Table 2. The spectrum of fatty acids found corresponds, in general, to an aquatic pattern, where the acids of linolenic series (ω3) prevail over those of the linoleic series (ω6).

It is important to remark that, although the 22:6ω3 acid is a typical component of aquatic organisms, specially those of marine origin, only small quantities of an acid with its gas-chromatographic characteristics were detected. Instead, the 22:5ω3 acid, which is a possible direct precursor of the 22:6ω3 acid, is the major C_{22} fatty acid of the α-linolenic family. Other relatively important components were, as expected, 20:5ω3, 16:0 and 18:1.

TABLE 2

Seasonal Fatty Acid Composition (% wt/wt) Found in Total Lipids of *Phymactis clematis*

Fatty acid	Autumn	Spring
14:0	1.1[a] (0.1)[b]	1.0 (0.2)
15:0	1.1 (0.0)	1.5 (0.0)
X	0.5 (0.0)	0.6 (0.0)
16:0	9.8 (0.1)	9.9 (0.4)
16:1	6.0 (0.2)	6.0 (0.2)
16:2?	2.1 (0.0)	2.5 (0.0)
17:0	4.9 (0.1)	2.1 (0.0)
18:0	6.9 (0.1)	5.9 (0.0)
18:1ω9	10.9 (0.2)	7.1 (0.3)
18:2ω6	7.1 (0.7)	2.7 (0.1)
18:3ω3	1.5 (0.1)	1.8 (0.1)
18:4ω3	3.0 (0.1)	2.8 (0.1)
20:1ω9	5.5 (0.1)	5.6 (0.2)
20:2ω6	2.4 (0.4)	1.0 (0.1)
20:2ω3	2.4 (0.2)	1.3 (0.0)
20:3	0.7 (0.1)	— —
20:4ω6	4.8 (0.1)	4.7 (0.4)
20:4ω3	3.9 (0.1)	4.1 (0.1)
20:5ω3	8.2 (0.5)	13.9 (0.2)
22:2	2.2 (0.1)	1.8 (0.1)
22:3	0.4 (0.4)	1.0 (0.1)
22:4ω3	4.6 (0.1)	4.5 (0.3)
22:5ω3	8.7 (0.1)	16.6 (1.1)
22:6?	0.4 (0.4)	0.5 (0.5)
24:2	0.9 (0.0)	1.1 (0.1)

[a]Average of 2 lots of 4 animals each.
[b]Extreme deviation of the mean.

In general, aquatic animals show quantitative seasonal changes in their fatty acids. As regards to *P. clematis*, the most significant variations are produced in the 20:5ω3 and 22:5ω3 acids. Quantitative variations observed in fatty acids at different periods of the year, could be attributed to the sexual cycle, diet or proportions of different lipid classes. In this respect, conclusions obtained from data for only two seasons of the year would be merely speculative.

On the other hand, the possibility of fluctuations in the biosynthesis of fatty acids in the animal tissues, or the contribution of the symbiont algae located in the polyp tentacles, cannot be discarded. With respect to this last point, it is well established (21,22) that zooxanthellae can translocate fatty acids to the cells of their animal hosts.

Incorporation of Fatty Acids into Lipids

Some aspects of the dynamics of fatty acids in the animal were studied with labeled fatty acids. When the ammonium salts of ^{14}C-labeled linoleic and α-linolenic acids were incubated with the anemones, it was found that after 2 hr, significant radioactivity was incorporated into the lipids (Table 3). The amount incorporated

TABLE 3

[1-^{14}C] Linoleic and [1-^{14}C] Linolenic Acids Incorporation into Lipids of *Phymactis clematis*

Lipid[a]	[1-^{14}C]18:2ω6		[1-^{14}C]18:3ω3	
	Radioactivity (%[b])	dpm / mg lipid	Radioactivity (%)	dpm / mg lipid
WE + SE	3.9 ± 2.5		4.9 ± 1.5	
DGE	2.6 ± 1.1		6.1 ± 1.9	
TG	41.9 ± 3.6		52.6 ± 5.1	
FFA	31.7 ± 3.5		10.8 ± 3.0	
FA + FS + DG	6.0 ± 2.4		1.1 ± 0.8	
MG	13.9 ± 4.1		18.8 ± 2.9	
PL	traces		5.7 ± 0.7	
TL	100.0	11869		14457

[a]WE: wax esters; SE: sterol esters; DGE: diacylglycerol ethers; TG: triacylglycerols; FFA: free fatty acids; FA: fatty alcohols; FS: free sterols; DG: diacylglycerols; MG: monoacylglycerols; PL: phospholipids; TL: total lipids.

[b] Results are the mean of 4 determinations ± standard deviation.

was dependent on the fatty acid added. Anemones incubated with linolenate showed an incorporation higher than those incubated with linoleate. After incubation, ca. 15% and 30%, respectively, of the radioactivity "incorporated" remained as free fatty acids.

When the lipids were separated after incubation with linoleate and analyzed by TLC, the labeling was found principally in the neutral lipids, especially triacylglycerols and monoacylglycerols. The same neutral lipids were labeled by α-linolenate, but in this case it must be added that the phospholipids were also labeled significantly. The area near the origin was scraped from the plates, eluted and rechromatographed by TLC using the solvents appropriate for the phospholipid development, as described in the experimental part. In this circumstance, it was observed that a remarkable proportion of the labeling belonged to the ceramide aminoethyl phosphonate and phosphatidylcholine area. No radioactivity was detected in other phospholipids.

These results would suggest that lipid synthesis in *P. clematis* follows the common pathways whereby linoleic and α-linolenic acids or their metabolic products would be converted into acyl-CoA, and rapidly incorporated into lipids. Although polyunsaturated acids, they were preferentially stored in the triacylglycerols. Similar results were found by Hill-Manning and Blanquet (12).

The plate zones corresponding to free sterols, fatty alcohols, wax esters and sterified sterols were separated from the plates and saponified as described previously. When the unsaponifiables were rechromatographed by TLC, some labeling was observed in free sterols, fatty alcohols and wax esters.

Several factors should be taken into account relating to wax ester biosynthesis. Sargent et al. (23) have demonstrated that the limiting factor in wax ester synthesis is the fatty alcohol availability, produced by reduction of the corresponding fatty acids or by the de novo synthesis from carbohydrates and dietary proteins (24). They have also suggested that the biosynthesis of triacylglycerols and that of wax esters may be reciprocally exclusive and the results obtained in the anemones would agree with this suggestion.

As regards the incorporation of substrates in diacylglycerol ethers, similar conclusions may be drawn. However, the high levels of diacylglycerol ethers in *P. clematis* seem rather difficult to explain by this mechanism.

Labeling found in free sterols suggests de novo synthesis from acetate produced by the fatty acid β-oxidation. A similar conclusion may be drawn from the results of fatty alcohol labeling. Linoleic and α-linolenic acid reduction to their corresponding fatty alcohols does not seem reasonable, since the specificity of the acyl-CoA reductase to saturated and monoenoic substrates has been demonstrated (13).

When the anemone tentacles including zooxanthellae were separated after whole animal exposure and analyzed separately, no difference was found in the lipid radioactivity distribution. Nevertheless, the labeled precursor incorporation was much higher in tentacles than in the remaining parts of the animal. The relative specific activities of tentacles:body were 3.56 and 3.58 for the linoleic and α-linolenic acid

incubations, respectively.

Biosynthesis of Fatty Acids

After incubating the anemones with [1-^{14}C]-linoleate and [1-^{14}C] α-linolenate, the fatty acids were analyzed by gas-liquid radiochromatography. The results are presented in Table 4. It is shown that both precursors not only are activated and incorporated into lipids, but also are converted into other fatty acids.

TABLE IV

Labeling Distribution in the Fatty Acids of the Anemone after Administration of [1-^{14}C] Linoleic and [1-^{14}C] Linolenic Acids

Fatty acid	Radioactivity (%[a])	
	[1-^{14}C] 18:2ω6	[1-^{14}C] 18:3ω3
16:0	4.4 ± 1.7	–
18:2	87.7 ± 3.8	–
18:3	–	94.0 ± 1.7
18:4	–	6.0 ± 1.7
20:2	traces	–
22:2	4.2 ± 0.7	–
24:2	3.7 ± 1.5	–

[a]Results are the mean of 4 determinations ± standard deviation.

Linoleic acid was elongated to 20:2, 22:2 and 24:2 acids, but no radioactivity was detected in the 18:3ω6 acid. This result indicates that Δ6-desaturase was inactive in the conditions of the experiment and only the elongating enzymes that convert the substrate into its long-chain homologs were active. These results agree with other reports that also showed that linoleic acid elongation rather than desaturation takes place in some marine animals (25). Some oxidation of the linoleic acid and de novo synthesis of fatty acids apparently took place, since the radioactivity was also detected in palmitic acid.

The incubation with α-linolenic acid showed no radioactivity in shorter chain acids. After 2 hr, most of the radioactivity was still found in the substrate. However, the conversion of the 18:3ω3 acid into 18:4ω3 acid showed that a Δ6-desaturase actively desaturates α-linolenic acid. There exists the possibility that the Δ6-desaturation of 18:3ω3 to 18:4ω3, an important fatty acid in zooxanthellae (26), may be plant-oriented and not part of the animal system.

If Δ6-desaturase is the same enzyme for the 18:2ω6 acid and 18:3ω3 acid desaturation, as is generally agreed and has been reported for higher animals by Brenner (27), a very low affinity would exist in *P. clematis* for the 18:2ω6 acid but a very strong one would exist for 18:3ω3 acid. The presence of a Δ6-desaturase specifically active for α-linolenic acid and apparently inactive for linolenic acid was also observed in *Acanthamoeba castellanii* (28), *Chlamys tehuelcha* (Pollero, R.J., and Brenner, R.R., unpublished results), and cultured HTC cell (29,30).

In conclusion, 18:2ω6 and 18:3ω3 acids are rapidly incorporated by the algae host system of *P. clematis*, especially in triacylglycerols. They are also converted into other acids by elongating enzymes and by a desaturase specific for 18:3ω3 acid. An important question may be posed about the reasons and mechanisms of substrate specificity of the Δ6-desaturase in the aforementioned cells, that is not shown in rat liver, human liver (31), fish (32) and many other animals.

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Cholesterol Esterifying Capacity of Various Organs in Cholesterol-Fed Guinea Pigs

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ABSTRACT

In guinea pigs fed a diet enriched with 1% cholesterol, the liver, adrenals, spleen, and small intestine accumulated cholesterol much more than the lungs and kidneys. The cholesterol content of the aorta, stomach and colon was not increased by the diet. Cholesteryl ester was the predominant form of cholesterol deposited in the organs richest in this sterol and the total cholesterol content of a tissue tended to increase with the proportion of cholesteryl ester. Cholesterol esterifying activity (ACAT) was found in most tissues and paralleled the cholesteryl ester content of these tissues, being highest in the adrenals, liver, spleen and the proximal part of the small intestine. ACAT activity was enhanced by the cholesterol diet and its elevation was fairly well correlated with the increase in the cholesterol content of the organs. However, the liver and adrenals tended to accumulate more cholesterol than anticipated from their cholesteryl ester content and their ACAT activity. Cholesterol esterification may play a major role in the ability of organs to accumulate cholesterol.
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INTRODUCTION

Feeding of cholesterol to animals results in metabolic alterations which vary from one animal species to another. Cholesterol-fed guinea pigs develop hypercholesterolemia with serum lipoprotein alterations (1,2), fatty liver (3) and hemolytic anemia (4-7). In cholesterol-fed guinea pigs as in other animal species, several mechanisms have been shown to be operating in processing the cholesterol load supplied by a diet enriched with cholesterol, such as increased excretion of bile acids (8) and decreased cholesterol synthesis (9).

Most of the increase in total body cholesterol in cholesterol-fed guinea pigs can be accounted for by an increase in liver esterified cholesterol content (10).

Preliminary studies in our laboratory have shown that in normocholesterolemic guinea pigs, there is a good correlation between the total cholesterol content of tissue and the proportion of esterified cholesterol in the same tissue and between the last parameter and the cholesterol esterifying capacity of the same tissue (Heller, F.R., unpublished results).

The aim of the present study was to estimate the cholesterol esterifying capacity of various tissues by assaying the acyl-CoA cholesterol acyltransferase (ACAT; EC 2.3.1.26) in guinea pigs fed a cholesterol enriched diet (1%), in relationship with the amount of cholesterol deposited in these tissues.

MATERIALS AND METHODS

Chemicals

[4-^{14}C] Cholesterol was obtained from the Radiochemical Center, Amersham, England. Bovine serum albumin (fraction v, Sigma Chemical Co.) was essentially fatty acid free. All other chemicals and solvents were standard commercial high purity materials.

Animals and Diets

Forty-seven male guinea pigs weighing 300-400 g were fed a semisynthetic diet enriched with cholesterol (cholesterol-fed guinea pigs; CHOL GP). Cholesterol (1%) (USP grade) was added to the standard diet (Table 1). The diet was prepared by Hope Farms BV, Woerden, The Netherlands. The animals had free access to food and water containing ascorbic acid (1 g/ℓ) and were weighed every 14 days. Preliminary studies have shown that weight plateauing immediately preceded an impairment in the clinical state of the animals; hence, to obtain a maximum load of cholesterol, the animals were sacrificed after the weight had stabilized for 4-6 weeks.

Sixteen animals died, most from a pulmonary infection. So 31 guinea pigs were submitted to metabolic investigations. Most animals (27/31) were sacrificed between 4 and 10 months after the start of the experiment; one animal was sacrificed at 7 weeks as rapid

TABLE 1

Composition of the Standard Diet

Chemical analysis		Trace elements	
Protein total (%)	22.3	Iron (mg/kg)	277.0
Protein-digestible (%)	21.1	Manganese (mg/kg)	87.4
Arginine (%)	1.40	Zinc (mg/kg)	28.2
Lysine (%)	1.21	Copper (mg/kg)	19.5
Methionine (%)	0.44	Cobalt (mg/kg)	0.22
Cystine (%)	0.35	Iodine (mg/kg)	0.72
Tryptophane (%)	0.29	Vitamin A (iu/kg)	32,400.0
Phenylalanine (%)	1.11	Vitamin D3	3,400.0
Tyrosine (%)	0.89	α-Tocopherol (E) (mg/kg)	92.0
Leucine (%)	1.62	Vitamin K (mg/kg)	12.1
Isoleucine (%)	1.07	Thiamin (B1) (mg/kg)	11.6
Histidine (%)	0.52	Riboflavin (B2) (mg/kg)	13.2
Threonine (%)	0.77	Niacin (mg/kg total)	68.4
Valine (%)	1.12	Niacin (mg/kg available)	38.2
Fat (%)	4.4	Pantothenic acid (mg/kg)	34.3
Linolenic acid (vitamin F) (%)	2.1	Folic acid (mg/kg)	9.4
Fiber (%)	13.2	Pyridoxine (mg/kg) (B6)	5.8
Minerals (%)	5.8	Choline (mg/kg)	2,082.0
Calcium (%)	0.84	Vitamin B12 (mcg/kg)	20.0
Phosphorus (%)	0.44	Biotin (H) (mcg/kg)	± 200.0
Potassium (%)	1.54	Vitamin C (mg/kg)	1,600.0
Magnesium (%)	0.41		
Sodium (%)	0.32		

weight loss occurred at this time. Three apparently resistant guinea pigs were sacrificed only after 21 months (21) and 23 months (2) on the diet. These apparently hyporesponding guinea pigs displayed, like the other guinea pigs, the same impressive cholesterol storage in their tissues as shown by the microscopic examination and the determination of the cholesterol content; thus, their data were pooled with those of the responding animals.

At the moment of sacrifice, the weight of the CHOL GP was from 325 to 1020 g (685 ± 32; mean ± SEM). Twenty-four male guinea pigs were used as control (control guinea pigs; CONT GP); their weight was between 225 and 1100 g (471 ± 52; mean ± SEM) and the animals were on the standard diet for periods extending from 1 to 24 months. Most animals were sacrificed by exsanguination (carotid artery) between 8 and 10 A.M. and were not fasted. Blood was collected in the presence of EDTA for red blood cell (RBC) count and heparin for cholesterol determination. In some animals killed by decapitation, sufficient blood could not be obtained for chemical analysis. The tissues were quickly excised, placed in ice-cold 0.9% saline and weighed.

The following organs were studied: stomach, small intestine (divided in 3 equal portions named proximal, middle and distal parts), colon, liver, kidneys, adrenals, lungs, spleen and aorta. All regions of the gastrointestinal tract were carefully freed from surrounding mesentery, opended longitudinally and washed thoroughly in ice-cold saline. Tissue samples were used for pathological examination, free and esterified cholesterol determination and cholesterol esterification capacity. In some animals, the size of the entire organs was sometimes not great enough for performing all tissue analysis and frequently organs such as aortas, adrenals and spleens had to be pooled. Details concerning the number of organs studied will be given in the accompanying tables. For cholesterol determination, tissue samples were homogenized in 0.9% saline and extracted with petroleum ether. For measurement of cholesterol esterifying capacity, tissue samples were homogenized in 0.25 M sucrose; the homogenate was centrifuged at 4 C (4500 g for 10 min) and the supernatant was kept frozen or immediately used for enzyme activity measurement.

Analysis

Plasma and tissue total and free cholesterol was measured by gas liquid chromatography (11). The number of red cells was obtained by means of an electronic particle counter (Coulter Counter). Histopathology of the various organs

was studied on stained sections (hematoxylin-eosin) of formalin-fixed tissue. CoA-dependent cholesterol esterifying activity of the liver was estimated by using the method of Goodman et al. (12) as modified by Beck and Drevon (13). The incubation mixture contained in a volume of 0.75 ml, cytoplasmic extract from 50 mg liver, bovine serum albumin 8 mg/l, ATP 26.7 mM, CoA 1.07 mM and about 0.05 μCi of [4-^{14}C] cholesterol.

The radiolabeled cholesterol was added to the incubation tube as a [4-^{14}C] cholesterol-albumin solution which was prepared by adding slowly the [4-^{14}C] cholesterol dissolved in acetone to a solution of 100 mg bovine serum albumin in 1 ml of 0.1 M phosphate buffer pH 7.4; acetone was removed by evaporation. The tubes were incubated in a shaking water bath maintained at 37 C. The incubation time was 90 min, as preliminary experiments showed that the reaction rate was linear during this period of time. The reaction was stopped by the addition of 10 ml of chloroform/methanol 2:1, v/v) and lipids were extracted by the method of Folch et al. (14).

Free cholesterol and cholesteryl esters were dissolved in a small volume of chloroform (500 μl) and separated by thin layer chromatography on Silica Gel H using light petroleum/diethyl-ether/glacial acetic acid (85:15:3, v/v/v). The spots containing the radiolabeled free cholesterol and cholesteryl esters were visualized with iodine vapor, scraped into vials containing 4 ml of mixture containing naphthalene 320 g, POPOP 2 g, PPO 20 g, toluene 1540 ml, dioxane 1540 ml, and absolute ethanol 920 ml. For the whole procedure, recovery of cholesterol was 85.2%. The radioactivity was quantitated in a liquid scintillation counter (Intertechnique SL 31).

The ACAT activity in the other organs were assayed by the technique used for the liver. Esterification rate was calculated as fractional esterification rate (FER) (percentage of cholesteryl ester formed per hour and per g of tissue). For statistical analysis, the Student t-test and correlation coefficient were used.

RESULTS

Blood Parameters and Pathology

In CONT GP (N=23), the total plasma cholesterol concentration and percentage of free cholesterol were, respectively, 42.8 ± 32.6 mg/100 ml and 25.2 ± 11.6 % (mean ± 2 standard deviations). In CHOL GP (N=31), the total plasma cholesterol concentrations were between 60 and 550 mg/100 ml (268.3 ± 246.8) and the percentage of plasma free cholesterol between 30 and 95% (51.6 ± 35.0) (mean ± 2 standard deviations).

Twenty-two of the 31 CHOL GP (71%) developed anemia (RBC count less than 4.475 x 10^6/mm^3). In CHOL GP, the weights of the liver, the spleen and the lungs were 3, 7 and 2 times higher, respectively, than in the CONT GP. A microscopic examination revealed the presence of fatty infiltration, intra- and extracellular cholesterol crystals and sometimes hematopoiesis foci, particularly in the liver, spleen, adrenals, lungs and kidneys.

Tissue Cholesterol Content

Tables 2 and 3 show the total cholesterol concentration and the percentage of esterified cholesterol in the organs of CONT GP and CHOL GP.

When compared with the CONT GP, and taking into account the weight of the organs, the cholesterol content in the CHOL GP increased in the liver (x 16.6), in the spleen (x 16.3), the adrenals (x 7.8) and in the lungs (x 2.1). So, in the CHOL GP, the liver was the organ that accumulated by far the greatest amount of cholesterol.

In the CONT GP, cholesteryl esters represented 9-14% of the total cholesterol in most organs (Table 3), except for the liver and adrenals. The percentage of esterified cholesterol in CHOL GP was 2-5 times higher in most organs, except for the stomach and aorta in which the percentage of esterified cholesterol was lower and did not differ from the CONT GP. There was a significant correlation between the cholesterol content of each organ and the percentage of esterified cholesterol in the same organ, in the CONT GP: $r = +0.73$; $p = 0.01$ and in the CHOL GP: $r = +0.88$; $p<0.001$; for all animals, the correlation coefficient was +0.81 ($p<0.001$). Except for the liver and adrenals, the relative amount of cholesterol accumulation in each organ tends to increase with the relative percentage of esterified cholesterol of the same organ. In the liver and the adrenals, the amount of cholesterol deposited was much higher than would be predicted by the change in the percentage of esterified cholesterol (Fig. 1).

Tissue ACAT Activity

Table 4 shows the values of the FER in various organs of the CONT GP and of the CHOL GP. Values of the FER of each organ showed significant correlation with the values of the percentage of esterified cholesterol in the same organ. In the CONT GP: $r = +0.87$, $p<0.001$; and in the CHOL GP: $r = +0.74$,

TABLE 2

Total Cholesterol Content (mg/g Organ) of Various Organs in Control (CONT) and Cholesterol (CHOL) Guinea Pigs (GP)

	CONT GP	CHOL GP
Liver	2.5 ± 0.2 (16)[a]	13.4 ± 1.2 (17)[c]
Spleen[b]	3.6	8.4
Adrenals[b]	5.9	34.2
Kidneys	3.6 ± 0.2 (14)	4.0 ± 0.5 (10)
Lungs	4.3 ± 0.3 (16)	5.1 ± 0.8 (10)
Aorta[b]	1.6	2.0
Stomach	1.9 ± 0.2 (9)	2.1 ± 0.2 (10)
Small intestine proximal	1.4 ± 0.1 (17)	3.5 ± 0.3 (10)[c]
middle	1.5 ± 0.1 (17)	4.0 ± 0.3 (10)[c]
distal	1.5 ± 0.1 (17)	3.0 ± 0.3 (10)[c]
Colon	2.0 ± 0.2 (18)	1.8 ± 0.2 (10)

[a]In brackets, the number of animals studied.

[b]Due to their small size, the spleens, the adrenals and the aortas had to be pooled on several occasions. For the spleen, the values represent the means of 6 determinations in CONT GP (corresponding in 4 cases to the pool of 2 spleens and in 2 cases to the pool of 3 spleens) and the means of 6 determinations in CHOL GP (corresponding in 3 cases to isolated spleens, in 2 cases to the pool of 2 spleens and in 1 case to the pool of 3 spleens). For the adrenals, the values represent the means of 4 determinations in CONT GP (corresponding in 1 case to the pool of adrenals from 8 animals, in 1 case to the pool of adrenals from 5 animals, and in 2 cases to the pool of adrenals from 4 animals) and the means of 4 determinations in CHOL GP (corresponding in 2 cases to the pool of adrenals from 5 animals, in 1 case to the pool of adrenals from 4 animals, and in 1 case to the pool of adrenals from 3 animals). For the aortas, the values represent the means of 7 determinations in CONT GP (corresponding in 4 cases to 1 aorta, in 1 case to the pool of 4 aortas, in 1 case to the pool of 5 aortas and 1 case to the pool of 8 aortas) and the means of 4 determinations in CHOL GP (corresponding in 1 case to the pool of 3 aortas, in 1 case to the pool of 4 aortas and in 2 cases to the pool of 5 aortas).

[c]Means that the difference between CONT GP and CHOL GP is significant with a p value inferior at least to 0.0005.

TABLE 3

Cholesterol Esterified Content (%) of Various Organs in Control (CONT) and Cholesterol (CHOL) Guinea Pigs (GP)

	CONT GP	CHOL GP
Liver	22.6 ± 2.6 (16)[a]	59.6 ± 3.2 (17)[c]
Spleen[b]	11.8	50.1
Adrenals[b]	60.0	85.4
Kidneys	11.5 ± 2.1 (17)	25.1 ± 5.6 (10)[d]
Lungs	12.4 ± 2.4 (16)	40.2 ± 2.1 (10)[c]
Aorta[b]	10.3	6.1
Stomach	14.1 ± 4.0 (17)	13.3 ± 3.9 (10)
Small intestine proximal	11.2 ± 2.5 (24)	38.6 ± 1.6 (10)[c]
middle	11.5 ± 2.6 (24)	38.6 ± 2.3 (10)[c]
distal	8.9 ± 2.0 (24)	32.6 ± 2.6 (10)[c]
Colon	12.1 ± 2.8 (18)	25.7 ± 5.4 (10)[c]

[a]In brackets, the number of animals studied.

[b]Due to their small size, the spleen, the adrenals and the aortas had to be pooled on several occasions. For more detail, see legend of Table 2.

[c]Means that the difference between CONT GP and CHOL GP is significant with a p level value < at least to 0.0005.

[d]Means that the difference between CONT GP and CHOL GP is significant with a p level value < at least to 0.001.

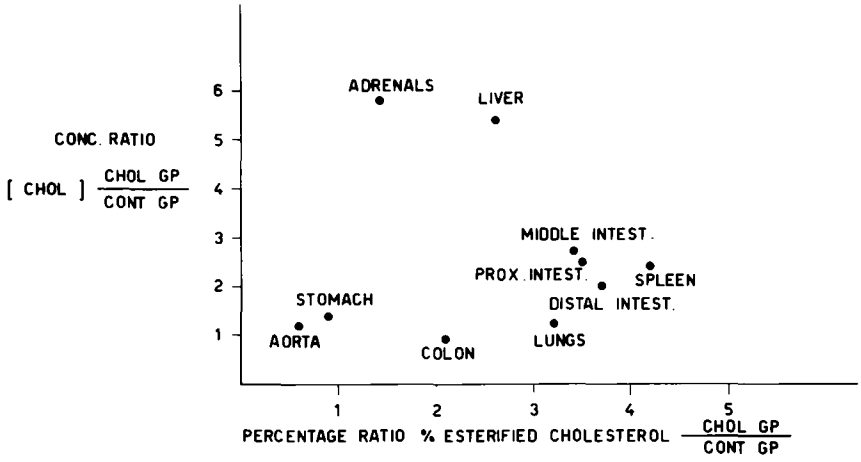

FIG. 1. Relation between cholesterol accumulation and changes in esterified cholesterol content in various tissues of guinea pigs.

TABLE 4

ACAT Activity of Various Organs of Control (CONT) and Cholesterol (CHOL) Guinea Pigs (GP)

	FER (% h^{-1})	
	CONT GP	CHOL GP
Liver	13.8 ± 1.1 (20)[a]	22.8 ± 2.1[c] (17)
Spleen[b]	14.3	8.0
Adrenals[b]	30.7	17.0
Kidneys	8.8 ± 1.1 (11)	8.2 ± 2.6 (10)
Lungs	1.2 ± 0.5 (9)	0.7 ± 0.2 (10)
Aorta[b]	1.5	0.6
Stomach	2.1 ± 1.1 (11)	0.2 ± 0.04 (10)
Small intestine proximal	11.5 ± 2.2 (20)	15.1 ± 2.8 (11)
middle	3.3 ± 0.8 (18)	8.8 ± 3.0[d] (10)
distal	4.4 ± 0.7 (18)	5.6 ± 0.7 (10)
Colon	7.3 ± 1.6 (13)	9.0 ± 1.1 (10)

[a]In brackets, the number of animals studied.

[b]Due to their small size, the spleens, the adrenals and the aortas had to be pooled on several occasions. For more details, see legend of Table 2.

[c,d]Means that the difference between the CONT and the CHOL GP is significant with p values inferior at least, respectively, to 0.025 and 0.0025.

$p<0.01$.

The relative amount of cholesterol accumulated in each organ is also fairly well correlated with the relative increase in the FER, but only when the liver and the adrenals are not taken into account (all organs minus liver and adrenals: $r = +0.69$; $p<0.05$: all organs: $r = +0.28$; NS). Thus, the amount of cholesterol accumulated in the liver and adrenals is higher than would be predicted by the increase in the cholesterol esterifying activity of these organs.

DISCUSSION

It is known that a cholesterol-enriched diet induces striking metabolic alterations in guinea pigs, such as marked tissue and plasma cholesterol accumulation and hemolytic anemia. Numerous studies have been conducted to understand the mechanisms which control the expansion of the cholesterol pool in these animals (8,9,15-17).

Preliminary studies performed in our labora-

tory on normocholesterolemic guinea pigs have demonstrated that the cholesterol esterifying capacity of tissue could be an important factor for determining the amount of cholesterol deposited in the tissue.

The anemia usually observed in guinea pigs after 10-15 weeks on a cholesterol diet (RBC less than 3-3.5 x $10^6/mm^3$) (5,18) was not frequently attained in our animals. No overt hemolytic anemia developed in guinea pigs fed cholesterol and saturated fat in contrast to those fed cholesterol and polyunsaturated fatty acids (19). As the diet used in our work did not contain arachidonic acid, the low degree of anemia observed in our guinea pigs could be explained by the relative enrichment of saturated fatty acids in the diet.

As previous studies have shown (4,5,10,16), the liver, spleen and adrenals are organs which accumulate the greatest amount of cholesterol when the diet is enriched with this sterol, and cholesteryl ester is the predominant state of the cholesterol deposited in these organs (3,4,7, 10,16). Our study shows that the amount of cholesterol deposited tends to increase with the percentage of esterified cholesterol in the same organs, except for the liver and the adrenals which seem to accumulate more cholesterol than would be predicted by the degree of esterification.

The increase in the cholesteryl ester content of the tissues could be due to a diminished activity of the cholesterol esterase; however, at least in the organs studied by Drevon, this does not apply to cholesterol-fed guinea pigs (16). Preliminary work in our laboratory has demonstrated that a coenzyme A-dependent esterification mechanism of cholesterol previously demonstrated in some organs of guinea pigs (13,16,20) is present in most tissues, with variable activity depending on the organ studied (Heller, F.R., unpublished results). The present work shows that, in response to dietary cholesterol, the ACAT activity expressed as fractional esterification rate was found to be increased only in the liver and intestine (Table 4). This suggests that the enhancement is not due simply to increased substrate concentration. As sterol synthesis in the liver seems to be far less sensitive to cholesterol accumulation than in other organs (9), this can account for the impressive amount of cholesterol deposited in the liver of cholesterol-fed guinea pigs. Compared with the control animals, the ACAT activity is increased in the 3 parts of the small intestine in cholesterol-fed guinea pigs, although the difference is significant only for the middle part of the intestine. It has recently been suggested that the ACAT of the small intestine can play a role in the regulation of cholesterol absorption, or, at least, in chylomicron formation (21). Therefore, one reason for the inability of guinea pigs to limit absorption of dietary cholesterol (22) is that the middle part of the small intestine can increase its ACAT activity, allowing more cholesterol to be absorbed.

In several organs, such as adrenals, spleen and probably proximal and distal parts of the small intestine, the decrease of the ACAT activity as expressed by FER can be explained by the dilution of the radiolabeled cholesterol by the high free cholesterol content of these organs. The absence of morphological changes of the cardiovascular system noted in the cholesterol-fed guinea pigs in the present study as in the study of Yanamaka et al. (5) can be related to the low cholesterol content and possibly to the low ACAT activity of the aorta.

Despite the fact that changes in ACAT activity vary according to the organ, it was found that the cholesterol esterifying activity is relatively well correlated with the degree of cholesterol esterification and the total cholesterol content in the various organs of guinea pigs. Thus, it is suggested that ACAT can play a major role in the disposition of excess cholesterol supplied by the diet in addition to the other mechanisms reported to be operating by other researchers (8,9). It remains to be determined whether the primary event is the ability of the cell surface receptors to bind more cholesterol containing lipoproteins, or the capacity of the cells to increase cholesterol esterification or (and) to suppress steroidogenesis.

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Heterogeneous Labeling of Adipocytes during in vivo-in vitro Incubation of Epididymal Fat Pads of Aging Mice with [1-^{14}C] Palmitate

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ABSTRACT

We have hypothesized that the in vivo-in vitro technique of Stein and Stein for studying free fatty acid incorporation into adipose tissue triglycerides and phospholipids may introduce artifacts due to diffusion barriers such as collagenous membranes, especially in fat pads of old animals. By using this technique in young and old mice and peeling the external cells, either physically or by collagenase treatment, we were able to show that the outer adipocytes are preferentially labeled. However, this pattern of heterogenous labeling occurred in fat pads of both young (10-14 weeks) and old (80 weeks) mice. Fat pads are known to develop thicker, collagenous outer membranes during aging. Therefore, it seems likely to us that the marked decrease in free fatty acid esterification in fat pads of old mice, using the in vivo-in vitro method that we have described prevously and confirmed here, could have been due to greater diffusion barriers in the tissues of the older mice.
Lipids 18:25-31, 1983.

INTRODUCTION

One of the potentially useful techniques for studying fatty acid turnover in adipose tissue is the in vivo-in vitro technique first introduced for that purpose by Stein and Stein using rats (1). We have applied this technique in a study of PLFA and TGFA turnover in adipocytes of aging mice (2). In analyzing these data, we noted that the incorporation of labeled palmitate into lipid esters, when expressed per unit membrane (based upon cell PL mass), fell markedly with age. We wondered whether this might be an artifact of decreased FFA penetrability into the fat pads of aging mice. If an external barrier existed, it seemed conceivable that the labeled palmitate added to the incubation medium might have made contact with only the most superficial cells of the fat pad, while we might expect the distribution of FFA within the smaller and more delicate fat pad of young mice to be much more uniform.

Earlier studies of possible heterogeneity within the fat pad have focused on intracellular compartmentation (1,3,4, and references cited therein), which Stein and Stein could demonstrate at early times following the in vivo-in vitro incubation of rat adipose tissue with labeled palmitate. In that study, the authors did not address the question of whether the peripherally and centrally located adipocytes were uniformly labeled by this technique.

However, this point was addressed briefly, as a methodological side issue, by Hollenberg who used the in vivo-in vitro approach (1) to study lipogenesis from glucose-^{14}C in rat epididymal fat pads (5). Hollenberg concluded that the outermost cells were labeled to the same extent as the innermost adipocytes, as evidenced by "peeling" of the adipocytes from the whole fat pad with collagenase and analyzing successive yields of cells for TG specific activity (Sp.A.). However, we have reanalyzed these data (below) and conclude that the cells may not have been homogenously labeled.

In the present study, we have used a similar peeling technique to determine whether adipocytes in epididymal fat pads of young and old mice are labeled in a homogeneous fashion following incubation with [1-^{14}C] palmitate complexed to bovine serum albumin using the in vivo-in vitro technique of Stein and Stein (1).

MATERIALS AND METHODS

Mice

Mice (C57B1/6) were obtained from Simonsen Labs (Chatsworth, CA). Older mice (40 weeks or more) were either maintained at the VA Wadsworth Medical Center Animal Research Facility from 4 to 6 weeks of age or obtained as retired breeders and then aged further. The

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Abbreviations: FFA = free fatty acids; PL = phospholipids; PLFA = phospholipid fatty acids; TG = triglycerides; TGFA = triglyceride fatty acids; Sp.A. = specific activity; and SE = standard error.

mice were fed Purina Lab Chow and water ad libitum at all times. Experiments were conducted between 8:00 A.M. and 12:00 noon to insure that the mice would be in a fed or early post-absorptive nutritional state during our studies (6).

In Vivo-In Vitro Incubation of Fat Pads

The in vivo incubation technique developed by Stein and Stein (1) was adapted for labeling epididymal fat pads of mice. As previously described (2), fat pads of anesthetized mice were exteriorized and incubated by placing the mouse in a prone position with the fat pads distended into wells containing incubation medium. The pads' innervation and blood supply remain intact. Surgery and incubations were carried out under Diabutal® (Diamond Laboratories, Inc., Des Moines, IA; 0.11 mg pentobarbital/g BW) anesthesia. After incubation for 20 min, pads were rinsed briefly with 3 changes of warm buffer.

The medium for labeling the pads consisted of Krebs-Ringer Phosphate buffer (KRB) with ½ the normal $CaCl_2$ concentration, 2.5% albumin (essentially fatty acid free, Sigma Chemical Co.), and 0.35 μeq palmitate/ml. The palmitate was complexed to the albumin as described previously (2). [1-^{14}C] palmitate was obtained from Rosechem Products (Los Angeles, CA). The final Sp.A. of the media for all experiments ranged from 14.3 to 39.1 x 10^6 cpm/μeq palmitate, i.e. from 5.0 to 13.7 x 10^6 cpm/ml. Counting efficiency for doses and experimental samples was ca. 60% (60 cpm/100 dpm) using a Beckman Model LS 3133P scintillation spectrometer. The complex was made in 100-ml amounts and frozen (once only) in 10-ml aliquots.

Preparation of Adipocytes

Following incubations, pads were chilled to 0-4 C. This chilling has been reported (Bruckdorfer, personal communication) to inhibit irreversibly the lipolysis of TG. We are aware, however, of reports by other authors that only adrenalin-stimulated lipolysis is inhibited irreversibly by lowering the temperature of fat pads (7,8). In any case, very little of the newly formed TGFA broke down to FFA during the subsequent preparation of adipocytes using a modification of Rodbell's procedure (9).

In order to demonstrate the possible heterogeneity of labeling, fat pads were excised after incubation, and the cut edge of the pads tied off with waxed dental floss. The pads were then lowered into 50-ml Erlenmeyer flasks containing 3 ml of collagenase medium, the flasks were shaken at 37 C at a speed of ca. 30 cycles/min. The tied, cut ends were not exposed to the collagenase. The collagenase medium consisted of Krebs-Ringer phosphate buffer (½ Ca^{2+} concentration and containing 5% albumin and 5 mg collagenase/3 ml medium). The pad was removed to a fresh flask of collagenase medium at 5-10 min intervals. For the last sample of each pad, the floss was removed and the entire remaining piece of tissue was shaken with collagenase until all the cells were released. The last fraction contained 20-40% of the total cells of the pad and consisted primarily of cells from the interior portion of the pad. Although some peripheral cells at the cut end of the pad were included in this last fraction, they would have constituted only a minor portion of it.

Cell sizes were measured (2), using the pad contralateral to the one used for the incubation. In some cases, cell size and number were estimated from known pad size and cell size relationships (2).

Lipid Analyses

Each fraction of cells from each pad was analyzed for its TG and PL content and radioactivity; Analyses were done as described previously (2).

RESULTS

When the Stein and Stein (1) in vivo-in vitro method of labeling rat epididymal fat pad lipids was used by Hollenberg to study ^{14}C-glucose metabolism (5), he considered the possibility that the outermost layers of adipocytes might be labeled differently from inner layers. Thus, using collagenase, he attempted to "peel" successively larger layers of adipocytes from the pads to determine whether these different populations of cells had similar specific activities. In 3 experiments, he obtained cumulative fractions consisting of 1.1, 3.6, and 9.9% of the total pad lipid; these fractions contained 0.4, 2.8, and 12.3% of the total pad lipid radioactivity, respectively. He interpreted these data to mean that the fractions were not different from one another and concluded that the cells were homogeneously labeled throughout the fat pad. When we reanalyzed his data, however, by subtracting the smaller from the larger fractions, we obtained the graph shown in Figure 1, which shows that the lipid Sp.A. of successive adipocyte fractions were not equal; thus, the Sp.A. of each fraction increased markedly (from 0.36 Sp.A. units in the first cell fraction to 1.5 Sp.A. units in the third fraction).

Since this reanalysis of Hollenberg's data suggested a possible heterogeneous labeling of

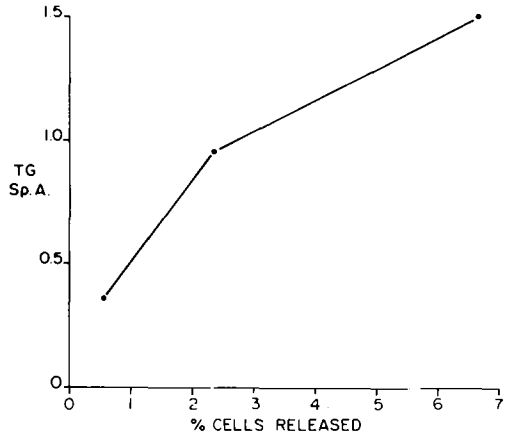

FIG. 1. Increase in lipid Sp.A. of successively larger fractions of adipocytes from rat epididymal fat pads labeled with ^{14}C-glucose by the in vivo-in vitro method of Stein and Stein (1). Horizontal abscissa represents the percent of cells released relative to the whole pad. The units of Sp.A. are % TL-^{14}C/% TL mass. Graph was obtained by reanalysis of data obtained by Hollenberg (5), as discussed in text.

adipocytes, we did a preliminary experiment to further examine such a possibility. Mouse epididymal fat pads were incubated for 10 min in [1-^{14}C] palmitate as described in Materials and Methods. After incubation, they were frozen on dry ice; thin slices (~ 10 mg TG) were taken from the outermost edges of the pad, the intermediate areas were cut away and discarded (~ 470 mg), and the approximate centers (~ 40 mg TG) of the pads were saved. The Sp.A. of these samples are shown in Table 1. The outer adipocytes of the fat pads had incorporated 20 times more label than those in the center of the pad. These results may seem contrary to our analysis of Hollenberg's data, which showed that the first cells released contained less radioactivity in newly synthesized lipids than did the last batch of adipocytes released by collagenase. However, in his study, precautions were not taken to insure that the first cells released represented outer cells (see Discussion).

To confirm our results, we did experiments using Hollenberg's "peeling" approach, but with precautions to prevent escape of inner cells from cut fat pads. Mouse epididymal fat pads were incubated for 20 min in [1-^{14}C] palmitate, chilled, and then incubated in collagenase medium, as described in Materials and Methods. Figure 2 shows the results of one of these experiments with one group of 4 mice. The Sp.A. of the TG in the adipocytes released during the first incubation are represented by the first bar. The first collagenase incubation thus yielded about 13% of the total pad lipid, and the mean TG Sp.A. of this fraction was 260 cpm/mg. The last fraction collected, consisting of 38% of the total pad lipid, had a TG Sp.A. of only 60 cpm/mg, ca. one-fourth that of the first fraction. Figure 2B shows these same data presented more conveniently, i.e. only the midpoints of the percent of total pad lipid are plotted with the SE of the midpoints indicated by the horizontal lines and the SE of the Sp.A. indicated by the vertical lines. The presentation in Figure 2B is used in Figures 3 and 4, except that the S.E. of the percent of cells released are shown only in Figure 3A, since the percent cells released for a particular age-group remained the same in all figures.

Figure 3 shows the extent of [1-^{14}C] palmitate incorporation into TG in 4 collagenase-derived cell fractions of pads from mice of varying ages. The data are expressed as in our earlier study (2), i.e., per unit mass (mg TG), per adipocyte, and per unit membrane (μg PL). In all cases and for all ages of mice and sizes of pads, the activity was less in the last fraction representing the innermost portion than in that derived from the outermost part of the fat pad, confirming the result we obtained in the slicing experiment (Table 1). As reported earlier (2),

TABLE 1

Heterogeneity of Cell Labeling after in vivo-in vitro Incubation of Mouse Epididymal Fat Pads with [1-^{14}C] Palmitate

Adipocyte fraction	Approximate fraction of total pad TG	Sp.A. of TG in fraction[a]
Outer, peripheral cells	0.02	6.1 ± 1.8 (4;2)
Inner, central cells	0.08	0.3 ± 0.04 (3;2)

[a]Mean ± SE (number of pads; number of mice). The fat pads contained 520 ± 100 mg TG/pad. Fractions were obtained by slicing as described in Results.

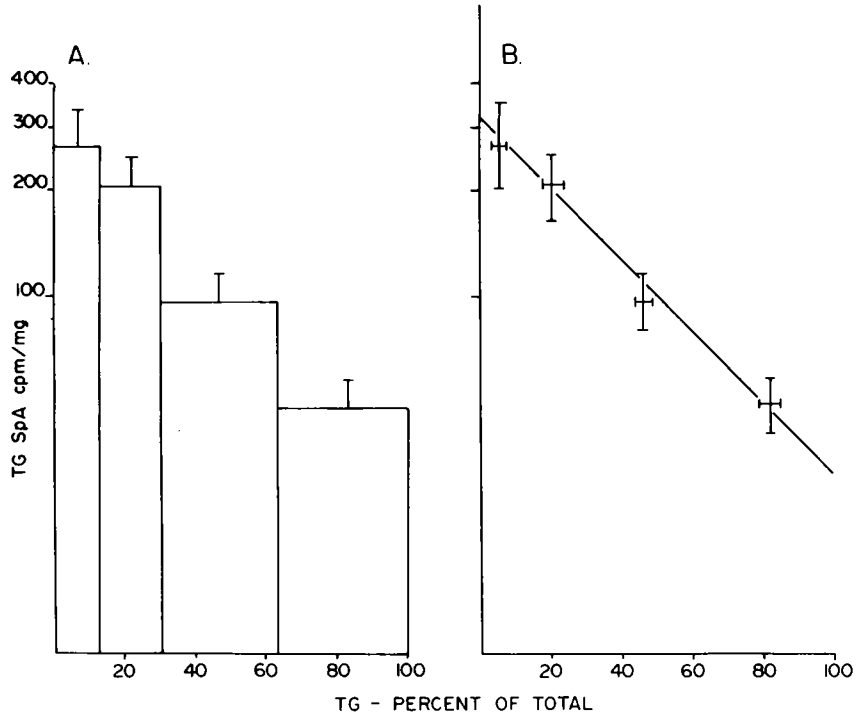

FIG. 2. TG Sp.A. of samples of adipocytes isolated by the "peeling" method (see Materials and Methods). Epididymal fat pads of 80-week old mice were incubated 20 min in [1-^{14}C] palmitate (Sp.A. 14.4 cpm/μeq) by the in vivo-in vitro method prior to the peeling of adipocytes. (A) Each bar represents the mean TG Sp.A. (±SE) (4 mice/bar) of the fraction; the size of the fraction is indicated by the width of the bar on the x-axis (percent of pad total lipid). (B) This is the same data as shown in Figure 2A, but here the percent of pad total lipid is represented only by the mean of the midpoints of the lipid in the fraction, expressed as percent of total pad TG lipid. For example, the first bar in Figure 2A shows that ca. 12% (± SE of 4 mice) of the total lipid was collected in the first fraction; in Figure 2B, this is represented by the point of intersection of the TG Sp.A. ± SE line and the line indicating the midpoint ± SE of the percent pad lipid i.e., ca. 6% in this case.

the mean Sp.A. (cpm/mg TG, Fig. 3A) obtained with pads from the youngest mice were an order of magnitude greater than those of the larger pads from the older mice. The ratio of the TG Sp.A. (Fig. 3A) of the outermost adipocytes to the innermost ranged from ca. 2 (14-week old mice) to 5 (80-week old mice). The heterogeneity of TG labeling is evidenced whether expressed as sp.A., per adipocyte, or per unit membrane (μg PL), as seen in Figures 3B and 3C.

In general, incorporation of [1-^{14}C] palmitate into phospholipids of the adipocytes also showed a logarithmic decrease from the outside to the inside of the fat pad, as shown in Figures 4A-C. This was the case in mice of all ages and whether the PL activity was expressed per unit mass (mg TG), per adipocyte, or per unit membrane (μg PL). An inconsistency in the apparent heterogeneity of the released cells based upon their degree of labeling in the TG and PL moieties was found in 2 groups of mice. Thus, in both the 14- and 54-week old mice, the two outermost fractions representing the outer third of the cells appeared to be homogeneous with respect to their PL labeling (Fig. 4A-C). Reexamination of the data for these two groups with respect to their TG labeling (Figs. 3A-C– shows that there were no significant differences in TG Sp.A. of the two fractions in the outer third of cells released.

The effects of age on PL labeling were complex, as reported previously (2). When expressed per unit adipocyte (Fig. 4B), the cell PL radioactivity of the 54-week old mice was significantly higher than that of all the other age groups.

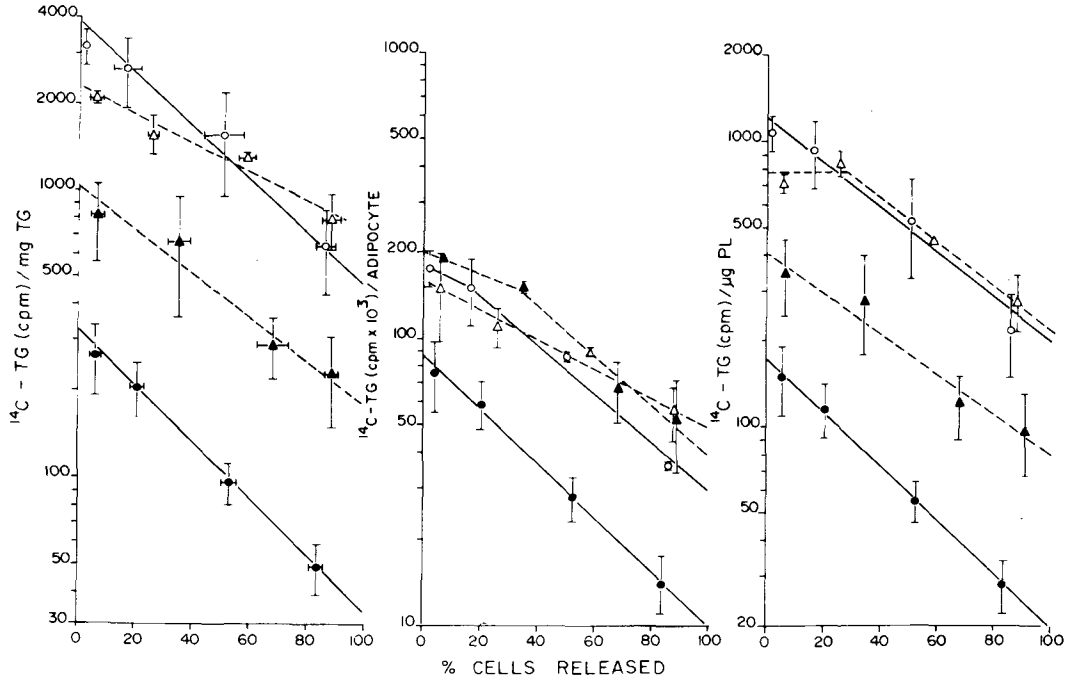

FIG. 3. Incorporation of [1-^{14}C]palmitate (dose Sp.A. 14.4 × 10^6 cpm/µeq) into TG in adipocytes of epididymal fat pads of mice of various ages, expressed (A) per unit mass (mg TG), (B) per adipocyte, and (C) per unit membrane (µg PL). Pads were incubated 20 min by the in vivo-in vitro method. Adipocytes were subsequently isolated in fractions representing cell populations from outer (first fractions) and inner (later fractions) regions of the pads ("peeling" method, see Materials and Methods). Data are from 2 experiments (indicated by triangles or circles) with 2 ages of mice per experiment (○, 12 weeks; △, 14 weeks; ▲, 54 weeks; ●, 80 weeks). Each point (4 mice/point) represents the mean TG activity ± SE (y-axis) of fractions containing the mean of the midpoints of the percent of the pad total lipid recovered in that fraction (x-axis).

When expressed per unit membrane (Fig. 4C), the cells from the 54-week old mice had the highest and the 80-week old mice the lowest PL Sp.A.

DISCUSSION

We have shown here that the in vivo-in vitro incubation of mouse epididymal fat pads with [1-^{14}C]palmitate results in a heterogeneous pattern of adipocyte labeling. Those cells that have greater access to the label, i.e., the most peripherally located adipocytes, are the ones that incorporate the most fatty acids from the medium into TG and PL. This phenomenon was seen in both small and large fat pads of mice varying in age from 12 to 80 weeks.

We are unable to determine from these studies whether the heterogeneous pattern of labeling that we have observed is due to metabolic differences between the peripherally and centrally located cells or to simple physical aspects such as diffusion gradients. The most likely and simplest explanation is that the outermost cells of the pad are exposed to the highest concentration of labeled FFA and are, therefore, selectively labeled. Based on evidence in other species (10,11), the peripherally located cells are probably smaller than the central ones. If this is true in mouse epididymal fat pads, then it is unlikely that the outer (smaller) cells would be more active metabolically than the inner (larger) adipocytes. We base this reasoning on a large body of evidence showing that, for a given age group of animals, the larger the cell, the greater the rate of TG synthesis/cell from glucose or from FFA (12-14), even though hormone responsiveness is clearly diminished in the larger adipocytes (15). Further studies based upon the present techniques for collecting the outer cells could be used to resolve these questions (relative size and metabolic activity of peripheral adipocytes in mice). Tentatively, we conclude that the heterogeneous pattern of FFA incorporation

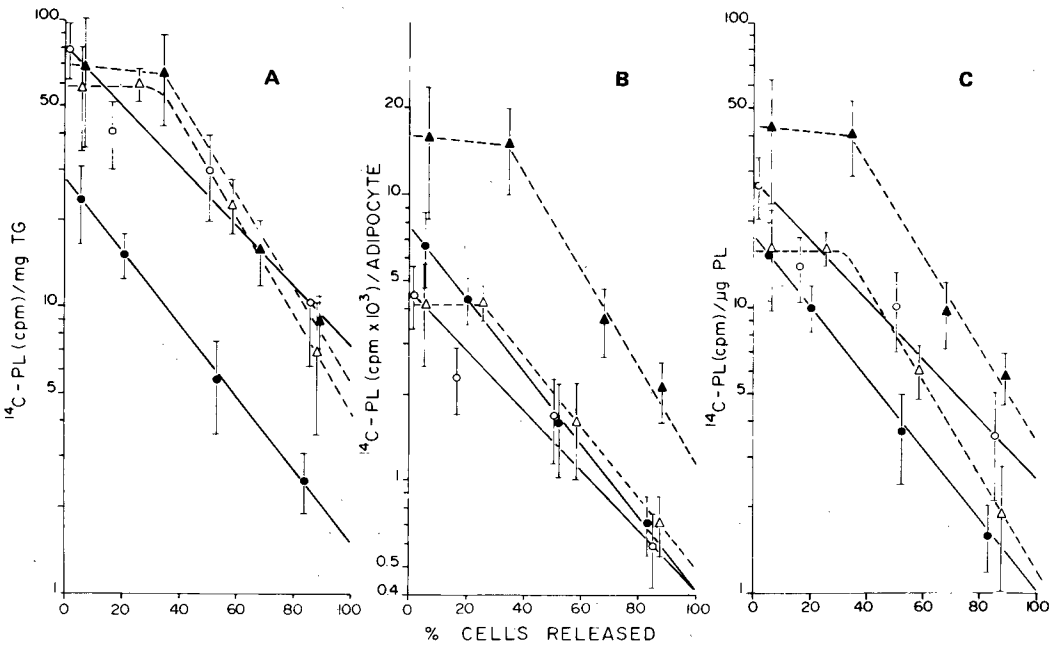

FIG. 4. Incorporation of [1-^{14}C]palmitate (dose Sp.A. 14.4 × 10^6 cpm/μeq) into PL in adipocytes of epididymal fat pads of mice of various ages expressed as (A) per unit mass (mg TG), (B) per adipocyte, and (C) per unit membrane (μg PL). Pads were incubated 20 min by the in vivo-in vitro method. Adipocytes were subsequently isolated in fractions representing cell populations from outer (first fractions) and inner (later fractions) regions of the pads ("peeling" method, see Materials and Methods). Data are from 2 experiments (indicated by triangles or circles) with 2 ages of mice per experiment (○, 12 weeks; △, 14 weeks; ▲, 54 weeks; ●, 80 weeks). Each point (4 mice/point) represents the mean PL activity ± SE of fractions containing the mean of the midpoints of the percent of the pad total lipid recovered in that fraction (x-axis).

into TG and PL of adipocytes in mouse epididymal fat pads when the in vivo-in vitro technique (1) is used results from simple diffusion gradients.

A different pattern of labeling can be seen in a study of lipogenesis from labeled glucose carbon in rat epididymal fat pads (5). In this study it was concluded that heterogeneous labeling of cells did not occur. However, our reanalysis of those data shows that the cells were clearly heterogeneous with respect to their total lipid Sp.A. The first batch of cells released by collagenase treatment (9) had only about one-fourth the Sp.A. compared to the third batch of cells released. This is in apparent direct contradiction to our results, but we think that the most likely explanation of Hollenberg's data is that the first batch of cells released were those derived from the central, cut portion of the pad. We have obtained evidence (N. Baker, V. Hill and M. Jacobson, manuscript in preparation) that the epididymal fat pads of mice are surrounded by a thick membrane which could be a significant barrier to collagenase action at an uncut surface. Therefore, we would expect the first cells to be released by collagenase when a pad is dissected from the animal and placed in a buffer, as in Hollenberg's study (5), would be the centrally located cells at the cut surface. In our studies, the pads were cut, but precautions were taken to preclude the exposure of the centrally located cells to collagenase until after the outer thick collagen layer and peripheral cells were first removed. If this rationalization of the apparent differences between our findings and those of Hollenberg is correct, then it would appear that the heterogeneous pattern of labeling that we have observed using the in vivo-in vitro approach occurs in both mice and rats and is seen when the label is either a rapidly diffusing, water soluble substance (glucose) or a lipid (pamitate) bound to albumin as a complex.

In an earlier study using the Stein and Stein technique (1), we reported marked differences in the esterification of palmitate by adipocytes of epididymal fat pads from young and old mice.

Small cells from younger mice synthesized TG from FFA much faster (per unit cell) than large adipocytes from older mice (2). In that paper, we noted that labeled FFA complexed to albumin and added to the incubation medium may have had more difficulty gaining access to the bulk of the adipocytes in large pads from older mice than in small pads from younger animals. The present study does not resolve that question since the phenomenon of heterogeneous labeling of cells occurred in fat pads of both young and old mice. It remains to be established whether FFA that enter adipocytes from the capillaries that are in contact with all the cells are incorporated more readily into adipocytes of young than of old mice.

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Dietary Squalene Increases Tissue Sterols and Fecal Bile Acids in the Rat

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ABSTRACT

Feeding 1% squalene increased markedly the concentrations of squalene and methyl sterols in each serum lipoprotein class, intestinal mucosa, liver and also in adipose tissue. It also increased cholesterol concentration of the liver and serum VLDL, and esterified cholesterol in serum LDL as well as fecal bile acids. The results suggest that absorbed dietary squalene contributes to some extent to the squalene content of adipose tissue, effectively increases the overall cholesterol synthesis and enhances cholesterol elimination preferentially as fecal bile acids.
Lipids 18:32-36, 1983.

Squalene, a water-insoluble precursor of cholesterol, is a widely distributed lipid in common western diets, especially in fish and plant oils (1-5). Liu et al. have estimated that the average daily intake of squalene is about 25 mg/2000 calories in the USA, but that it can reach up to 200 mg if olive oil is used (5). Dietary squalene can affect the serum level of squalene in man (5,6) and cholesterol concentration of the rat liver (7). However, the metabolic fate of dietary squalene has not been investigated in detail.

Squalene is also present in most mammalian tissues (5,8). Postmortem analysis of human tissues have revealed that the highest squalene concentrations are found in adipose and muscle tissues (8). In serum, squalene is principally transported with VLDL and its concentration is closely correlated with that of triacylglycerols (9). Radioactive squalene administered intravenously is only negligibly taken up by adipose tissue of the rat (10). Whether the raised serum squalene could affect the squalene concentration of adipose tissue is still unknown.

In this study, sterols of serum lipoproteins, tissues and feces were analyzed in rats on high and low squalene diets. Special emphasis was paid to serum methyl sterols, which reflect the activity of overall cholesterol synthesis in many experimental and clinical conditions (11) and to sterol balance of the animals in order to evaluate changes in cholesterol synthesis.

MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley rats (Anima Ltd., Finland), weighing 230-260 g, were fed for 10 days with "fat-free test diet" (Nutritional Biochemicals, Cleveland, OH). This diet contained virtually no squalene and sterols. Squalene-rich diet was prepared by dissolving 10 g squalene (Sigma Ltd., St. Louis, MO) in 50 ml olive oil (Fischer Scientific Company, Fairlawn, NJ) and the solution was mixed with 1 kg of "fat-free test diet". On this diet, the daily intake of squalene of the rats was 190 ± 30 mg. The animals were housed one in the cage and had free access to tap water.

Determination of Tissue Lipids

After exsanquination of the rats liver, intestine and epidymal fat pads were removed and washed with saline at room temperature. The mucosa of washed jejunum was scraped off with a scalpel blade to the muscle layer. The samples were homogenized with Ultra-Turrax^{R} and the lipids were extracted with chloroform/methanol (2:1, 12). After addition of coprostanol (Sigma Ltd, USA) as an internal standard, squalene, free and esterified methyl sterols and cholesterol were determined using the thin layer chromatography-gas chromatography procedure (8). The chemical structure of different methyl sterols was studied by mass spectrometry (8).

Fecal neutral sterols and bile acids were extracted and quantitated by the methods described earlier (13,14). Chromic oxide was used for correction of fecal flow.

The significance of difference was estimated by the Student's two-tailed t-test.

RESULTS

Squalene-rich diet for 10 days raised the squalene concentrations 106-fold in the intestinal mucosa, 51-fold in serum, 15-fold in liver and 2-fold in adipose tissue (Table 1). The increase was detectable in each serum lipoprotein,

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TABLE 1

Effects of Squalene Feeding on Squalene and Sterol Concentrations

Specimen	Number of animals	Squalene (μmol/l[b] or kg)		Methyl sterols (μmol/l[b] or kg)		Cholesterol (mmol/l[b] or kg)	
		Control	Squalene-fed	Control	Squalene-fed	Control	Squalene-fed
Serum	12						
free				21 ± 4	320 ± 11[a]	29 ± 3	37 ± 2
ester				64 ± 11	1598 ± 282[a]	103 ± 5	117 ± 6
total		67 ± 11	3407 ± 1105[a]	85 ± 11	1918 ± 276[a]	132 ± 11	154 ± 8
VLDL							
free				5 ± 1	101 ± 15[a]	4 ± 1	12 ± 3[a]
ester				14 ± 2	756 ± 287[a]	4 ± 1	13 ± 2[a]
total		35 ± 6	2802 ± 1298[a]	19 ± 2	857 ± 284[a]	8 ± 1	25 ± 2[a]
LDL							
free				6 ± 1	88 ± 15[a]	9 ± 3	10 ± 1
ester				44 ± 9	740 ± 209[a]	17 ± 3	28 ± 3[a]
total		21 ± 5	325 ± 124[a]	50 ± 9	828 ± 208[a]	26 ± 3	38 ± 3[a]
HDL							
free				10 ± 2	131 ± 14[a]	16 ± 2	15 ± 2
ester				5 ± 1	102 ± 9[a]	82 ± 5	76 ± 6
total		11 ± 3	280 ± 108[a]	15 ± 1	233 ± 12[a]	98 ± 5	91 ± 6
Liver	6	30 ± 6	432 ± 132[a]	13 ± 5	196 ± 48[a]	4 ± 1	10 ± 2[a]
Intestinal mucosa	6	11 ± 3	1163 ± 417[a]	27 ± 5	76 ± 8[a]	6 ± 1	4 ± 1
Adipose tissue	6	68 ± 9	161 ± 11[a]	8 ± 2	29 ± 3[a]	1 ± 1	2 ± 1

Mean ± SEM.

[a]$p \leq 0.05$. The animals were pair-fed for 10 days with fat-free test diet (control or with fat-free test diet supplemented with squalene (1%).

[b]All serum values multiplied by 100.

the most significant elevation being found in VLDL (80-fold). Under the base-line conditions, 52% of serum squalene was transported by VLDL. During the squalene feeding, the value increased to 82%.

The total methyl sterol concentrations were also markedly increased in serum (23-fold), liver (15-fold), intestinal mucosa (3-fold), and even in adipose tissue (3-fold). Both the free and esterified methyl sterol concentrations were markedly elevated in each serum lipoprotein class, especially in VLDL (Table 1). The increase in esterified methyl sterols was even more marked than in free methyl sterols. The bulk of methyl sterols was transported by LDL (59%) on the control diet and by VLDL (45%) and LDL (43%) on the squalene diet.

All the individual free and esterified methyl sterol subfractions were significantly increased in each serum lipoprotein class. The highest increase of free methyl sterols was found in methostenol (fraction III) and fraction V, the increase of methostenol being predominant in LDL and HDL (Fig. 1). Of the individual esterified methyl sterols, the two monomethyl sterols, especially methostenol, increased most significantly in each lipoprotein by squalene feeding. The proportion of the two dimethyl sterols (fractions II and V) was surprisingly low especially on the high squalene intake.

Fraction V was virtually unesterified in all lipoproteins and, in contrast to cholesterol, esterification percent of all methyl sterols was low (5-50%) in HDL (Table 2). Squalene feeding increased the esterification percentage of all methyl sterols in VLDL, less consistently in LDL and in HDL.

The pattern of hepatic total methyl sterols resembled that of esterified VLDL methyl sterols, in that methostenol was the major compound. Squalene feeding increased all the individual methyl sterols, the proportion of methostenol being increased from ca. 50% to 75% by dietary squalene. Methyl sterol patterns of jejunal mucosa and adipose tissue were different from those of serum lipoproteins. Squalene feeding changed the pattern of the two organs similarly. Thus, only lanosterol (fraction IV) and diunsaturated dimethylsterol (fraction V) were consistently increased, the others remained unaltered.

In contrast to methyl sterols, the serum level

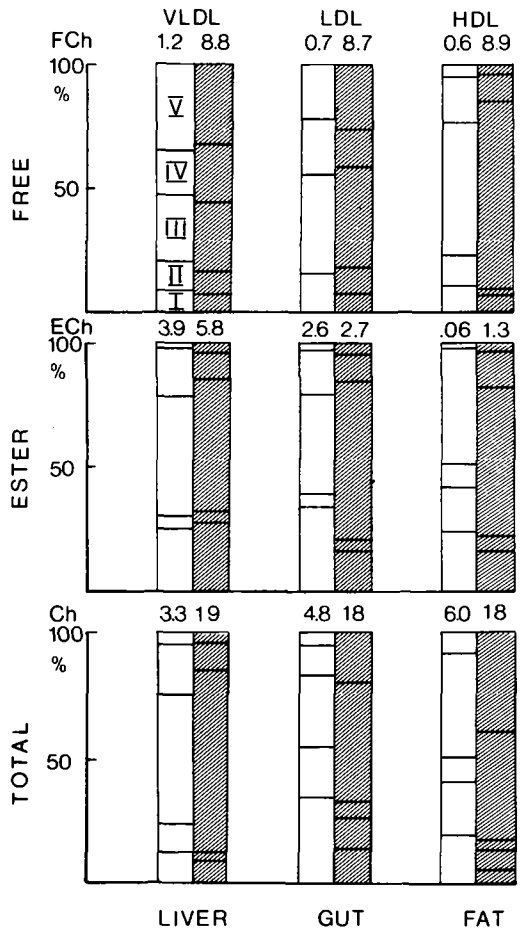

FIG. 1. Percentage distribution of individual methyl sterols in serum lipoproteins, liver, intestinal mucosa, and adipose tissue of the rat on squalene-free (□) and squalene-rich (■) diet. Upper panel: free methyl sterols of serum VLDL, LDL and HDL; middle panel: esterified methyl sterols of VLDL, LDL and HDL; lower panel: total methyl sterols of liver, intestinal mucosa and adipose tissue. On the top of each column, the sum of the 5 methyl sterols (I-V) is expressed in terms of mmol/mol free (upper panel), esterified (middle panel), or total cholesterol (lower panel). On the ordinate, the sum of the 5 methyl sterols is indicated by 100. The chemical structures of different methyl sterols are indicated in footnote to Table 2.

of total cholesterol was not significantly affected by squalene feeding (Table 1). However, a marked redistribution of cholesterol was observed in serum lipoproteins. Thus, free and esterified cholesterol was tripled in VLDL on the squalene-rich diet. The three triglyceride measurements revealed inconsistent decrease in VLDL (1.33 mmol/l in controls and 0.92 mmol/l in squalene-fed rats). Furthermore, cholesteryl esters were almost doubled in LDL, whereas the level of LDL-triglycerides did not rise, and the total amount of cholesterol in HDL tended to decrease. In the liver, the concentration of total cholesterol was markedly increased (3-fold).

The changes in the lipoprotein concentrations, as indicated by cholesterol, might have contributed to the increase in the cholesterol precursor levels during squalene feeding. Data on the top of the columns in the upper panel of Figure 1 show that, in terms of mmol/mol of free cholesterol, VLDL, LDL and HDL are almost equally rich in free methyl sterols under the base-line conditions and during squalene feeding, respectively. On the squalene diet, the values are 7-15 times higher, however. The less polar squalene and methyl sterol esters exhibit highest contents in VLDL and very low ones in HDL, both off and on squalene; squalene feeding increased the content mainly in VLDL, though the relative increase was highest in HDL.

In order to evaluate the effect of squalene feeding on overall cholesterol synthesis, the fecal steroids of the rats were analyzed (Table 3). In spite of the similar weight gain of the animals, squalene feeding increased significantly total fecal steroids mainly as bile acids.

DISCUSSION

Markedly increased concentrations of serum and tissue squalene and methyl sterols, and fecal elimination of cholesterol primarily as bile acids indicate that dietary squalene has been absorbed and contributed to overall cholesterol synthesis. In fact, our unpublished studies showed that 30% of dietary squalene was observed and cholesterol synthesis from endogenous precursors was suppressed, dietary squalene being virtually the only source of newly found cholesterol.

Despite marked increase of dietary squalene in intestinal mucosa, lanosterol and diunsaturated dimethyl sterols were the only methyl sterols which accumulated as a consequence of enhanced cholesterol synthesis. These two sterols are the next successive intermediary sterols after squalene in the synthesis pathway of cholesterol. The levels of the subsequent methyl sterols were not increased, suggesting that demethylation of methyl group at carbon 14 and especially dihydrogenation of the side-chain double bond were rate-limiting. The hepatic methyl sterol pattern differed from that

TABLE 2

Effects of Squalene Feeding on the Percentage Esterification of Sterols in Serum Lipoproteins

Diet	Individual methyl sterols						Cholesterol
	I	II	III	IV	V	Sum	
				VLDL			
Control	88 ± 6	50 ± 3	93 ± 2	75 ± 5	5 ± 1	75 ± 4	48 ± 3
Squalene	95 ± 4	78 ± 6	95 ± 2	78 ± 4	38 ± 3	88 ± 4	53 ± 2
				LDL			
Control	85 ± 3	75 ± 4	86 ± 3	86 ± 3	4 ± 1	88 ± 2	67 ± 2
Squalene	84 ± 3	76 ± 6	95 ± 4	89 ± 4	46 ± 4	89 ± 2	73 ± 2
				HDL			
Control	54 ± 5	37 ± 4	13 ± 6	45 ± 2	5 ± 2	33 ± 4	83 ± 1
Squalene	46 ± 6	40 ± 2	34 ± 4	48 ± 3	25 ± 6	43 ± 5	84 ± 1

Mean ± SE (N=6).

Subfraction I = 8-en-monomethylsterol (4α-methyl-5α-cholest-8-en-3β-ol), contains also trace amounts of dihydrolanosterol (4,4,14-trimethyl-5α-cholest-8-en-3β-ol); II = monounsaturated dimethylsterol (4,4-dimethyl-5α-cholest-8-en-3β-ol); III = methostenol (4α-methyl-5α-cholest-7-en-3β-ol); IV = lanosterol (4,4,14α-trimethyl-5α-cholest-8,24-dien-3β-ol); and V = diunsaturated dimethylsterol (4,4-dimethyl-5α-cholest-8(7), 24 dien-3β-ol).

TABLE 3

Effect of Squalene Feeding on Weight Gain and Fecal Steroids

Diet	Weight gain (g/day)	Fecal steroids (μmol/day)		
		Neutral sterols	Bile acids	Total
Control	2.1 ± 0.2	10.4 ± 1.6	8.3 ± 1.6	18.7 ± 2.1
Squalene	1.8 ± 0.1	14.5 ± 1.9	16.9 ± 2.6[a]	31.4 ± 4.2[a]

Mean ± SEM (n = 6).

[a]$p \leq 0.05$. The average daily intake of squalene was 509 ± 80 μmol/day. The feces was collected on the 9th day after the start of feeding.

in the mucosa, indicating that biliary sterols apparently play a minor role in the genesis of mucosal methyl sterols. The similar change in the intestinal and adipose tissue methyl sterol patterns suggests that chylomicrons and VLDL transported intestinal methyl sterols to adipose tissue. In fact, labeled squalene administered orally appears in thoracic lymph as squalene and to a small extent as methyl sterols and cholesterol (15). In addition, labeled squalene administered intravenously in VLDL or chylomicrons is found in adipose tissue (10). Thus, the conversion of squalene to cholesterol could be similarly rate-limited in the two tissues.

The increase in all hepatic methyl sterols during the squalene-induced activation of cholesterol synthesis indicates limited hepatic demethylation at both carbon 14 and 4. The predominant elevation of the methostenol level with less prominent Δ^8-methostenol (fraction I) points especially to rate-limiting 4α-demethylation of Δ^7-methyl sterols. The pattern of free methyl sterols in different lipoproteins was not very much different and resembled more that of the liver than of the intestine or fat tissue. Thus, the increase in the serum methyl sterol levels was mainly caused by enhanced release of these sterols from the liver and reflected squalene-induced activation of hepatic cholesterol synthesis. Quite marked diurnal fluctuation of serum free methyl sterols (16) suggests that they are equilibrated on and off lipoproteins and that they are metabolized to a lesser extent via synthesis and catabolism of whole lipoprotein molecules.

The low esterification percentage of methyl sterols especially in HDL can be explained by the finding that, in contrast to cholesterol, methyl sterols are apparently formed by ACAT, mainly in the liver, probably, and released with

the newly formed VLDL into the blood. Catabolism of VLDL may transfer the esters to LDL and HDL. The marked increase of esterified methyl sterols and cholesterol especially in VLDL, but also in LDL by dietary squalene, is most likely caused by enhanced hepatic esterification of those sterols.

It has been suggested that VLDL production is linked with cholesterol and bile acid synthesis (18). Squalene feeding enhanced hepatic cholesterol and bile acid synthesis, and increased VLDL cholesterol. However, the few analyses of VLDL and LDL triglycerides indicated a lack of the squalene effect. Thus, increased cholesterol synthesis caused by squalene feeding was apparently associated with a formation of hepatic VLDL that was quite rich in cholesterol. Subsequent extrahepatic catabolism of this VLDL may have enriched LDL with esterified cholesterol. It remains to be shown whether the squalene-induced increase in cholesterol synthesis is actually associated with enhanced turnover of VLDL.

Earlier studies have indicated that newly synthesized cholesterol serves as a preferential precursor for bile acid synthesis for cholic acid production, in particular (18-21). The marked increase in fecal bile acids of the present study may actually be associated with the squalene-induced increase in newly formed hepatic cholesterol.

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Peroxidative Formation of C_3-Hydrocarbons from an ω-4 Polyunsaturated Fatty Acid(16:3ω4) in the Alga *Bumilleriopsis*

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ABSTRACT

Under peroxidative conditions (i.e., illumination in the presence of Cu^{2+} or a *p*-nitro diphenylether herbicide), the xanthophycean microalga, *Bumilleriopsis filiformis*, evolves C_2 and C_5 hydrocarbons besides substantial amounts of propane and propene. Fatty acids were separated as methyl esters by argentation and reversed-phase thin layer chromatography and the fractions subsequently peroxidized by illuminated and copper-supplemented *Anacystis* thylakoids. These membranes do not contain polyunsaturated fatty acids and are, therefore, unable to evolve volatile hydrocarbons by itself. The C_2 and C_5 hydrocarbons formed by the fractions added match with their content of ω-3 and ω-6 fatty-acid species having 2-4 double bonds. The fractions yielding C_3 hydrocarbons contain a fatty acid hitherto unknown for *Bumilleriopsis*, which was isolated and identified as 16:3ω4.

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INTRODUCTION

Degradation of fatty acids can be achieved using chemical (1,2) or biological systems (see ref. 3 for review). Several attempts have been made to determine fatty acid sources of short-chain hydrocarbons formed under peroxidative conditions. Formation of short-chain hydrocarbons by organisms requires polyunsaturated fatty acids with at least two double bonds. Furthermore, the chain length of the evolving hydrocarbons is determined by the position of the double bond most distant from the carboxyl group. For example, thylakoid membranes from blue-green algae form C_2 hydrocarbons from endogenous ω-3 polyunsaturated and C_5 hydrocarbons from ω-6 polyunsaturated fatty acids (4).

Recently, it has been reported that peroxidation of fatty acids in the xanthophycean alga, *Bumilleriopsis*, caused by peroxidative herbicides like oxyfluorfen (2-chloro-4-trifluoromethylphenyl-3′-ethoxy-4′-nitrophenylether) or paraquat (1,1′-dimethyl-4,4′-bipyridlium dichloride) resulted in the formation of propane and some ethane (5,6).

In this paper, we report the formation of C_3 and C_5 hydrocarbons in addition to those with 2 carbons in *Bumilleriopsis*. In particular, the formation of propane and propene is quite substantial, which led us to determine the source of the C_3 volatile hydrocarbons.

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MATERIALS AND METHODS

Cultivation and Spheroplast Preparation

The microalga *Bumilleriopsis filiformis* (from our own stock) was grown in sterile liquid medium as described previously (7,8). The marine diatom, *Skeletonema costatum* (strain no. LG 1077/1, Algal Culture Collection, University of Cambridge) was used to extract the ω-4 hexadecatrienoic-acid (16:3ω4); its cultivation was carried out in enriched seawater medium as described by Darley and Volcani (9) under continuous fluorescent light (10 W/m^2) for 4 weeks at 22 C. Spheroplasts were prepared from the blue-green alga, *Anacystis nidulans* (strain 1402-1, Algae Culture Collection, University of Göttingen), and used for biological peroxidation of isolated fatty acid methyl esters. For growth of this algae, see Sandmann and Böger (4). Spheroplast preparation by lysozyme treatment was done according to Spiller (10). Active thylakoids were obtained by osmotically shocking the spheroplasts, i.e., by transferring them into the incubation medium which was used by Sandmann and Böger (4) to determine volatile-hydrocarbon evolution as described.

Extraction and Separation of Fatty Acids from Algae

The wet algal pellet from a culture suspension of *Bumilleriopsis* (4 batches, 1.25 ℓ each) was extracted first with methanol (80 ml) for 15 min at 65 C and then with a chloroform/methanol mixture (2:1, v/v, 80 ml) for 15 min

at 40 C. The combined extracts were pooled and poured into saturated NaCl solution (100 ml). After phase separation, the chloroform layer was collected, dried over anhydrous Na_2SO_4, and chromatographed on activated silica gel plates (Merck AG, Darmstadt, W. Germany) up to a front distance of 5 cm, using chloroform/methanol/water (65:2:4, v/v/v). The part between the origin and the lowest colored band was scraped off and extracted twice with methanol (30 ml for 5 min at 65 C). Saponification and esterification was performed as described (4). The methyl esters were then partitioned into petroleum ether (bp 40-60 C, 50 ml) and reduced under nitrogen to a volume of about 1 ml. The ester mixture was spotted as a line on $AgNO_3$-impregnated silica gel plates which were prepared by spraying with a 10% (w/v) $AgNO_3$ solution in acetonitrile (10 ml) and heating for 30 min at 130 C. The plates were developed twice in a solvent system containing hexane/diethyl ether/acetic acid (94:4:2, v/v/v) (11), dried under a stream of nitrogen, and sprayed with a 1:1 (v/v) mixture of 0.03% rhodamine 6G and 8% NaOH. Bands made visible under ultraviolet (UV) light were scraped off and extracted twice with diethyl ether (1 ml each). Fraction no. 4, from bands with and R_f value from 0.3 to 0.5, was rechromatographed on silica gel plates impregnated with silicone oil (Merck AG, Darmstadt) by immersing them in a solution of 5% silicone oil in diethyl ether. The solvent system for chromatography was acetonitrile/acetic acid/water (70:10:20, v/v/v) (12). The 16:3ω4 fatty acid, Δ6,9,12-hexadecatrienoic acid methyl ester from *Skeletonema costatum*, was prepared in the same way.

Analysis of Fatty Acids

Fatty acid methyl esters were separated and identified by gas chromatography on either a 15% Reoplex column at 200 C or a 15% DEGS column at 180 C using a Perkin-Elmer F22 gas chromatograph. Identification and quantification of fatty acid methyl esters was performed by using standards purchased from Sigma, Munich. The 16:2ω6 fatty acid was a generous gift of Dr. P.G. Gülz. The 14:1 and 18:4ω3 acids were identified by determination of their equivalent chain length values by the procedure of Ackman (13).

Fatty Acid Peroxidation

The ether solution containing the fatty acid methyl esters was pipetted into vessels fitting into the automatic head-space sampler of a Perkin-Elmer gas chromatograph, mod. F22. Then the organic solvent was removed in a stream of nitrogen and the residue redissolved in an 0.2% Triton X-100 solution (100 μl). Incubation medium (phosphate buffer, pH 7.8, 10 mM; NH_4Cl, 5 mM; $MgCl_2$, 5 mM, $CuSO_4$, 50 mM), and *Anacystis* thylakoids equivalent to 200 μg of chlorophyll were added to a final volume of 2 ml. Then, the samples were incubated in the sealed head-space vessels over 2 hr at 25 C in the light.

For the experiment in Table 1, cultures of *Bumilleriopsis* were inoculated with a packed cell volume (pcv) of 1 μl/ml and grown autotrophically for 18 hr with 100 μM Cu^{2+} or 10 μM oxyfluorfen present. Then the suspension was concentrated to 40 μl/ml pcv by centrifugation and 2-ml samples were incubated in head-space vessels as described above. The volatile hydrocarbons produced were analyzed after 4 hr. The detailed procedure of automatic sample-withdrawing and chromatography of the volatile hydrocarbons produced on activated alumina has been described (4).

TABLE 1

Volatile Hydrocarbons (pmol) Evolved in the Light by *Bumilleriopsis* Cultures Treated with Cu^{2+} or the *p*-Nitrodiphenylether Oxyfluorfen

Hydrocarbons	Cu^{2+}	Oxyfluorfen
Ethane	20.2	11.6
Ethylene	126.9	7.6
Propane	23.2	38.0
Propene	97.6	3.4
Pentane	20.4	15.3
Pentene	41.4	11.9

Evolution of each hydrocarbon gas in untreated controls was below 1 pmol.

RESULTS AND DISCUSSION

When cells of *Bumilleriopsis* had been treated with Cu^{2+} or oxyfluorfen (a peroxidative herbicide, 2-chloro-4-trifluoromethylphenyl-3′-ethoxy-4′-nitrophenylether), formation of various hydrocarbons was observed (Table 1). With the temperature program used for gas chromatography, it was possible to determine all saturated and unsaturated hydrocarbon gases with chain lengths of 2 to 5 C-atoms, if present. Using either Cu^{2+} or oxyfluorfen to induce peroxidation, pentane and pentene were detected in addition to the C_2 and C_3 hydrocarbons previously reported to be formed in *Bumilleriopsis* (3,6). The evolution of any saturated or unsaturated C_4 hydrocarbons can be ex-

cluded. Cu^{2+} was the stronger peroxidative agent. Also, the relative amounts of C_2, C_3, and C_5 hydrocarbons varied with the peroxidative agents. When Cu^{2+} was used, C_2-hydrocarbon formation dominated over C_3, followed by evolution of C_5. When oxyfluorfen was present, most of the volatile hydrocarbons evolving had 3 carbon atoms, followed by C_5 hydrocarbons and by C_2 species. This variation in hydrocarbon evolution can be explained by different mechanisms by which (starter) radicals are formed through Cu^{2+} (14) and oxyfluorfen (15), respectively, which initiate peroxidative chain reactions with fatty acids and keep them going.

The dominance of unsaturated volatile hydrocarbons vs saturated ones in the presence of Cu^{2+} is seemingly due to the redox activity (valence state) of the copper ion (14). Also, in paraquat-mediated peroxidation with intact *Bumilleriopsis* cells, addition of Fe^{2+} increased the rate of evolution of unsaturated vs saturated hydrocarbon gases (6).

Dumelin and Tappel (1) discussed the formation of propane from CCl_4-treated rats by β-scission of pentane which is the major hydrocarbon formed under these conditions. *Anacystis* thylakoids have no polyunsaturated fatty acid and do not produce short-chain hydrocarbons by themselves when illuminated in the presence of Cu^{2+}. Experiments in which various polyunsaturated fatty acids were added exogenously showed that a β-scission reaction is of minor importance (4). Therefore, ω-6 fatty acids which are degraded to C_5 hydrocarbons apparently are not the source of propane and propene in *Bumilleriopsis*.

To study the origin of C_3 hydrocarbons, individual fatty acid fractions were isolated as methyl esters from *Bumilleriopsis* and incubated with isolated thylakoid material (see Methods). Argentation thin layer chromatography (TLC) resulted in 6 distinct fractions (nos. 1-6) in which the fatty acids were enriched according to their degree of desaturation (Table 2). Fractionation helped to identify new fatty acids from *Bumilleriopsis* as 16:2, 18:0 and 18:2 species, which extends previous findings (16). Chain length and number of double bonds of these fatty acids match with their expected accumulation in the corresponding fractions.

In contrast to fatty-acid fractions nos. 1 and 2 of Table 2, fractions 3-6 gave rise to volatile hydrocarbons under peroxidative conditions (Table 3). From fraction 3, C_5 hydrocarbons were predominantly obtained in addition to some C_2 and C_3 species. Fractions nos. 5 and 6 yielded high amounts of C_2 and C_5 hydrocarbons. Peroxidation of fraction no. 4 by *Anacystis* thylakoids led to formation of substantial propane and propene. Therefore, this fraction was further purified to identify the fatty acid(s) responsible for C_3-hydrocarbon evolution. Reversed-phase TLC separated the methyl esters into 3 bands (nos. 4-1/4-2/4-3 of Table 2). Most of the band (97%) with the highest R_f value (no. 4-1) was a fatty acid "X", which could not be identified with commercially available standards. Its accumulation in the fraction containing the unsaturated species with 3 double bonds (no. 4, Table 2) and the

TABLE 2

Composition of Fatty-Acid Fractions from *Bumilleriopsis*

Fraction no.	R_f regions[a]	Total fatty acid esters (mg)	% Composition of fatty acids of each fraction
1	0.74-0.85	61.3	14:0 (52%), 14:1 (1%), 16:0 (45%), 16:1 (1%), 18:0 (1%)
2	0.55-0.75	96.1	14:1 (7%), 16:1 (88%), 18:1 (5%)
3	0.50-0.55	30.5	"X" (5%), 16:1 (10%), 16:2ω6 (63%), 18:2ω6 (11%), 18:3ω3 (4%), 18:3ω6 (7%)
4	0.35-0.50	28.7	"X" (30%), 16:1 (4%), 16:2 (3%), 18:3ω3 (7%), 18:3ω6 (21%), 20:4ω6 (35%)
4-1	0.7-0.9[b]	8.0	"X" (97%), 18:3ω6 (2%), 20:4ω6 (1%)
4-2	0.5-0.7[b]	9.1	18:3ω3 (27%), 18:3ω6 (57%), 20:4ω6 (16%)
4-3	0.3-0.5[b]	8.6	18:3ω3 (1%), 18:3ω6 (9%), 20:4ω6 (90%)
5	0.25-0.35	23.9	"X" (2%), 18:4ω3 (24%), 20:4ω6 (52%)
6	0.05-0.25	41.4	18:4ω3 (11%), 20:5ω6 (89%)

[a]R_f values of fractions nos. 1-6 refer to argentation TLC.

[b]R_f values from reversed-phase TLC of fraction; see Methods for details.

TABLE 3

Formation of Volatile Hydrocarbons (nmol) over 2 hr from Fatty Acid Methyl Ester Fractions Peroxidized with Illuminated *Anacystis* Thylakoids in the Presence of 100 μM Cu^{2+}

Addition of fractions from table 1 (no.)	Ethane	Ethylene	Propane	Propene	Pentane	Pentene
3	0.32	0.74	1.01	1.98	7.60	12.24
4	1.09	1.95	4.09	5.90	5.28	6.11
5	6.39	6.19	0.54	0.88	8.57	2.89
6	3.09	3.37	0.02	0.03	1.63	2.00
4-1	0.05	0.08	3.46	4.82	0.18	0.11
4-2	0.91	1.22	0.03	0.04	1.02	1.46
4-3	0.01	0.02	0.07	0.08	4.88	5.44

From fractions nos. 1 to 6, 5 mg were used per peroxidation assay; in the case of the more enriched fractions 4-1 to 4-3, 2 mg were added. Fractions 1 and 2 evolved only traces of hydrocarbons. Addition of 2 mg of 16:3ω4 isolated from *Skeletonema* yielded about 2 nmol of propane and 4 nmol of propene.

C_{16} fraction (no. 4-1) is indicative of a 16:3 fatty acid, although it did not show the retention times of a 16:ω3 species, either using a 15% DEGS or a 15% Reoplex column.

Eventually, we isolated a 16:3ω4 fatty acid from *Skeletonema costatum*, an alga in which a 16:3ω4 unsaturated fatty acid had been found previously (17). The corresponding fatty acid methyl ester cochromatographed with fraction no. 4-1 in the TLC systems. The separation behavior on both gas-chromatography columns is shown in Figure 1. Using either a 15% DEGS column or a 15% Reoplex column, identical retention times for "X" and for the 16:3ω4 species from *Skeletonema* were obtained. The data prove identity of the 16:3ω4 species with the fatty acid "X" from *Bumilleriopsis* enriched in fraction no. 4-1. Peroxidative formation of C_3 hydrocarbons from 16:3ω4 and "X" were also about the same (Table 3).

In conclusion, we have postulated previously that the chain length of hydrocarbons formed during peroxidative degradation of fatty acids by biological systems is determined by the position ω of the double bond most distant from the carboxyl group (3,4). This was based on experiments with thylakoids from several blue-green algae with various patterns of endogenous fatty acids. A position of ω should result in $C_{\omega-1}$ saturated or unsaturated hydrocarbons. This was demonstrated for C_2 hydrocarbons originating from ω-3 and C_5 hydrocarbons from ω-6 polyunsaturated fatty acids (4). The present study has proved that C_3 hydrocarbons originated from an ω-4 polyunsaturated fatty acid.

ACKNOWLEDGMENTS

This investigation was supported by the Deutsche Forschungsgemeinschaft (grant Bo 310/12-4). The authors are indebted to Dr. P.G. Gülz from Botanisches Institut, Universität Köln, Germany, for a sample of a 16:2ω6 fatty acid, and to R. Lambert from this laboratory for assistance in analyzing the fatty-acid fractions. The excellent technical assistance of Mrs. Silvia Kuhn is gratefully acknowledged. Rohm and Haas, Spring House, PA, USA, supplied a sample of pure oxyfluorfen.

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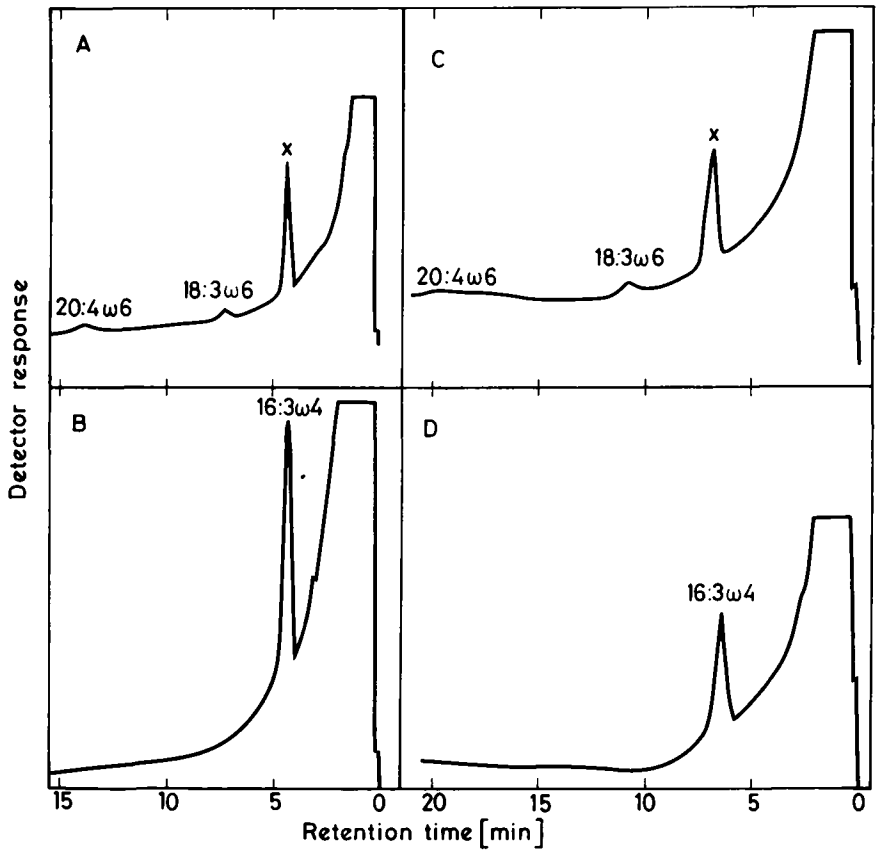

FIG. 1. Identification of "X", the major component of fraction 4-1 of Table 2, as a 16:3ω4 species. Separation of fraction 4-1 (A) and 16:3ω4 prepared from *Skeletonema* (B) by gas chromatography on a 15% Reoplex column. The same fatty acid methyl esters were identified additionally on a 15% DEGS column (see parts (C) and (D), respectively).

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Effects of Purified Eicosapentaenoic Acid on Arachidonic Acid Metabolism in Cultured Murine Aortic Smooth Muscle Cells, Vessel Walls and Platelets

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ABSTRACT

The effects of highly purified eicosapentaenoic acid (97% pure) on the arachidonic acid cascade in isolated murine vascular cells and platelets were studied. The incorporation of eicosapentaenoic acid was not as active as that of arachidonic acid in platelets. The ratio of incorporation of eicosapentaenoic acid to arachidonic acid into platelet phospholipids was about 0.7. Analysis of the phospholipid fractions of platelets after labeling with ^{14}C-eicosapentaenoic acid and ^{14}C-arachidonic acid revealed that the incorporation of ^{14}C-eicosapentaenoic acid into the phosphatidylinositol fraction is significantly less than that of ^{14}C-arachidonic acid, while the incorporation of both fatty acids into other phospholipid fractions was almost the same. On the other hand, no significant difference between either fatty acid in incorporation rate, kinetics or distribution in cellular phospholipids was found in cultured aortic smooth muscle cells. Following treatment with eicosapentaenoic acid, cells produced less prostacyclin from endogenous arachidonic acid than did control cells. This was not due to the decrease in fatty acid cyclooxygenase activity, but rather, due to the decrease in arachidonic acid content in cellular phospholipids. In addition, eicosapentaenoic acid was neither converted to prostaglandin I_3 by the vascular cells nor to thromboxane A_3 by platelets. Furthermore, similar results were also obtained by in vivo experiments in which rats were fed with eicosapentaenoic acid enriched diet. *Lipids* 18:42-49, 1983.

INTRODUCTION

Greenland Eskimos have been demonstrated to have a low incidence of myocardial infarction by epidemiological studies (1). Dyerberg et al. (2) showed that high content of all *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) in their food is connected with low incidence of myocardial diseases. Since then, some investigators have reported that EPA has an inhibitory effect on platelet aggregation (3,4). However, all the reports published to date were investigated using crude EPA or fish oil instead of purified EPA. The crude EPA and fish oil contain other fatty acids having an inhibitory effect on platelet aggregation, such as docosahexaenoic acid (5). Therefore, to know the exclusive effect of EPA alone on vascular cells and platelets, studies using purified EPA must be done.

In the present studies, we showed that purified EPA was easily incorporated into phospholipids of cultured murine smooth muscle cells as well as vessel walls, and the biosynthesis of prostacyclin (prostaglandin I_2, PGI_2) was inhibited by the incorporated EPA, both in vivo and in vitro. The effects of purified EPA on the platelet arachidonic acid (AA) cascade were also examined.

MATERIALS AND METHODS

Chemicals

[1-^{14}C] Arachidonic acid (58 Ci/mol) was purchased from Radiochemical Centre, Amersham, U.K. [1-^{14}C] Eicosapentaenoic acid (50 Ci/mol) was purchased from New England Nuclear, Boston, MA. Arachidonic acid and standard fatty acid methyl esters for gas chromatography were purchased from Sigma Chemical Co., St. Louis, MO. Authentic prostaglandins (PG) and PGI_2 sodium salt were generous gifts of Ono Pharmaceutical Co., Ltd., Osaka, Japan. Purified EPA (97% pure, the other components were 1.0% 18:3, ω3; 1.0% 16:0; 0.5% 18:1, ω9; 0.5% 18:2, ω6) was kindly supplied by Nippon Oil and Fats Co., Ltd., Tokyo, Japan. Thin layer chromatographic plates of Silica Gel G $60F_{254}$, 0.25 mm

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Abbreviations: EPA, eicosapentaenoic acid; AA, arachidonic acid; PG, prostaglandin; TX, thromboxane.

in thickness, were purchased from Merck, Darmstadt, F.R.G.

Animals

Male Sprague-Dawley rats aged 8 weeks, with body weight ca. 230 g, have been housed in Sizouka Experimental Animals Agricultural Corp., Hamamatsu, Japan, prior to experimental use. Commercial laboratory chow (CRF-1, Oriental Yeast Co., Ltd, Japan) and water were provided ad libitum.

Isolation and Culture of Rat Aortic Smooth Muscle Cells

Rat aortic smooth muscle cells were isolated from medial explants of thoracic aortas and maintained and subcultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum according to the methods described in our previous paper (6). Cells displayed multilayered growth and abundant myofilament characteristic of smooth muscle cells. Cells under passage 8 were used in this series of experiments.

Incorporation of AA and EPA by Cultured Cells and Platelets

Aortic smooth muscle cells in a petri dish (30-mm diameter) were incubated with ^{14}C-labeled AA or EPA in 1 ml of culture medium supplemented with 10% fetal calf serum at 37 C under normal culture conditions. Radioactivity incorporated into the cells was estimated by counting that remaining in the medium by liquid scintillation counter.

Platelet-rich plasma was prepared by centrifuging citrated rat blood at 1200 rpm for 15 min at room temperature. Platelet-rich plasma (1 ml) was incubated with ^{14}C-labeled AA or EPA at 30 C several times. After incubation, platelet-rich plasma was centrifuged at 3000 rpm for 15 min, and the radioactivity incorporated into the platelets and that remaining in the platelet-poor plasma were measured, respectively.

PG Production by the Homogenates of Smooth Muscle Cells

Cells in a petri dish (60-mm diameter) were incubated with 10 or 20 μg of EPA or AA in 4 ml under the conditions mentioned below. After 18-hr incubation, cells were washed twice with 0.05 M phosphate buffer (pH 8.2) and then reharvested using a teflon sheet. Cells were sonicated and the resulting homogenates were used for PG synthesizing activity assay. The homogenate (1 ml) was incubated with 0.2 μCi of ^{14}C-AA or ^{14}C-EPA for 15 min at 37 C. The extraction, separation and identification of PG were performed as described in our previous papers (7,8). In brief, PG were extracted by ethyl acetate at pH 3 and separated by thin layer chromatography (TLC). The solvent system was the organic phase of a solvent of ethyl acetate/2,2,4-trimethylpentane/water/acetic acid (11:5:10:2, v/v/v/v). The radioactive products were detected by a Dünnschicht radiochromatoscanner.

Release of PGI_2 from Smooth Muscle Cells

The release of PGI_2 from cultured aortic smooth muscle cells was determined by radioimmunoassay of 6-keto $PGF_{1\alpha}$ (a hydrolyzed product of PGI_2). Details of the procedure for radioimmunoassay have been described in our previous paper (9). In brief, EPA- and AA-treated cells and control cells were washed twice with serum-free Eagle's minimum essential medium and recultured with 1 ml of medium supplemented with 10% fetal calf serum for 18 hr under normal conditions. The medium was then collected and 6-keto $PGF_{1\alpha}$ in the medium was measured by radioimmunoassay.

Lipid Extraction and Fractionation

Cells in a petri dish (30-mm diameter) were harvested into 0.8 ml of 1% methanol containing 0.1% EDTA to which were added 2 ml of methanol. The mixture was sonicated by a model W185 Branson Sonifier. Platelets in which radioactive fatty acids were incorporated were mixed with 1 ml of methanol, 0.5 ml of chloroform and 0.4 ml of water. A whole lipid extraction was then carried out by the method of Bligh and Dyer (10). Separation of neutral lipid, free fatty acid and phospholipid was carried out by TLC in a solvent system of *n*-hexane/diethyl ether/acetic acid (90:10:1, v/v/v). Separation of the subclass of phospholipid was carried out by TLC using a solvent system of chloroform/methanol/acetic acid/water (75:45:12:3, v/v/v/v) (11). Separation of phosphatidylinositol and phosphatidylserine was carried out by TLC using a solvent system of chloroform/methanol/acetic acid/formic acid/water (45:10:1:5:1, v/v/v/v/v). Rf values of phosphatidylinositol and phosphatidylserine were 0.16 and 0.29, respectively.

Fatty Acid Analysis by Gas Chromatography (GC)

Analysis of acylated fatty acids in phospholipids was carried out by GC of their corresponding methyl esters. The samples for the analysis were obtained by transesterifying with 1 ml of 5% methanolic hydrochloric acid solu-

tion at 80 C for 2 hr. Internal standard heptadecanoic acid (17:0) was added before the esterification. The methyl esters were extracted with *n*-hexane and dried under an N_2 gas stream and then dissolved in 20 μl of chloroform for GC analysis. The fatty acid methyl esters were separated on a column of 16% polyethylene glycol succinate coated on Celite 545 at 185 C with an N_2 gas flow rate of 40 ml/min in a Shimadzu Model 7A gas chromatograph. The data were computerized by a Shimadzu Chromatopac E1A. The identification of EPA and AA was carried out by a capillary column (50 m PEG-HT, purchased from Gasukuro Kogyo Inc., Tokyo, Japan) gas chromatograph.

EPA Feeding in Animals

Rats were divided into two groups. Each group consisted of 6 rats with 230-g body weight. One group received free EPA (75% pure) at 0.7% (wet weight % of the total diet) while the control group received the same % of linoleic acid instead of EPA. The diet was prepared every day in order to prevent autoxidation. The basal diet was the commercial diet as mentioned above. The individual food consumption and body weight were measured daily. Both groups consumed about 20 g/day of food. EPA treatment was continued for 2 consecutive weeks. Rats were fasted one day prior to experimental use. The average body weight of control and EPA-fed rats was 280.3 and 272.3 g, respectively.

Preparation of Aortas

Thoracic aortas were removed immediately after killing. Surrounding fat tissues were carefully trimmed off. The aortas were then washed with 15 mM Tris-HCl buffer (pH 7.6) containing 140 mM NaCl and 5.5 mM glucose.

PGI_2 Production by Aortas

Aortic rings (ca. 5 mg) were incubated with 1 ml of Tris-HCl buffer (pH 8.6) at 37 C for 5 min with shaking. Platelet-rich plasma was prepared by centrifuging citrated rabbit blood at 1200 rpm for 15 min at room temperature. Platelet-rich plasma (200 μl) was preincubated with 20 μl of each sample or standard PGI_2 solution, for 1 min at 37 C in constant stirring, in a Model DP-247E aggregation meter prior to initiation of aggregation induced by 20 μl ADP, at a final concentration of 100 μM. The concentration of PGI_2 in the sample was calculated from the standard curve by using authentic PGI_2 sodium salt.

RESULTS

Incorporation of AA and EPA into Murine Aortic Smooth Muscle Cells in Culture and Platelets in vitro

Murine aortic smooth muscle cells and platelet-rich plasma were incubated with 0.1 μCi of ^{14}C-AA or ^{14}C-EPA. The time courses of the incorporation of the labeled fatty acids into these cells were shown in Figure 1. There was no difference in the incorporation rate between AA and EPA in smooth muscle cells (Fig. 1a), but AA was incorporated more easily than EPA throughout the experimental times in platelets (Fig. 1b).

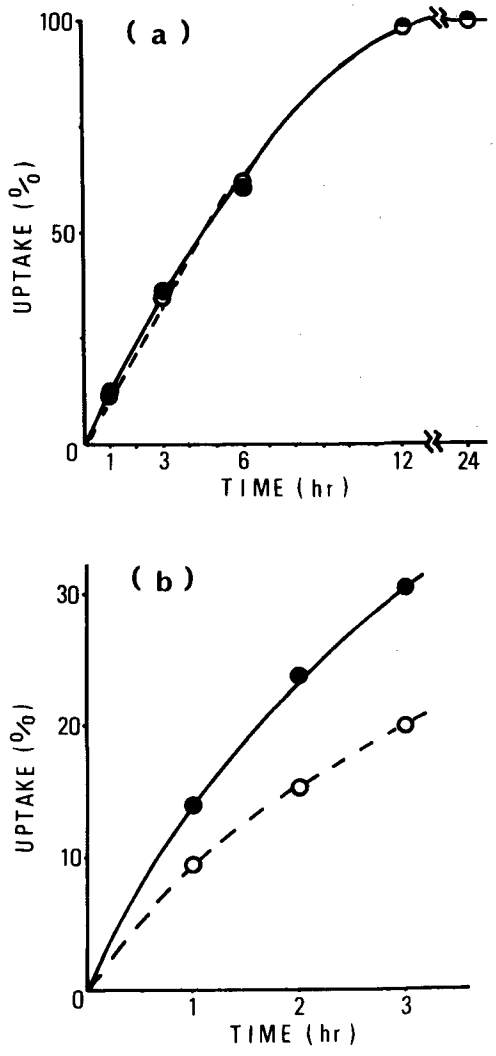

FIG. 1. Time course of the incorporation of ^{14}C-AA (•—•) and ^{14}C-EPA (○—○) into rat (a) aortic smooth muscle cells and (b) platelets.

Next, the effects of dose of AA or EPA on the incorporation of each fatty acid were examined. As shown in Figure 2, both AA and EPA behaved exactly in the same manner and were incorporated linearly into the cultured vascular cells. On the other hand, both AA and EPA were linearly incorporated into the platelets when up to 6 μg of fatty acids were used. However, the increase in incorporation rate declined gradually in a concentration range of 6-21 μg. At any dose of fatty acids used, the incorporation of EPA was always about 70% of that of AA in platelets (Fig. 2b).

Total lipids were then extracted from the labeled cells and platelets. The extracted lipids were fractionated into individual lipids. The ^{14}C-AA and ^{14}C-EPA were found to be distributed largely into the phospholipid fractions in both cultured vascular cells and platelets. Ca. 92% and 93% of the radioactivity incorporated into total lipids were found in the phospholipid fractions of the cultured cells and platelets, respectively. The phospholipids were then subfractionated by TLC analyses. As indicated in Table 1, both AA and EPA had nearly the same distribution pattern in the phospholipid fractions of cultured vascular cells. However, less EPA was incorporated into the phosphatidylinositol fraction as compared with AA in platelets.

Effects of EPA on PG Synthesizing Activity in Cell-Free Homogenates

Cultured murine aortic smooth muscle cells were treated with EPA and AA for 18 hr. Fatty acid concentrations of 10 and 20 μg/dish did not elicit any significant effects on cell proliferation (data not shown). Cell-free homogenates were then prepared as the enzyme source from the control, AA-treated and EPA-treated cells. PG synthesizing activity in cell-free homogenates was investigated by using ^{14}C-AA and ^{14}C-EPA as substrates. The typical scanning profiles showing the conversion of ^{14}C-AA and ^{14}C-EPA are shown in Figure 3. 6-Keto $PGF_{1\alpha}$

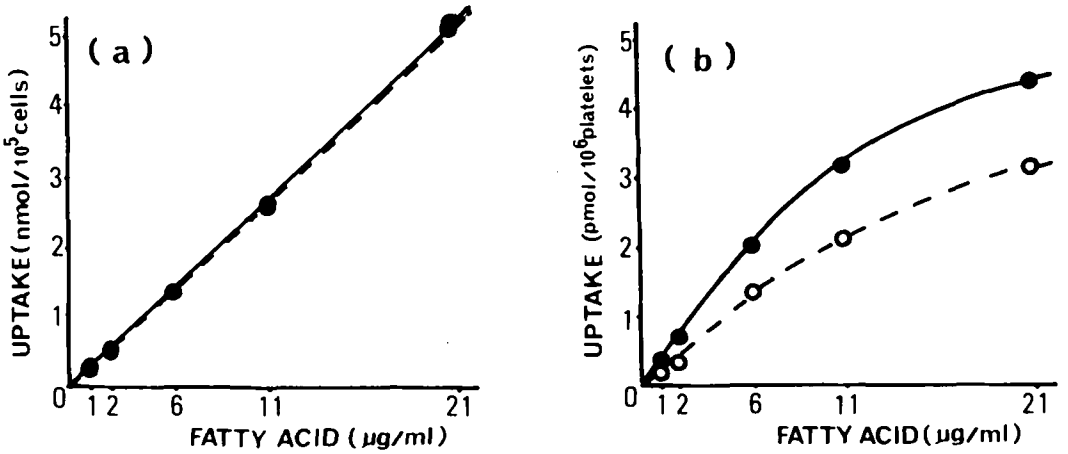

FIG. 2. Incorporation of added AA (•—•) and EPA (○ ··· ○) into (a) aortic smooth muscle cells and (b) platelets.

TABLE 1

Distribution of ^{14}C-Labeled AA and EPA in Phospholipid Fractions

Cells	^{14}C-Fatty acids used	Phospholipid fraction (%)					
		PC	PE	PI	PS	LysoPC	LysoPE
Smooth muscle cells	AA	42.8	45.3	–	–	7.9	4.0
	EPA	42.3	45.7	–	–	8.8	3.2
Platelets	AA	61.2	21.4	13.1	4.3	–	–
	EPA	66.3	21.4	8.9	3.3	–	–

Values are the percentages of total ^{14}C-labeled fatty acids distributed in the total cellular phospholipid fractions. They represent the mean 4 individual experiments and the deviation was less than 5%.

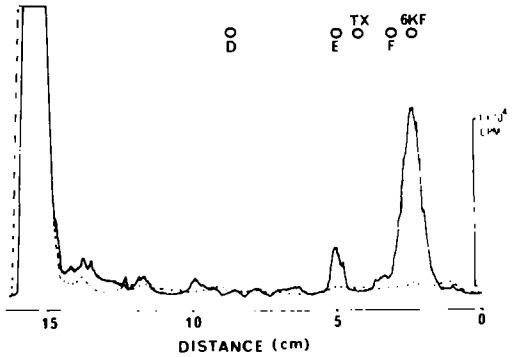

FIG. 3. Radiochromatoscans showing the PG-synthesizing activity in smooth muscle cells. ——, ^{14}C-AA; – – –, ^{14}C-EPA. The reaction mixture (1 ml) contained 0.05 M phosphate buffer (pH 8.2), 0.2 μCi of ^{14}C-AA or ^{14}C-EPA and the sonicated cell-free enzyme source. The TLC plates were developed with the organic phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (11:5:2:10, v/v/v/v). Abbrevations used: D, E, F, 6KF and TX are PGD_2, E_2, $F_{2\alpha}$, 6-keto $PGF_{1\alpha}$ and TXB_2, respectively.

and small amounts of PGE_2 were formed from ^{14}C-AA by cell homogenates prepared from all 3 groups of cells. However, none of the 3 groups of cells were able to convert ^{14}C-EPA into any significant products. As indicated in Table 2, the conversion of ^{14}C-AA into ^{14}C-6-keto $PGF_{1\alpha}$ by cells homogenates prepared from 3 groups of cells was not significantly different. These results indicated that the PG cyclooxygenase activity in cells is not affected by EPA treatment.

TABLE 2

Effects of AA and EPA on PGI_2 Synthesizing Activity in Smooth Muscle Cells

Groups	6-Keto $PGF_{1\alpha}$ produced (cpm/mg prot)
Control	7245 ± 242
EPA 10 μg/dish	7421 ± 186
20 μg/dish	7454 ± 316
AA 10 μg/dish	7677 ± 323
20 μg/dish	6876 ± 298

Values represent the mean ± SE from 5 individual dishes.

Effects of EPA and AA on PGI_2 Production by Intact Aortic Smooth Muscle Cells

After treatment with EPA and AA for 16 hr, cells were again cultured with fresh medium in the absence of additional fatty acids for 18 hr. The amount of 6-keto $PGF_{1\alpha}$ released into the medium during 18-hr incubation was measured by radioimmunoassay. The result is shown in Figure 4. EPA treatment apparently inhibited the production of 6-keto $PGF_{1\alpha}$ by cells, while AA treatment stimulated the 6-keto

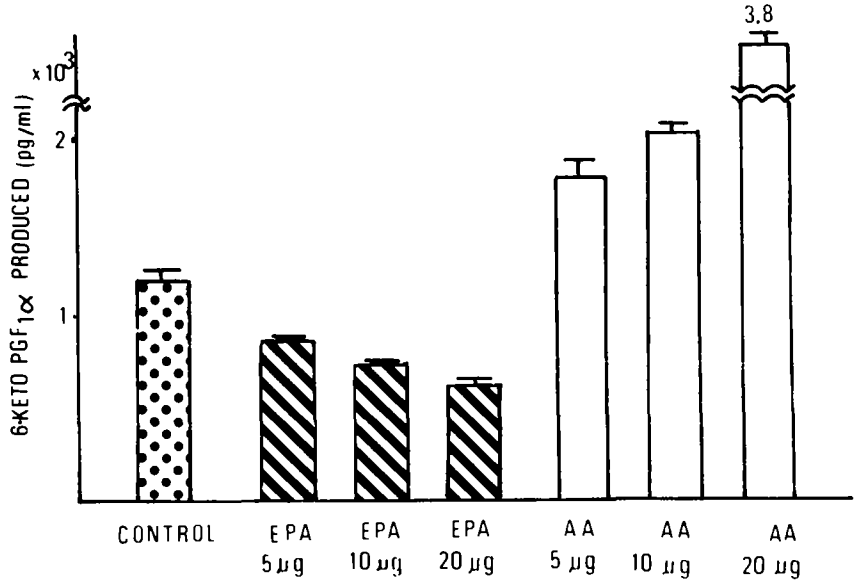

FIG. 4. Effects of incorporated EPA and AA on the release of 6-keto $PGF_{1\alpha}$ from rat smooth muscle cells. The amounts of 6-keto $PGF_{1\alpha}$ produced endogenously were measured by radioimmunoassay. Details were described in the text.

$PGF_{1\alpha}$ production in a dose-dependent manner. In these experiments, the principle fatty acid composition in cellular phospholipid fractions was also analyzed by TLC and GC. The principal fatty acid composition in cellular phospholipids is indicated in Table 3. In EPA-treated cells, the content of AA in phospholipids decreased, while the content of EPA increased. On the other hand, the content of AA increased in AA-treated cells. The increase in AA content in cellular phospholipids in AA-treated cells and the decrease in AA content in EPA-treated cells might provide a plausible explanation of the changes in 6-keto $PGF_{1\alpha}$ production by EPA- and AA-treated cells shown in Figure 4.

Transformation of ^{14}C-EPA by Platelets and the Direct Effect of EPA on Platelet Aggregation

Since ^{14}C-AA is converted to thromboxane (TX) B_2, 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) by washed rat platelets (12), we therefore studied the conversion of ^{14}C-EPA by washed rat platelets. When ^{14}C-EPA was incubated with washed platelets, no formation of TXB_3 and 12L-hydroxy-5,8,10,14-heptadecatetraenoic acid was observed. The direct effect of EPA on platelet aggregation in vitro was then studied. Platelet aggregation in platelet-rich plasma was induced by ADP and collagen in the presence of 5-20 μg/ml of EPA. No significant change in platelet aggregation and time delay of platelet aggregation by EPA were observed (data not shown).

Effects of EPA Diet on PGI_2 Production by Intact Aortas and Platelet Aggregation in Rats

In order to study the effects of EPA on PGI_2 biosynthesis in aortas and platelet aggregation in vivo, rats were fed with EPA-enriched diet. PGI_2 production by aortas was assayed by the antiplatelet aggregation method. The antiaggregatory factor released from aortas was identified to be PGI_2, since its activity was lost after acidification and its formation was inhibited by the preincubation of 2×10^{-5} M indomethacin for 10 min at 37 C. The effect of EPA diet on PGI_2 production by aortas is indicated in Table 4. The aortas from EPA-fed rats produced significantly less PGI_2 than those of control rats. The conversion of ^{14}C-AA and ^{14}C-EPA to PG by aortas from both control and EPA-treated rats was also studied. As in the case of cultured vascular cells described above, the transformation of exogenous ^{14}C-AA by aortas from both groups of rats did not show any difference, and the exogenous ^{14}C-EPA was not converted to any PG in either group of rats (data not shown).

After EPA treatment in rats in vivo, the platelet aggregation of both control and EPA-treated groups was also compared. Platelet aggregation in platelet-rich plasma was induced by ADP and collagen. The aggregation of plate-

TABLE 4

Effect of EPA-feeding on PGI_2 Biosynthesis in Rat Aortas

	PGI_2 produced (ng/mg aorta)
Control	3.59 ± 0.39
EPA feeding	1.90 ± 0.30

Values represent the mean ± SE from 6 individual rats.

TABLE 3

Composition of the Principle Fatty Acids in Phospholipids of Smooth Muscle Cells after EPA and AA Treatment

	Fatty acid (%)						
		EPA			AA		
	Control	5 μg	10 μg	20 μg	5 μg	10 μg	20 μg
16:0	27.5	28.1	27.9	33.1	29.2	27.2	27.4
16:1	4.5	5.6	6.8	5.2	5.3	5.4	5.2
18:0	24.8	20.9	20.7	22.5	20.0	21.2	20.3
18:1	29.3	31.2	31.4	26.1	30.2	28.0	26.4
18:2 ω6	1.5	2.6	2.0	2.0	1.2	1.5	1.8
20:4 ω6	12.5	11.8	10.8	8.9	14.1	16.9	19.0
20:5 ω3	–[a]	–	0.6	2.8	–	–	–

[a]Not detectable.

Values are the percentages of total fatty acids in phospholipid fractions. They represent the mean from 4 individual dishes, and the deviation was less than 10%.

lets was slightly inhibited (about 10% inhibition) by EPA treatment, but the inhibition was not statistically significant (data not shown).

DISCUSSION

Since Dyerberg et al. (1,2) suggested that EPA-enriched food may prevent thrombosis and atherosclerosis according to their epidemiologic study results, many investigations on the effects of EPA on platelet aggregation have been done. Regarding the possible mechanism on the inhibitory effects of EPA on thrombosis, the following two speculations can be made: (a) EPA feeding may cause a shift from PGI_2 and TXA_2 production to PGI_3 and TXA_3 production (13). Since TXA_3 is not a proaggregating substance and PGI_3 has antiaggregating properties similar to PGI_2, the shift from TXA_2 to TXA_3 by EPA feeding in platelets may decrease the platelet aggregation. (b) EPA may competitively block the oxidation of AA by fatty acid cyclooxygenase in platelets (14). The competitively inhibitory effect of EPA on the oxidation of AA by cyclooxygenase in platelets may result in a decrease in TXB_2 formation, which favors antiaggregation in platelets. The reality of these two speculations are still being challenged. But the antiaggregatory effect of EPA was not direct. In our experiments, EPA did not affect the magnitude and time course of ADP-aggregation, which is inconsistent with the report by Needleman et al. (15). The difference may be due to the dose of EPA used. Their dose was as high as 125 μg/ml, while ours was only 5-20 μg/ml. Even in that high dose, they could demonstrate that EPA was less than 50% of inhibition of ADP-aggregation. The reality of these two speculations is still being challenged.

Whitaker et al. (16) and Hornstra and Henker (17) have independently demonstrated that rat endothelial cells are not able to convert EPA to PGI_3. Hamazaki et al. (18) also reported that rat aorta is not able to convert EPA to Δ-17-6-keto $PGF_{1\alpha}$ (a hydrolyzed product of PGI_3) in control or EPA-fed animals. Moreover, no one has been able to detect TXB_3 and Δ-17-6-keto $PGF_{1\alpha}$ in blood and urine in EPA-treated animals and humans. In the present studies, we could find neither conversion of EPA to Δ-17-6-keto $PGF_{1\alpha}$ by cultured murine aortic smooth muscle cells and rat aortas, nor to TXB_3 by rat platelets. However, Dyerberg et al. (19) recently reported that human umbilical blood vessel walls transform EPA to PGI_3. We still do not know whether the difference is due to the species difference or tissue difference. Therefore, the first speculation on the antithrombotic characteristic of EPA is very unlikely at least in the case of rats according to the observations mentioned above.

If EPA acts as a competitive inhibitor of the oxidation of AA by fatty acid cyclooxygenase in platelets, this phenomenon should be applicable to any other cells which produce PGI_2. Since the balance between PGI_2 produced by vascular cells and TXA_2 produced by platelets plays an important role in the development of atherosclerosis and arterial thrombosis, the change in the ratio between PGI_2 and TXA_2 production is crucial to thrombus formation. The incorporation rate of EPA and AA in cultured vascular cells and platelets was compared in the present investigation. In platelets, EPA is incorporated into cellular phospholipids to a lesser degree than is AA (Figs. 1b and 2b).

The result may be explained by the recent findings by Wilson et al. (20) who reported on AA specific acyl-CoA synthetase in human platelets. The enzyme shows a higher Km value with EPA than with AA. We also found that the incorporation of EPA into the phosphatidylinositol fraction is less than that of AA in platelets (Table 1). Therefore, we could not find any results showing a preferential incorporation of EPA as compared with AA into platelets and vascular cells. Cells treated with EPA apparently produce less PGI_2 from endogenous AA than do control cells (Fig. 4). The inhibition of PGI_2 production by EPA is not due to the inhibition of fatty acid cyclooxygenase activity in cultured vascular cells (Table 2). In EPA-treated cells, the content of AA in phospholipid fractions decreases, while the content of EPA increases (Table 3). Upon stimulation, EPA might be released from phospholipids in the same manner as is AA, and the amount of cellular free AA released from phospholipids in EPA-treated cells should be less than that in control cells. Since EPA is not a good substrate for the fatty acid cyclooxygenase in cultured vascular cells (Fig. 3), the inhibition of PGI_2 production by EPA might be due to the competitive inhibition of the oxidation of AA in cells. The inhibition of PGI_2 production by EPA treatment in cultured vascular cells was well produced in intact aortas of animals fed with EPA-enriched diet. In agreement with our results, Hornstra et al. (21) also reported that the vessel walls in rats fed cod oil synthesize less PGI_2 than those in control rats. However, Hamazaki et al. (18) recently reported that aortas of rats fed with ethyl ether of EPA produce more PGI_2 than those of control rats, though the mechanism of the increase in PGI_2 production by ethyl ester of EPA has not been described yet. It is difficult to explain the dis-

crepancy between the results because we used free EPA rather than ethyl ester of EPA. Therefore, the second speculation on the antithrombotic characteristic of EPA is also questionable, at least in the case of rats.

Since EPA has been suggested to have a possible antithrombotic characteristic in cardiovascular systems, we tried to find the beneficial effects of EPA on the balance between PGI_2 and TXA_2 in vessel walls and platelets in rats in the present studies. We were unable to demonstrate any significant effects of EPA on improving the ratio of PGI_2 to TXA_2 in favor of its possible antithrombotic characteristic. However, the effects of EPA may vary with its type (e.g., free acid or esters, etc.) and animal species used in the studies. More investigations are abolutely necessary to explain the possible antithrombotic properties of EPA in the future.

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Unsaturated Fatty Acids in the Postnatally Developing Rat Lung

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ABSTRACT

Fatty acid desaturase activity specific for the C-9 position is present in lung microsomes prepared from rats of all ages. This activity is significantly lower in neonatal rat lung compared with adult lung. A rapid increase in C-9 fatty acid desaturase activity seen at the approximate time of weaning may be related to a decrease in the polyunsaturated fatty acid (PUFA) content of the diet as the rat begins to consume laboratory chow instead of mother's milk. The 900 × g supernatant fraction of rat lung parenchymal cell homogenates is capable of incorporating linoleate, linolenate, and arachidonate into both triacylglycerols and phospholipids. Lung tissue from rats less than 20 days old incorporates these PUFA into phospholipids at a greater rate than lung tissue from adult rats. The incorporation of these PUFA into phospholipids in neonatal lung tissue occured at a greater rate than their incorporation into triacylglycerols. In contrast, lung tissue from adult rats incorporated PUFA into triacylglycerols at a greater rate than into phospholipids. These data show that PUFA, known to be elevated in neonatal rat lungs, are used primarily for phospholipid biosynthesis in neonatal lung tissue whereas in adult lung tissue they are preferentially esterified to glycerol.
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The fatty acid composition of pulmonary triacylglycerols changes rapidly during the first 3 weeks of life in the rat (1,2). This change primarily involves an increase in the quantity of arachidonic acid (20: 4) and docosahexaenoic acid (22: 6) found in triacylglycerols from birth to 5 days of age. Thereafter, the quantity of these fatty acid moieties decreases until adult levels are reached about 20 days of age. A similar change in the polyene content of diacylglycerols has been reported by other researchers (3) while the fatty acid composition of pulmonary phospholipids has been shown to be unchanged during postnatal development (1).

The origin of these polyunsaturated fatty acid (PUFA) moieties of pulmonary triacylglycerols and the reason for their rapid decline after 5 days of age is not clear. They may originate either from PUFA in the diet of mothers' milk or as a consequence of an enhanced capacity to synthesize unsaturated fatty acids. Mammalian lung can catalyze the synthesis (4,5), desaturation (6), and elongation (7) of various fatty acids. Monodesaturated fatty acids may serve as substrates for subsequent desaturation reactions required for the synthesis of PUFA (8). Although it is known that C-6 fatty acid desaturase directly catalyzes the synthesis of PUFA, measurement of this activity was not possible in our lung preparations. C-9 fatty acid desaturase activity has been measured in pulmonary microsomes (6) and lung slices from adult rats, however (9). The C-9 fatty acid desaturase system catalyzes the monodesaturation of palmitate (16:0) to palmitoleate (16: 1) and stearate (18: 0) to oleate (18:1). Thus, C-9 desaturase activity was used in this study as an index of the overall capacity of lung tissue to desaturate fatty acids, and an age-related change in C-9 fatty acid desaturase activity in pulmonary microsomes is described. Age-dependent changes in the in vitro incorporation of linoleate, linolenate, and arachidonate into pulmonary triacyglycerols and phospholipids were also measured in an attempt to determine the ultimate destination of free PUFA.

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MATERIALS AND METHODS

Materials

Stearoyl-CoA [1-^{14}C] (50.4 Ci/M) was obtained from New England Nuclear (Boston, MA). Silica Gel G and silica Gel GF with 10% $AgNO_3$ thin layer chromatography (TLC) plates were obtained from Analtech. Amersham Corporation supplied linoleic acid [1-^{14}C] (60 Ci/M), linolenic acid [1-^{14}C] (60 Ci/M) and arachidonic acid [1-^{14}C] (61 Ci/M). Stearoyl CoA, coenzyme A, unlabeled fatty acids, BF_3 (14%) in methanol, NADH, NADPH, and ATP were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were reagent grade.

Animals

Sprague-Dawley rats obtained from either BioLab (St. Paul, MN) or ARS-Sprague-Dawley

(Madison, WI) were used in these studies. Mature female rats were bred at the University of Iowa as described elsewhere (10). Lung tissue was obtained from nonpregnant adult females (300–350 g) and neonates of both sexes. The animals were decapitated and the lungs perfused through the pulmonary artery with isotonic saline.

Tissue Preparation

Lung tissue was minced with scissors into pieces of ca. 1–2 mm^3 and stirred vigorously at 4 C in isotonic saline for 6 min. The suspension was filtered through HC-160 nylon cloth (TETKO, Inc.) and washed twice with isotonic saline to remove extracellular lung fluid and pulmonary alveolar macrophages (11). After weighing, the remaining tissue was homogenized (1: 3, w/v) in a glass homogenizer using a motor driven teflon pestle. The homogenization buffer was composed of 0.02 M Tris-HCl, 0.15 M KCl, 4 mM $MgCl_2$, 0.02 M sodium citrate and 4 mM sodium acetate at pH 7.4. The homogenate was filtered through gauze to remove large particles and unbroken cells and centrifuged at 900 × g for 5 min.

PUFA Incorporation Into Phospholipids and Triacylglycerols

The final reaction mixture in which the rates of linoleic, linolenic, or arachidonic acid incorporation were measured contained 10 μmol ATP, 2 μmol NADH, 2 μmol NADPH, 0.6 μmol coenzyme A, 10 μmol glucose 6-phosphate, 2 μmol fatty acid and 900 × g supernatant fraction containing 4 mg of protein in a total volume of 2 ml. Protein concentrations of the 900 × g supernatant fraction were determined by the microbiuret method (12). Fatty acids were stored desiccated under nitrogen at –20 C and added to the incubation mixture from a solution made up immediately before its use. This preparation was made by dissolving 0.055 μmol of the labeled fatty acid and 11.1 μmol of the unlabeled fatty acid in 0.2 ml of methanol and adding this to 3 ml of buffer; 0.5 ml of the resulting emulsion was added to each reaction.

The final reaction mixture was incubated at 37 C for 30 min. The reaction was terminated by the addition of 2 ml of chloroform/methanol (2: 1). The lipids were then extracted by the method of Folch et al. (13) and the phospholipid and triglyceride fractions isolated by thin layer chromatography (1). Lipid fractions were located by exposure to iodine vapor. The amount of radioactivity in each lipid fraction was determined by liquid scintillation methods. The incorporation of each fatty acid into the appropriate lipid fraction was calculated as nmol/min/mg lung protein. The assumption was made that there were no significant changes in the fatty acid specific activity during the incubation period.

Liquid Scintillation Counting

Radioactivity measurements were done on a Beckman LS 250 liquid scintillation counter. Lipids were identified on TLC plates by comparison with commercial standards and then scraped directly into counting vials. Phospholipids were solubilized by the addition of 1 ml of methanol. Triglycerides and fatty acid methyl esters were soluble in the scintillation fluid which contained 0.4% 2,5-diphenyloxazole and 0.025% p-*bis*-[2-(5-phenyloxazolyl] benzene in toluene. Each sample was counted 3 times for 10 min in 10 ml of scintillation fluid and quench corrections were made.

C-9 Fatty Acid Desaturase

The monodesaturation of stearic acid to oleic acid was assessed to quantitate C-9 fatty acid desaturase activity in pulmonary microsomes. The method used has been described in detail by Montgomery (6). C-9 fatty acid desaturase activity was expressed as the nmoles of oleic acid formed/min/mg microsomal protein.

Statistics

All data were calculated as means ± SE. These data were analyzed by one-way analysis of variance and multiple comparisons were done with Student Newman-Keul's test (14). A p value at 0.05 or less was considered to be significant.

RESULTS

The C-9 fatty acid desaturase activity in pulmonary microsomes from rats 1–12 days of age remained constant and was significantly less than that measured in adult lung microsomes (Fig. 1). The activity measured in lung microsomes from rats 19 days old was significantly greater than that measured from rats 1–12 days of age but not significantly different from adult microsomes.

Optimal conditions for the incorporation of linoleic, linolenic, and arachidonic acid into lung phospholipids and triacylglycerols were determined. The incorporation of these PUFA into pulmonary phospholipids was a linear function of protein concentration to 3 mg/ml and time to 30 min (data not shown). Their incorporation into pulmonary triglycerides was a linear function of protein concentration to 2 mg/ml and time to 60 min (data not shown).

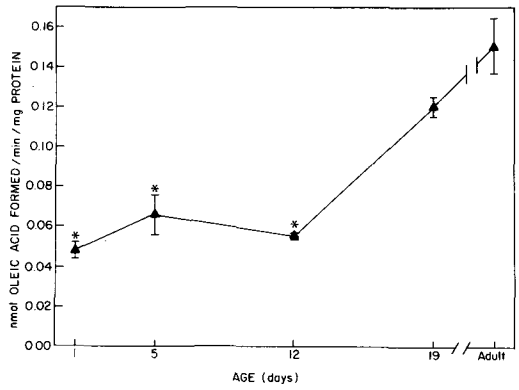

FIG. 1. Fatty acid desaturase activity in rat lung microsomes. Microsomes were isolated from rat lungs and assayed as described in Materials and Methods. Stearoyl CoA [1-^{14}C], 70 μm, was used as the substrate and C-9 fatty acid desaturase activity is expressed as nmol oleic acid formed min/mg microsomal protein.

*Significantly less than adult rat ($p < 0.05$).

Optimal conditions were identical in lung preparations from animals at all ages used in this study. Age-related changes in PUFA incorporation into pulmonary phospholipids and triacylglycerols were, therefore, examined after a 30-min incubation using a reaction mixture containing 900 × g supernatant fraction at a concentration of 2 mg protein/mg.

No difference in the maximal rate of incorporation of linolenate, linoleate, and arachidonate into lung phospholipids of rats of the same age was evident. However, rates of incorporation of all PUFA into phospholipids were significantly greater in lungs of rats 5–12 days of age compared with adult lung tissue (Fig. 2A-C). Compared to adult lung tissue, a significantly greater rate of arachidonate and linolenate incorporation into phospholipids was observed in lung tissue from 20-day old rats, although the difference was less than with the 5-12-day old age groups. This smaller increase was also observed with arachidonate in 1-day old rats.

As with incorporation into phospholipids, the rate of incorporation of linolenate, linoleate, and arachidonate into triacylglycerols was similar in lung tissue from rats of the same age. In contrast, however, significantly lower rates of incorporation into triacylglycerols were observed in lung tissue of rats less than 20 days of age compared with adult lung tissue. This was true for all PUFA tested.

The net rate at which linolenate, linoleate, and arachidonate were incorporated into lung phospholipids of animals 5 and 12 days of age was significantly greater than the rate of incorporation of these PUFA into triacylglycerols (Fig. 2A-C). Adult lung tissue incorporated these PUFA into triacylglycerols at a greater rate than into phospholipids.

DISCUSSION

The PUFA content of triacylglycerols in neonatal rat lung is much higher than in adult rat lung but begins a rapid decline toward the low adult level at 12–19 days of age (1). Possible explanations for the age-related changes in the fatty acid composition of pulmonary triglycerides include changes in the intestinal absorption of PUFA, changes in the fatty acid composition of the diet, changes in the de novo synthesis of PUFA, and changes in the pulmonary utilization of PUFA. Previous work has shown changes in the intestinal absorption of fatty acids (15) and in the PUFA content of rat milk at various days postpartum (2). Changes in the fatty acid composition of the diet fed to adult rats alters the fatty acid content of pulmonary triacylglycerols (16). The changes in rat milk fatty acids did not appear to be of sufficient magnitude, however, to account entirely for the profound alterations in the fatty acid composition of rat lung triacylglycerols associated with maturation.

Sinclair has reported that the PUFA in neonatal rat liver are derived directly from the diet rather than by synthesis from precursors (17). Because of the large, age-related changes in lung tissue, we considered it necessary to determine the capacity of lung tissue from rats of various ages to synthesize unsaturated fatty acids. Montgomery (6) reported that rat pulmonary microsomes catalyzed the monodesaturation of stearic acid to oleic acid. Our results confirmed this finding although the activity we measured was lower than previously observed, possibly as a result of sex and strain differences of the rats used (female Sprague-Dawley rats in our study compared with male Charles-River C-D rats used in the previous work) (6). Monodesaturase activity remained at a constant low level until the time of weaning when a rapid increase occurred. Other studies of liver C-9 fatty acid desaturase activity showed that fat free diets and low serum fatty acids enhanced the activity of this enzyme system (8,18,19), while the 9-desaturation of fatty acids in pulmonary tissue was inhibited by unsaturated fatty acids (20) The increase of pulmonary C-9 fatty acid desaturase activity observed at the time of weaning could, therefore, be due to a decrease in the

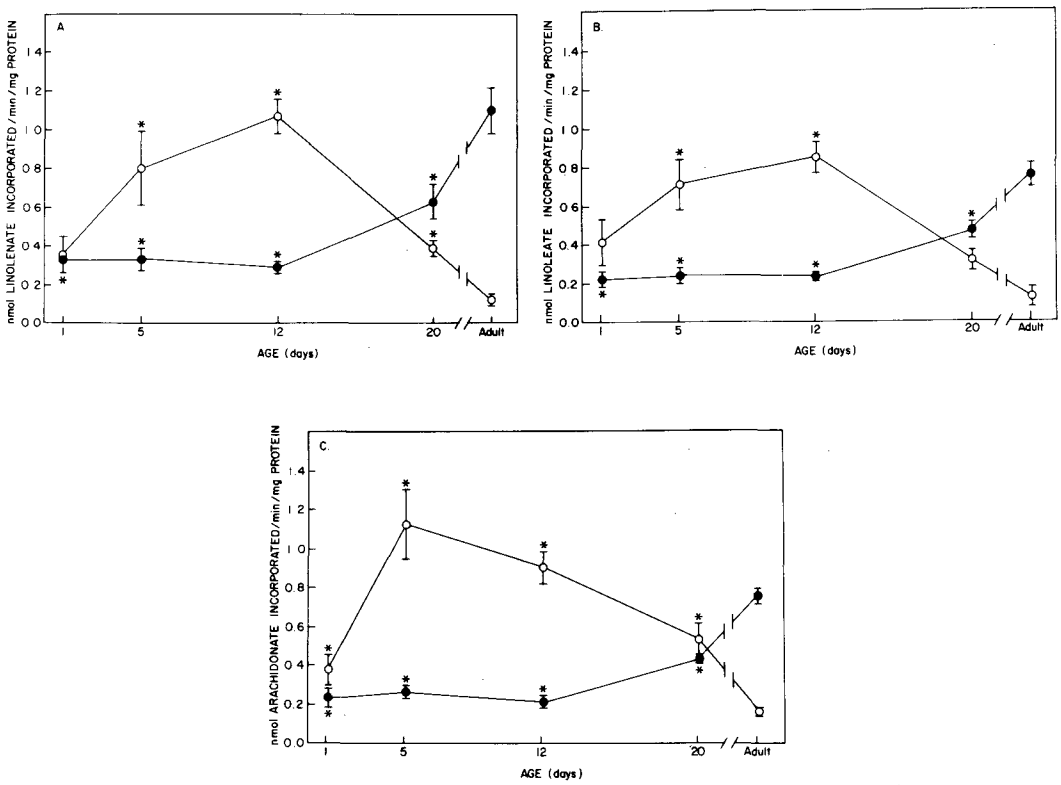

FIG. 2. Age-related changes in incorporation of PUFA into pulmonary phospholipids and triglycerides. Each fatty acid was incubated at a concentration of 1 mM with the 900 g supernatant fraction of a rat lung homogenate at 37 C for 30 min as described in Materials and Methods. The phospholipid and triglyceride fractions were isolated and the radioactivity in each determined by liquid scintillation counting. Data are expressed as nmol fatty acid incorporated/min/mg protein in the 900 g supernatant fraction. (A) Linolenic acid; (B) linoleic acid; and (C) arachidonic acid.

●–Triacylglycerols; ○–phospholipids.

*Significantly different from adult rats ($p < 0.05$).

total lipid and unsaturated fatty acid content of the diet at this time when rats change from a high fat milk diet to a low fat laboratory diet.

The biosynthesis of PUFA in rat liver microsomes is dependent primarily upon the 6-desaturation of monodesaturated fatty acids (8). Attempts to measure this activity in pulmonary microsomes from both neonatal and adult rats using linoleic acid and an acyl coenzyme A generating system were unsuccessful, however, and radioactive linoleoyl-CoA is not commercially available. Measurements of C-9 desaturase activity using stearic acid and an acyl coenzyme A generating system rather than commercial stearoyl-CoA were also unsuccessful suggesting that the formation of the fatty acyl CoA is a limiting step in the assay for desaturase activity in lung tissue.

The incorporation of linoleate, linolenate, and arachidonate into pulmonary phospholipids and triacylglycerols was assessed to determine any age related changes in the utilization of these PUFA. We found that neonatal rat lungs incorporate these PUFA into phospholipids at a significantly greater rate than do adult lungs. This age-related difference could be due to changes in competition by endogenous PUFA or to different metabolic requirements. Competition by endogenous fatty acids appears unlikely, however, since a 15-fold excess (compared to total free fatty acids) of PUFA were added to each incubation mixture (1). Although it is possible that the endogenous fatty acids were in a more available form, the large excess added should have minimized this factor.

Lung, liver, and heart tissue undergo rapid

growth during the first three weeks after birth. The biosynthesis of membrane phospholipids will require large quantities of PUFA which could be supplied by triacylglycerols in developing tissues. The presence of large amounts of arachidonic and docosahexaenoic acid in triacylglycerols from neonatal but not adult lung (1), liver (17), and heart tissue (21) suggest an important metabolic role for these PUFA.

Lung cell proliferation is minimal up to three days of age (22). The rapidly increasing pulmonary content of PUFA during this time period (1) could be the result of low utilization during a high dietary intake. As pulmonary hyperplasia resulting from maturational processes reaches a maximum between 5 and 12 days of age (22), the demand for PUFA to form membrane phospholipids will also increase. In conjunction with the gradually decreasing quantity of PUFA available from rat milk and the switch to laboratory food with increasing time after parturition (2), this demand could lead to the observed decline in PUFA esterified to glycerol.

In an extension of earlier data from our laboratory (1,2), Maksvytis et al. (23) have isolated and characterized triglyceride-containing interstitial cells from the developing rat lung. The time of appearance and disappearance of these cells correlates very well with the increase and decrease of PUFA content of total lung triglycerides. In addition, the fatty acid content of the triglycerides in those cells agrees with the fatty acid composition of total lung triglycerides except for palmitoleic acid which was found to be much higher in those cells (23) than in total lung tissue (1). We speculated on the basis of our earlier results (1,2) that the high PUFA in neonatal lung lipids could be involved in the rapid lung growth known to occur at this age. Similarly, Maksvytis et al. suggested that the lipid containing interstitial cells identified in neonatal lungs are involved in lung restructuring and alveolar formation during postnatal lung development (23).

The data reported here shows that C-9 fatty acid desaturase activity in neonatal lung tissue remains low until the time of weaning. These data also show that the majority of PUFA are incorporated into phospholipids in neonatal rat lung tissue and into triglycerides in adult lung tissue. This difference may be related to metabolic requirements of growth.

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Fatty Acid Composition of Polar Lipids in Goats' Milk

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ABSTRACT

Silicic acid column chromatography was used to separate the polar lipids of goats' milk into glycolipid, phosphatidylethanolamine, phosphatidylserine plus phosphatidylinositol, phosphatidylcholine, and sphingomyelin fractions. Each fraction was purified by column chromatography and its fatty acid profile determined by gas liquid chromatography and mass spectrometry. The glycerophospholipids each contained 18:1 as the predominant fatty acid (∿45%). The sphingolipids contained a high percentage of long-chain saturated fatty acids (C_{22} to C_{24} > 45%); the glycolipid fraction also contained ca. 2% 2-hydroxy fatty acids. The data represent a comprehensive cross-sectional study of the major polar lipids found in goats' milks.
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The fatty acid compositions of the total and neutral lipids in goats' milk have been studied (1,2); however, less is known regarding their polar lipids, which comprise about 1.6% of the total lipids (2,3). Although, as pointed out by Morrison (4), milk polar lipids do not constitute a large part of man's diet, they do represent a major fraction of the total phospholipid ingested and may be of dietary significance. For example, lecithin has recently been shown to inhibit cholesterol absorption in rats (5). The polar lipids also contain polyunsaturated acids which are essential for human nutrition and may serve as precursors for prostaglandins which regulate metabolic functions (6–8).

This paper is part of a study of the lipids of goats' milk (1) and describes the fatty acid composition of the polar lipids.

EXPERIMENTAL

Materials

Raw goats milk samples were obtained from a large commercial dairy goat company, and were maintained at 5 C in transit to the laboratory. Upon receipt, the samples were lyophilized and stored at −20 C. Before lipid extraction, equal weights of 5 samples obtained during the months of April through June were mixed together to minimize nutritional, environmental, and breed differences.

Reagents

All solvents were of nanograde quality. Unisil silicic acid, 100–200 mesh was from Clarkson Chemical Co. (Williamsport, PA). Precoated thin layer chromatographic plates SIL G-25 (Macherey-Nagel) were from Brinkmann Instruments, Inc. (Westbury, NY).

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Lipid Extraction

Free lipids were obtained by extracting the freeze-dried samples 4 times with petroleum ether; the bound lipids were obtained by 3 subsequent extractions with chloroform/methanol (2:1) as described previously (9). Lipid extracts and column fractions were analyzed by thin layer chromatography (TLC) (9). Developing solvents were petroleum ether/diethylether/acetic acid (90:10:1) for neutral lipids, and chloroform/methanol/water (65:25:4) for polar lipids (glycolipids and phospholipids). Iodine vapor was used to visualize the separated lipid classes. Identifications were made on the basis of migration relative to reference standards.

Silicic Acid Column Separations

Initial separations were carried out on Unisil columns (2.5 X 25 cm). Lipid classes were separated by modification of the procedures of Masoro et al. (10) and Rouser et al. (11). Routinely, 3.2 g of bound lipid were applied: fraction A, eluted with chloroform (575 ml), contains neutral lipids; fraction B, eluted with acetone (600 ml), contains glycolipids; fraction C, eluted with 700 ml chloroform/methanol (6:1), contains phosphatidylethanolamine; fraction D, eluted with 550 ml ethylacetate/methanol (7:4), contains phosphatidylserine and phosphatidylinositol; fraction E, eluted with 600 ml chloroform/methanol (1:1), contains most of phosphatidylcholine and sphingomyelin. Each fraction was rechromatographed on a smaller Unisil column (1 X 20 cm) using the same solvent systems to remove contaminants. Each separation step was monitored by TLC as described above. Weight distributions of the polar lipid classes were determined gravimetrically; recoveries were usually greater than 95%.

Preparation of Fatty Acid Methyl Esters

Glycerophospholipids (2 to 5 mg) were transesterified with 0.6 N NaOH in methanol (5 ml) for 1 hr at room temperature (12), after which 1 N HCl, chloroform, and water were added (for 10 ml total volume) and the methyl esters were recovered in the chloroform layer. If some fatty acids were not methylated, as judged by TLC, the fraction was treated with 5% HCl in methanol at 60 C for 1 hr. The methyl esters were extracted with petroleum ether.

Column fractions containing sphingomyelin and glycolipids (cerebrosides) were first purified (13) by mild alkaline hydrolysis (0.3 N NaOH, 1 hr, rt) to remove traces (if any) of glycerol containing lipids. The reaction mixture was chromatographed on a small Unisil column as described above. The purified lipids were refluxed in methanolic HCl (1 N) for 6 hr and methyl esters recovered by petroleum ether extraction.

Methyl esters of fatty acids were separated into nonhydroxylated and 2-hydroxylated esters by using a small (1 X 20 cm) Unisil column (14). Nonhydroxylated fatty acid methyl esters were eluted with petroleum ether/diethylether (96:4) and 2-hydroxylated esters with petroleum ether/diethylether (80:20).

Gas Liquid Chromatography (GLC) Mass Spectrometry (MS) of the 2-Hydroxy Fatty Acid Methyl Esters

A Hewlett-Packard Model 5922-B combination of GLC-low-resolution Quadropole MS interfaced to the Hewlett-Packard Model 9825-A data system was employed for GLC-MS analysis. The 70 eV electron impact mass spectra were obtained following separation on a 1.83 m X 0.64 cm (od) glass column packed with 3% OV-17 on 100/120 mesh Gas Chrom Q. The injection port was maintained at 150 C and the column temperature programmed from 140 C to 280 C at 4 C/min. Helium served as the carrier gas.

GLC of the Nonhydroxylated Fatty Acid Methyl Esters

GLC studies of the nonhydroxylated fatty acid methyl esters were performed on the Hewlett-Packard Model 5750 Gass Chromatograph equipped with a flame ionization detector. The methyl esters were separated isothermally at 180 C on a 1.83 m X 0.32 cm stainless steel column packed with 10% Silar 10C on 100/120 mesh Gas Chrom Q. The injection port and exit port were maintained at 200 C and 240 C, respectively, and helium served as the carrier gas. Peak identification was attained by reference fatty acid methyl ester retention times (C_{12} - C_{18} methyl esters) and extrapolation (C_{19} - C_{24} methyl esters). Identities of the high molecular weight methyl esters in the glycolipid and sphingomyelin fractions were confirmed by GLC-MS employing the same conditions outlined above for the 2-hydroxy fatty acid methyl esters.

RESULTS AND DISCUSSION

The milk samples in this study were obtained from a large commercial dairy which collects and processes goats' milk for human consumption. The 5 bulk milk samples are representative of commercially available goats' milk in that they were obtained from several breeds during the months of April through June when does usually are in full lactation.

The lipids were extracted first with petroleum ether (free lipid fraction) and then with $CHCl_3$/methanol ("bound lipid" fraction) as previously described (1). The first extract contains the majority of the neutral lipids as well as the free fatty acids of milk. The second extract consists of additional neutral lipid (46.8%) plus all of the polar lipids (53.2%). The bound lipid classes were separated by silicic acid column chromatography, and the weight distribution among the various polar lipid classes is given in Table 1.

The most notable figure in Table 1 is the relatively high glycolipid content found in this study. Early work on cows' milk (15) estimated the glycolipids to account for up to 6% of the total polar lipids. Kayser and Patton (16) subsequently estimated that the glycolipid content of milk membranes was closer to ~9% in both goats' and cows' milk. In this work, the value was found to be 16% of the total polar lipids of whole milk. The fatty acid

TABLE 1

Distribution of Polar Lipids of the "Bound Lipid" Fraction of Goats' Milk

	Weight (%)[a]
Phosphatidylethanolamine	29.7
Phosphatidylserine	2.7
Phosphatidylinositol	3.4
Phosphatidylcholine	23.7
Sphingomyelin	24.5
Glycolipids	16.0

[a]From chromatographic analysis of chloroform/methanol extract of goats' milk. Weight percentage was determined gravimetrically.

profiles associated with each class after purification have been determined by GC and GC-MS. The saturated and unsaturated fatty acids present in these polar lipids are shown in Table 2.

The predominant fatty acids associated with the glycerol-based phospholipids are 16:0, 18:0, 18:1, and 18:2 with the 18:1 accounting for about 50% of the fatty acids in each class. Phosphatidylcholine (PC) contained 36.9% 16:0, whereas phosphatidylethanolamine (PE) and phosphatidylserine (PS) + inositol (PI) contained only 12.9% and 7.0% of this acid. The fatty acid profiles found in this study for these glycerophospholipids are quite similar to those found for human and cows' milks (17). However, PE tended to have a lower polyunsaturated acid content, and a higher 18:0 content than reported by Moore et al. (18) for PE from goats' milk. These differences could be due to seasonal and dietary responses, but Moore's data was from a specific breed, while the data presented here may be more representative of goats' milk in general. The glycerophospholipids contained only trace amounts of hydroxy fatty acids.

The glycolipids or cerebrosides (GL) and sphingomyelin (Sph) fractions, in contrast to the phosphatidyl lipids contained significant amounts of 22:0, 23:0, and 24:0 fatty acids, indicating that these lipids are highly saturated in goats' milk. The 23:0 chain is somewhat unusual, but these data are in good agreement with the results previously reported for the glucosyl and lactosyl cerebrosides of cows' milk fat globule membrane (16), except that the 16:0 is somewhat higher in this work. The glycolipid fraction was not separated into glucosylceramide fractions because lactosylceramide was such a minor component of the glycolipid fraction of the goats' milk. The glycolipid fraction contained 2% 2-hydroxy fatty acids and the compositional profile of these acids is given in Table 3. The 2-hydroxy fatty acid content found for goats' milk is similar to that found for cows' milk glycolipids (19) with only the C_{25} and C_{26} present in significantly higher amounts in goats' milk.

The sphingomyelin fraction of goats' milk (Table 2), like that of cows' milk, contains a high percentage of long-chain fatty acids (19–21), but the goats' milk sphingomyelin has 16:0 and 18:0 at higher levels. The 2-hydroxy fatty acids were not quantitated because of insufficient amount of material. Hydroxy fatty acids comprised less than 2% of the total fatty acids in the sphingomyelin fraction.

Although the fatty acid composition of cows' milk has been studied in detail, the information on cows' milk has been more fragmentary. In this study, we report the fatty acid profiles for various polar lipids of goats'

TABLE 2

Weight Percentage of the Fatty Acids in the Polar Lipids of Goats' Milk[a]

Fatty acid[b]	Glycolipids	Phosphatidyl[c] ethanolamine		Phosphatidyl serine plus inositol	Phosphatidyl choline	Sphingo myelin
12:0	0.8	–		–	–	–
13:0	–	–		–	–	–
14:0	2.7	0.4	(0.6)	tr	2.2	3.1
15:0	tr	–		–	–	0.1
16:0	17.4	12.9	(10.0)	7.0	36.9	26.2
17:0	0.6	–		–	–	0.5
18:0	9.5	31.6	(9.9)	26.7	9.0	10.3
18:1	2.6	46.3	(52.0)	56.1	46.3	0.6
18:2	–	5.6	(18.4)	10.2	5.6	–
18:3	–	3.4	(1.7)	–	< 0.5	–
19:0	tr	–		–	–	tr
20:0	2.6	–	(2.8)	–	–	2.3
21:0	1.8	–		–	–	1.1
21:1	1.0	–	(4.5)	–	–	–
22:0	22.0	–		–	–	17.9
23:0	23.0	–		–	–	20.4
24:0	13.6	–		–	–	13.3
24:1	2.4	–		–	–	4.3

[a]Average of duplicate determinations.
[b]Minor fatty acids as branched-chain were identified in milk samples but were omitted from tables.
[c]Values in parenthesis reported by Moore et al. (18).

TABLE 3

Composition of the 2-Hydroxy Fatty Acids in the Polar Lipids[a] of Goats' Milk

Carbon number	Weight (%)[b]	
	Glycolipids	Sphingomyelin[c]
16	17.0	tr
18	11.9	tr
22	14.4	tr
23	22.4	tr
24	25.4	tr
25	4.0	–
26	5.0	–

[a]2-Hydroxy acids were not detected in phosphatidylcholine, phosphatidylethanolamine, or phosphatidylinositol plus phosphatidylserine fractions.
[b]Average of duplicate determinations.
[c]Carbon number identified by GLC-MS but quantitation not possible because of unsufficient amounts of material.

milk. The fatty acid profiles for only PE and PS from goats' milk have been previously reported (2,18); thus, those for PC, Sph, and GL are apparently reported for the first time. All of the values determined in this work are all from the same large, pooled commercial goats' milk sample. This information coupled with our report (1) on the triglyceride fatty acids from the same pooled samples present a comprehensive view of goat milk lipids.

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Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Hypolipidemic Effects of Clofibrate and Selected Chroman Analogs in Fasted Rats: II. High Sucrose-Fed Animals

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ABSTRACT

The hypolipidemic properties of ethyl 6-chlorochroman-2-carboxylate (II), 6-phenylchroman-2-carboxylate (III) and 6-cyclohexylchroman-2-carboxylate (IV) were compared to clofibrate (I) in sucrose-fed fasted male Sprague-Dawley rats. All compounds were administered at doses of 0.2 and 0.4 mmol/kg, orally, twice daily for 7 consecutive days. In this model, II was a more effective hypocholesterolemic drug than clofibrate, whereas III and IV were inactive. Chlorochroman II, like clofibrate, decreased serum α-lipoprotein cholesterol and pre-β-lipoprotein triglyceride concentrations and concomitantly increased serum β-lipoprotein triglyceride concentrations. In clofibrate-treated rats, serum free cholesterol concentrations increased concurrent with a reduction in serum lecithin:cholesterol acyltransferase activity, but no such correlation was observed for II. Only II lowered liver cholesterol levels and increased liver triglyceride levels. No consistent inhibition of liver microsomal 3-hydroxy-3-methylglutaryl-CoA reductase activity was observed with these analogs. The observed changes in triglyceride and cholesterol concentrations among serum lipoproteins were of a greater magnitude after chlorochroman II and clofibrate administration to sucrose-fed rats than in our previous studies using chow-fed fasted rats. These data suggest that chloro-substitution at the 6-position of the phenylchroman ring is important for hypolipidemic activity of these cyclic clofibrate analogs.

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INTRODUCTION

Thromboembolism and hyperlipoproteinemia are major clinical manifestations of ischemic heart disease (IHD;1,2). Clofibrate is one of the major drugs available for treatment of IHD which may be related to both antilipidemic (3,4) and antiaggregatory actions in vivo (5). Though clofibrate lowers serum lipids and reduces morbidity of patients suffering from IHD, prolonged treatment with clofibrate has also been reported to account for an increase in IHD-unrelated deaths (6). In addition, a potential carcinogenic action and higher incidences of gastrointestinal disorders in patients who have been treated with clofibrate for hyperlipoproteinemias has resulted in a restriction of the use of clofibrate in European countries and the United States (7-9). These reports indicate the necessity of developing selective and nontoxic hypolipidemic drugs for the treatment of IHD.

In recent years, we have reported the comparative antilipidemic (10-13) and antiaggregatory (14) activities of ethyl 6-chlorochroman-2-carboxylate (II), 6-phenylchroman-2-carboxylate (III) and 6-cyclohexylchroman-2-carboxylate (IV), in vivo and in vitro (see Fig. 1 for structures). Among these chromans, analog II was reported to be equal to or more effective than clofibrate in inhibiting human platelet function and lowering serum lipids. In similar studies, analog III showed only marginal hypolipidemic activity in vivo. However, analog III, when compared to clofibrate, was 20- to 50-fold more potent as an inhibitor of human platelet aggregation in vitro (14). These data suggest that it is possible to develop clofibrate-related analogs which are highly tissue selective and/or equally as active as clofibrate as antiplatelet or antilipidemic agents.

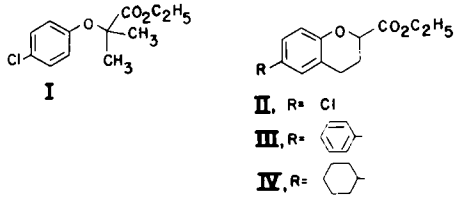

FIG. 1. Chemical structures of clofibrate (I), ethyl 6-chlorochroman-2-carboxylate (II), ethyl 6-phenylchroman-2-carboxylate (III) and ethyl 6-cyclohexylchroman-2-carboxylate (IV).

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It is well known that dietary status, lipemic state of animals, seasonal variations and experimental conditions (fasted vs nonfasted) significantly alter responsiveness of lipid lowering agents (13,15). Since these chroman analogs possess varying antilipidemic and antiaggregatory properties, it is important to evaluate the hypolipidemic activity of these compounds in several experimental models to consider variables such as the animal species, dietary status and experimental conditions. We have recently reported that analogs III and IV are ineffective in fasted normal chow-fed rats, whereas analog II and clofibrate were effective in lowering serum cholesterol levels and distribution of lipids among lipoproteins (13). In this report, we will examine the comparative effects of clofibrate and 3 chroman analogs (II-IV) on serum and liver lipid levels, serum LCAT activity, and lipid distribution among serum lipoproteins in fasted rats fed with an established synthetic diet containing a high proportion of sucrose. Assessment of antilipidemic activity of these analogs in diet-modified animal models could reveal changes in lipoprotein distribution as a function of underlying differences in mechanisms of action providing leads for development of new agents useful in the treatment of specific hyperlipoproteinemias.

MATERIALS AND METHODS

Chemicals

Clofibrate was a gift (Ayerst Laboratories, Rouses Point, NY). Ethyl 6-chloro-, 6-phenyl- and 6-cyclohexyl-chroman-2-carboxylates were synthesized as described previously (10,16).

Animals

Male, albino Sprague-Dawley rats (180–210 g), ca. 60 days old, obtained from Harlan Laboratories (Cumberland, IN) were used. Rats were housed in groups of 6 and given high sucrose-containing synthetic diet and water ad libitum.

Composition of the Synthetic Diet

The diet consisted of sucrose (63%); vitamin free casein (28%); vitamin fortification mixture (4%); USP XIV salt mixture (5%) and cellulose (5%). Composition of vitamin mixture/100 lb of diet is as follows:α-tocopherol (1000 IU/g), 9.10 g; L-ascorbic acid, 81.9 g; choline chloride, 136.5 g; calcium d-pantothenate, 5.46 g; inositol, 9.10 g; menadione, 4.09 g; niacin, 8.19 g; *p*-aminobenzoic acid, 9.1 g; pyridoxine HCl, 1.82 g; riboflavin, 1.82 g; thiamin HCl, 1.82 g; vitamin A acetate, 1,638,000 IU; calciferol (D2), 182,000 IU; biotin, 36.4 mg; folic acid, 163.8 mg and vitamin B12, 2.457 mg. This product was purchased from Nutritional Biochemicals Corporation (Cleveland, OH).

Experimental Design

Rats were fed a high sucrose diet for 4 days prior to initiating the treatment with clofibrate and analogs. Diet and drug treatment continued for 7 days. Rats were housed in a vivarium at 25–26 C on alternating 12-hr light and dark cycles. Animals were acclimatized to the housing conditions for a period of one week prior to these experiments. Animal selection was based upon the observation of Kritchevsky (15) who reported that serum CH and TG concentrations of 50–60 day old Sprague-Dawley rats are comparable to those of normal male human subjects between the age of 20–29 years.

Groups (n=6) of rats received various doses of clofibrate (0.1, 0.2, 0.4, 0.6 mmol/kg/day in 0.25% methyl cellulose) or 0.25% methyl cellulose orally by esophageal intubation twice daily for 7 consecutive days. In all experiments, drugs were given in a volume of 1.0 ml/100 g body wt. Following these initial dose-response studies, groups of animals were treated with vehicle or 0.2 and 0.4 mmol/kg/day of clofibrate twice daily for 7 consecutive days in 3 replicate experiments. Each of these 3 experiments also consists of groups of rats who received an equal dose of analogs II, III or IV.

All rats were fasted 16–18 hr before blood collection. Blood was drawn (orbital plexus) from rats under light ether anesthesia one day before (day -1) and after 4 days (day +4) of drug treatment. After 7 days (day +7) of drug treatment, blood was collected by exsanguination from the abdominal aorta. Livers were immediately excised, rinsed in ice-cold 0.1 M Tris-HCl buffer, pH 7.2 (containing 1.15% KCl, w/v), blotted, weighed, minced and homogenized in the same buffer using a glass homogenizer equipped with a Teflon pestle. Liver microsomes were isolated as described previously (11).

Liver Assay

Microsomal 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.34) activity was assayed by the method of Huber et al. (17), and protein content was determined by the procedure of Lowry et al. (18). CH and TG were extracted from liver homogenates (25% w/v) by the method of Abell et al. (19) and subsequently analyzed, respectively, by the methods of Parekh and Jung (20) and Soloni (21).

Serum Assay

Blood samples were placed on ice, and after clotting, serum was separated by centrifugation at 2000 g for 10 min. Serum samples were kept at 4 C and analyzed within 48 hr. Serum total CH was measured by the enzymatic method of Allain et al. (22) with A-Gent cholesterol reagent (Abbott Laboratories, Diagnostics Division, Chicago, IL) on an Abbott bichromatic analyzer (ABA-100) equipped with a 1:51 syringe plate. Serum total TG concentrations were measured with A-Gent TG reagent (Abbott Laboratories Diagnostics Division, Chicago, IL) on an ABA-100 according to the method of Sampson et al. (23).

α-Lipoprotein (α-LP)-CH (high density LP-CH), pre-β ("VLDL")-, β-("LDL")- and ("HDL") α-LP-TG were determined by electrophoresis on agarose gel at 90 volts for 25 min with 0.05 M universal barbital buffer, pH 8.6. The details of the staining and quantitation procedures of CH and TG contents in several electrophoretically migrated LP are described in our previous report (13).

LCAT activity was assayed according to the method of Glomset and Wright (24). Serum free CH was measured by the modification of the method of Nagasaki and Akanuma (25). Serum free CH was expressed as a percentage of serum total CH.

Statistical Evaluation

Since these experiments were designed to measure the performance trend over a period of time, the two-way Analysis of Variance (ANOVA) with repeated measurements was selected to analyze each variable (26). Seasonal and individual biological variations were observed over an 8-month period. To minimize error and to normalize data for comparative analysis, each animal was used as its own control whenever analytes were measured on day -1, day +4, and day +7, and all values were expressed as a percent of day -1. These percentages were used to compare results among the various treated groups of animals. When measurements were only made on day +7, analyte concentrations were expressed in absolute values. Post hoc analysis was performed using the Newman-Keuls procedure at $p<0.05$ (26).

RESULTS

Dose Response Relationships of Clofibrate on Serum Total Cholesterol, Triglycerides and α-LP-Cholesterol Concentrations

The effect of clofibrate in 0.2, 0.4 and 0.6 mmol/kg/day × 2 doses on the above parameters of serum lipids is shown in Figure 2. Dose-dependent reduction of serum total CH was observed for all doses on day +4. The percentage reduction ranged between 32 and 37% when compared to controls. Compared to the significant hypocholesterolemic effect of clofibrate on day +4 at all doses, the responses on day +7 were less pronounced. At 0.6 mmol/kg twice daily, clofibrate was ineffective at lowering serum CH (Fig. 2, Panel A). No change in serum total TG was noted following treatment with clofibrate (Fig. 2, Panel B). Serum α-LP-CH concentrations were reduced after +4 and +7 days of treatment with clofibrate at 0.1, 0.2 and 0.4 mmol/kg/day × 2 dose levels (Fig. 2, Panel C). Changes in α-LP-CH and serum CH by clofibrate were virtually identical. These dose-response relationships of clofibrate indicate that 0.2 and 0.4 mmol/kg/day × 2 doses are most appropriate for comparison of the hypolipidemic activity of analogs on several serum and liver parameters.

Effect of Drugs on Serum Parameters

Table 1 compares the effect of clofibrate and analogs II-IV on serum total CH and α-LP-CH levels in replicate experiments. Clofibrate showed significant hypocholesterolemic activity at 0.2 mmol/kg/day × 2 dose both on days +4 and +7 which averaged 67 and 72% of the corresponding control values. Similar hypocholesterolemia (62% of control) was also observed at 0.4 mmol/kg/day × 2 dose on day +4

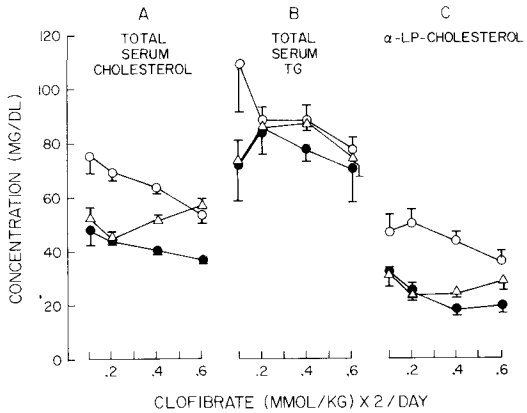

FIG. 2. Dose-response relationships of orally administered clofibrate on total serum CH (Panel A), TG (Panel B) and α-LP-CH (Panel C) concentrations in sucrose-fed fasted male Sprague-Dawley rats at days +4 (●——●), +7 (△——△) and control serum lipid concentrations at day -1 (○——○). Each point represents the mean ± SEM of pooled data for 4 replicate experiments (n=6-24).

TABLE 1

Effects of Clofibrate, Ethyl 6-Chlorochroman (Analog II), Ethyl 6-Phenylchroman (Analog III) and Ethyl 6-Cyclohexylchroman (Analog IV)-2-carboxylates on Serum Total Cholesterol and α-LP-cholesterol Levels in Male Sprague-Dawley Rats Fed a High Sucrose Diet [a]

Doses (mmol/kg/day × 2)	Serum total cholesterol		α-LP-cholesterol	
	Day +4	Day +7	Day +4	Day +7
Experiment 1				
Control	88.0 ± 11.4	98.6 ± 16.5	91.5 ± 14.4	94.0 ± 18.8
Clofibrate, 0.2	62.3 ± 15.0[c]	76.7 ± 15.0[c]	37.2 ± 11.5[c]	46.1 ± 19.6[c]
Clofibrate, 0.4	62.3 ± 9.0[c]	82.6 ± 6.3	44.6 ± 8.6[c]	68.1 ± 12.2
Experiment 2				
Control	99.0 ± 12.5	110 ± 15.8	107 ± 20.0	118 ± 21.9
Clofibrate, 0.2	70.1 ± 7.0[c]	68.6 ± 17.8[c]	65.0 ± 17.4[c]	45.3 ± 15.6[c]
Clofibrate, 0.4	67.0 ± 10.1[c]	83.0 ± 6.9[c]	44.4 ± 6.4[c]	53.7 ± 4.5[c]
Analog II, 0.2	79.7 ± 17.0[c]	75.3 ± 6.7[c]	90.9 ± 24.1[d]	72.5 ± 14.8[c]
Analog II, 0.4	60.0 ± 7.0[b,c]	80.7 ± 18.2[b,c]	56.4 ± 12.0[b,c]	60.9 ± 8.6[b,c]
Experiment 3				
Control	96.7 ± 14.2	80.4 ± 13.2	96.2 ± 15.8	88.6 ± 16.2
Clofibrate, 0.2	49.5 ± 8.0[c]	49.2 ± 4.7[c]	38.5 ± 11.0[c]	40.9 ± 5.3[c]
Clofibrate, 0.4	56.6 ± 6.2[c]	77.4 ± 16.0[d]	32.0 ± 6.1[c]	46.3 ± 9.0[c]
Analog III, 0.2	89.0 ± 10.0	88.5 ± 7.5	89.6 ± 12.4	102 ± 21.3
Analog III, 0.4	95.8 ± 7.0	85.0 ± 10.0	92.8 ± 9.5	95.7 ± 13.4
Experiment 4				
Control	110 ± 11.4	86.2 ± 6.0	104 ± 12.1	78.3 ± 7.9
Clofibrate, 0.2	70.0 ± 9.3[c]	63.0 ± 7.5[c]	59.2 ± 15.0[c]	50.2 ± 7.9[c]
Clofibrate, 0.4	70.4 ± 10.0[b,c]	80.0 ± 13.8[b,d]	52.1 ± 9.6[b,c]	57.3 ± 10.4[b,c]
Analog IV, 0.2	107 ± 9.4	82.0 ± 11.4	136 ± 35.8[c]	89.4 ± 27.6
Analog IV, 0.4	100 ± 9.3	91.0 ± 9.6	108 ± 15.0[d]	86.5 ± 18.1

[a]Data are expressed as percent of the day -1 value ± SD. n=6, unless otherwise noted.

[b]n = 5.

[c]Significant difference ($p<0.05$) between drug and nondrug treated means.

[d]Significant difference ($p<0.05$) between means at the 0.2 and 0.4 doses.

in all experiments. However, on day +7, this hypocholesterolemic effect was noted in only one out of 4 experiments. Chlorochroman II was effective in lowering total serum CH at both +4 and +7 days of treatment. Reductions in serum total CH concentrations were 28 and 31% at the 0.2 and 0.4 mmol/kg/day × 2 doses, respectively (Table 1). No effects on serum total CH were seen with the phenyl (III) and cyclohexyl (IV) chroman analogs at these doses.

Clofibrate and chlorochroman II also significantly lowered serum α-LP-CH concentrations. The average serum α-LP-CH levels in clofibrate-treated rats were 47–52% of the controls at 0.2 and 0.4 mmol/kg/day × 2 doses on days +4 and +7. Chlorochroman II treatment at the 0.2 mmol/kg/day × 2 dose lowered serum α-LP-CH concentration by 42% at day +7, whereas α-LP-CH concentrations were reduced on both days +4 (42%) and +7 (48%) at the 0.4 mmol/kg/day × 2 dose (Table 1). Phenylchroman III was ineffective in modifying α-LP-CH, and cyclohexylchroman IV was found to possess marginal effects in elevating serum α-LP-CH at day +4. This serum LP level, however, was not changed after treatment with analog IV for 7 days. As shown in Figure 3, reduction of α-LP-CH concentrations following clofibrate and chlorochroman II treatment was not due to the reduction in the circulating α-LP particles, but to a compositional change in α-LP. This resulted in a 55 and 38% lowering of the α-LP-CH: α-LP-TG ratio on days +4 and +7, respectively, after clofibrate treatment at the 0.4 mmol/kg/day × 2 dose. Chlorochroman II treatment at the equivalent dose also significantly reduced the α-LP-CH:α-LP-TG ratio on days +4 and +7 by 35 and 60%, respectively (Fig. 3).

The hypocholesterolemic effect of clofibrate and chlorochroman II on serum α-LP-CH levels may be due to an enhanced removal of α-LP-free CH from the α-LP particles mediated by an increase in serum LCAT activity. To assess this possibility, we simultaneously monitored the serum LCAT activities and free CH levels in

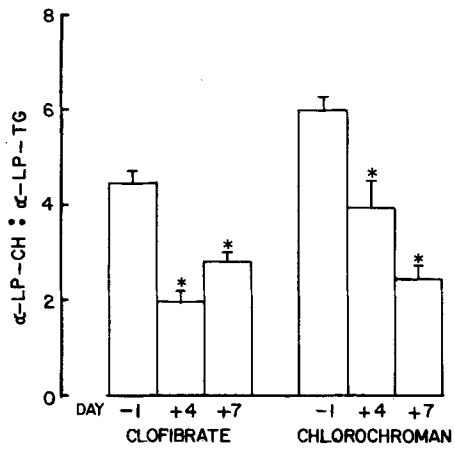

FIG. 3. Effect of various oral doses of clofibrate and ethyl 6-chlorochroman-2-carboxylate on the ratio of serum α-LP-CH to α-LP-TG on days -1, +4 and +7 following oral administration of a dose of 0.4 mmol/kg/day × 2 for 7 days. Bars represent the mean (n=6 except for 6-chlorochroman-2-carboxylate on day +7; n=5) and vertical lines represent the SEM. An asterisk indicates that the values were significantly different from day -1 values at $p<0.05$.

these experiments (Table 2). Serum free CH levels increased significantly after 7 days of clofibrate treatment at both the 0.2 (in 2 out of 4 experiments) and 0.4 mmol/kg/day × 2 (in 4 out of 4 experiments). Treatment with clofibrate at both doses also reduced LCAT activity in 2 out of 4 experiments. Chlorochroman II treatment at the 0.4 mmol/kg/day × 2 dose also increased serum free CH levels but had no effect on serum LCAT activity. Chromans III and IV had no effect on serum LCAT activity.

It is evident from the data presented in Figure 2 that clofibrate treatment at doses between 0.2 and 0.6 mmol/kg/day showed no hypotriglyceridemic effect in this rat model. However, both clofibrate and chlorochroman II treatment modified the TG content in pre-β-LP and β-LP (Table 3). This effect on serum TG distribution among LP was not found in phenylchroman (III) or cyclohexyl (IV) analog-treated rats.

Effect of Drugs on Liver Parameters

Increases in liver microsomal protein and liver to body weight ratios were noted with clofibrate treatment at the 0.2 (2 out of 4 experiments) and 0.4 (3 out of 4 experiments) mmol/kg/day × 2 dose levels (Table 2). Only II showed similar effects on liver microsomal protein and liver to body weight ratios at the 0.2 and 0.4 mmol/kg/day × 2 dose levels. Whereas clofibrate showed no change in liver TG levels, II increased liver TG by 52% at the 0.4 mmol/kg/day × 2 dose level. By contrast, II lowered liver total CH concentration by 28 and 33% at the 0.2 and 0.4 mmol/kg/day × 2 doses, respectively. A similar effect on liver CH was observed in clofibrate-treated rats at the higher dose level in 3 of 4 experiments. Moreover, clofibrate treatment lowered microsomal HMG-CoA reductase activity in only one out of 4 experiments, whereas II did not alter the activity of this enzyme system. Chromans III and IV did not change the activity of microsomal HMG-CoA reductase or liver TG and CH concentrations at either dose.

DISCUSSION

Our previous reports on the antilipidemic activity of analogs II, III, and IV (10–13) showed that their effect depended on the experimental conditions and diet used. In experiments with normal chow-fed, fasted male Sprague-Dawley rats, we have found that only II was effective in reducing serum CH concentrations (13). In the present study, we have examined the efficacy of these 6-substituted phenylchroman analogs in fasted, high sucrose-fed rats. Sucrose feeding per se did not produce significant changes in serum total TG levels. Similarly, an absence of hypertriglyceridemia in nonfasted sucrose-fed rats was reported by Tolman et al. (27). Even so, the effects of these analogs in normal chow-fed (13) and our high sucrose-fed rats were comparable. Analogs III and IV were inactive in these models, whereas clofibrate and II exhibited significant lipid lowering activities. Clofibrate and II administration in chow-fed (13) and sucrose-fed fasted (Fig. 2) or chow-fed unfasted (12) rats significantly lowered serum total CH concentrations. However, differences in the sensitivity to these agents were greater in the high sucrose-fed models than in normal chow-fed rats (10, 12,13). Whereas our studies have shown that only serum total CH levels were reduced by clofibrate treatment, Tolman et al. (27) found a reduction in both serum total CH and TG in sucrose-fed rats given clofibrate in the diet (0.25%). No effect of clofibrate on the serum total TG levels in fasted chow-fed rats has been noted in our laboratories (13). Thus, it is clear that drug-induced antilipidemic responses are highly dependent upon the nature of the experimental model employed.

Further insight into the antilipidemic action of these drugs was obtained by monitoring TG and CH distributions among the major LP.

TABLE 2

Effects of 7-Day Treatment with Clofibrate, Ethyl 6-Chlorochroman (Analog II), Ethyl 6-Phenylchroman (Analog III) and Ethyl 6-Cyclohexylchroman (Analog IV)-2-Carboxylates on Body Weight, Liver/Body Weight, Liver Triglyceride, Serum-free and Liver Cholesterol, Liver Microsomal Protein, Liver HMG-CoA Reductase and Serum LCAT Activities in Sucrose-fed Male Sprague-Dawley Rats

Drug treatment	Dose (mmol/kg/day X 2)	Final body wt (% of day -1)	Liver/body wt (%)	Liver triglycerides (mg/g)	Liver cholesterol (mg/g)	Microsomal protein (mg/g)	Serum free CH (% of serum total CH)	Liver HMG-CoA reductase (pmol/mg protein/hr)	LCAT (nmol CH esterified/ml/hr)
Control[a]	0.25% methyl cellulose	108 ± 1.5	2.82 ± 0.05	3.19 ± 0.24	4.42 ± 0.38	20.9 ± 0.7	17.5 ± 0.4	173 ± 41.8	42.8 ± 3.0
Clofibrate	0.2[a]	107 ± 1.4[b]	4.01 ± 0.07[b]	2.46 ± 0.20	3.41 ± 0.25	23.6 ± 0.6[b]	23.9 ± 1.4[b]	75.0 ± 24.9[c]	32.9 ± 1.0[b]
	0.4[d]	104 ± 1.3[b]	4.45 ± 0.08[e]	3.32 ± 0.30	2.90 ± 0.10[e]	23.9 ± 0.7[e]	28.4 ± 0.9[f]	91.9 ± 9.6[c]	31.7 ± 1.3[b]
Analog II	0.2[g]	113 ± 1.0	3.03 ± 0.02	3.65 ± 0.59	4.28 ± 0.15[h]	30.0 ± 0.9[h]	19.0 ± 1.6	54.0 ± 13.0	32.1 ± 1.7
	0.4[i]	102 ± 2.6[h]	3.69 ± 0.24[h]	6.39 ± 0.96[h]	4.03 ± 0.19[h]	26.8 ± 1.1	26.8 ± 2.7[h]	285 ± 160	49.1 ± 12.8
Analog III[g]	0.2	110 ± 1.2	2.78 ± 0.05	1.94 ± 0.14	3.88 ± 0.26	24.2 ± 0.8	17.1 ± 0.7	169 ± 28.8	30.2 ± 0.7
	0.4	109 ± 2.2	2.90 ± 0.09	2.57 ± 0.18	3.55 ± 0.14	25.8 ± 2.4	16.5 ± 0.4	80.3 ± 24.6	34.9 ± 0.7
Analog IV[g]	0.2	105 ± 0.4	2.80 ± 0.07	1.70 ± 0.37	4.09 ± 0.25	21.2 ± 0.9	15.1 ± 0.9	225 ± 58.8	46.9 ± 1.6
	0.4	103 ± 1.3	2.58 ± 0.09	2.27 ± 0.33	4.59 ± 0.60	21.2 ± 1.4	12.5 ± 0.4	246 ± 61.0	43.5 ± 0.8

[a]Pooled data of 4 experiments (n=24); values represent the mean ± SEM. Values at the level of $0.001 > p < 0.05$ were considered significant.

[b]Significantly different from the corresponding control group in 2 out of 4 experiments.

[c]Significantly different from the corresponding control group in 1 out of 4 experiments.

[d]Pooled data of 4 experiments (n=23); values represent the mean ± SEM.

[e]Significantly different from the corresponding control group in 3 out of 4 experiments.

[f]Significantly different from the corresponding control group in 4 out of 4 experiments.

[g]Values represent the mean ± SEM (n=6).

[h]Significantly different than the corresponding control group.

[i]Values represent the mean ± SEM (n=5).

TABLE 3

Effects of Clofibrate and Ethyl 6-Chlorochroman (Analog II)-2-carboxylate on Pre-β and β-Lipoprotein Triglyceride Contents in Male Sprague-Dawley Rats Fed a High Sucrose Diet [a]

Doses (mmol/kg/day X 2)	Pre-β-LP-TG		β-LP-TG	
	Day +4	Day +7	Day +4	Day +7
Experiment 1				
Control	109 ± 19.0	105 ± 20.3	112 ± 40.3	139 ± 40.0
Clofibrate, 0.2	51.0 ± 8.4[c]	69.4 ± 26.2[c]	374 ± 125[c]	256 ± 119
Clofibrate, 0.4	77.0 ± 23.6[c,d]	84.4 ± 16.0[d]	235 ± 86.2	183 ± 63.3
Experiment 2				
Control	91.1 ± 8.1	91.1 ± 13.0	109 ± 23.5	142 ± 71.3
Clofibrate, 0.2	71.5 ± 15.2[c]	68.6 ± 8.2[c]	223 ± 87.8[d]	328 ± 65.7[c]
Clofibrate, 0.4	64.2 ± 6.5[c]	52.5 ± 12.3[c]	365 ± 141[c]	710 ± 353[c,d]
Analog II, 0.2	91.6 ± 9.6	80.1 ± 9.4	128 ± 50.6	242 ± 122
Analog II, 0.4	92.7 ± 10.4	101 ± 21.6	154 ± 40.5	215 ± 114
Experiment 3				
Control	102 ± 14.5	106 ± 9.4	155 ± 49.4	144 ± 35
Clofibrate, 0.2	92.6 ± 6.6[c]	84.0 ± 15.0[c]	197 ± 38.6	256 ± 78
Clofibrate, 0.4	62.4 ± 20.0[c,d]	67.2 ± 11.2[c,d]	358 ± 118[c,d]	386 ± 60.8[c]
Experiment 4				
Control	77.0 ± 7.0	77.3 ± 16.0	138 ± 81.0	196 ± 118
Clofibrate, 0.2	73.0 ± 11.5	70.7 ± 11.7	103 ± 34.5	194 ± 79.4
Clofibrate, 0.4	58.3 ± 10.1[b,c,d]	57.0 ± 7.5[b,c]	247 ± 49.4[b]	281 ± 36[b]

[a]Data are expressed as the percent of serum total triglyceride relative to the pretreatment level (day -1) ± SD. n = 6, unless otherwise noted.

[b]n = 5.

[c]Significant difference ($p<0.05$) between drug and nondrug treated means (log transformation).

[d]Significant difference ($p<0.05$) between means at the 0.2 and 0.4 doses.

Serum total CH lowering by clofibrate and II, but not by III and IV, was directly correlated to a reduction in α-LP-CH. Segal et al. (28) reported that clofibrate treatment decreased the protein concentration of high density LP (HDL) in sucrose-fed rats, an observation which could be interpreted either as a modification of HDL particle composition or as a reduction in particle number. The observed decrease in α-LP-CH:α-LP-TG ratios after clofibrate and II treatment suggested that compositional changes in the lipid content of α-LP have occurred as opposed to a reduction in particle number (Fig. 3). Interestingly, despite an absence of serum total TG lowering by these compounds, both clofibrate and II lowered pre-β-LP-TG and increased β-LP-TG concentrations. In addition, it has been reported (29) that clofibrate treatment increased LDL at the expense of VLDL in Type IV patients. This parallelism supports the use of this fasted rat model as an evaluative tool for hypertriglyceridemia. Overall, the data with clofibrate and II are qualitatively similar, and the results with clofibrate agree with the earlier report of Segal et al. (28). Moreover, both clofibrate and analog II appear to enhance the catabolism of pre-β-LP in the sucrose-fed model, whereas only clofibrate exhibited similar effect in the normal fasted chow-fed rat model (13). This likely reflects a fundamental difference in the anabolic and/or catabolic lipid activities between these two animal models.

In clofibrate-treated normal fasted chow-fed rats, we reported a favorable correlation between serum α-LP-CH lowering and decreased serum LCAT activity (13). A similar relationship was obtained for clofibrate, and to a lesser degree for II in this study. Inactive analogs III and IV did not modify serum free CH concentrations or LCAT activity. These results may either (a) reflect drug-induced liver toxicity with concomitant LCAT lowering, free CH increases and enhanced catabolism of available free CH; or (b) reflect a direct consequence of lowered serum CH or α-LP-CH produced by these agents.

Changes in liver parameters after drug treatment to sucrose-fed rats were similar to those seen in chow-fed rats (13). Sucrose feeding alone increased liver TG (+37%) and CH (+56%). Whereas liver TG levels were not affected by

treatment with these compounds, liver CH concentrations were significantly lowered by clofibrate and II. This hypocholesterolemia could not be related to an inhibition of liver microsomal HMG-CoA reductase activity as had been suggested previously (13). The serum total CH lowering actions of these compounds most likely reflect an increased liver or serum CH catabolism and the mechanism for this effect remains to be elucidated.

Changes in serum CH and LP-lipid lowering properties of clofibrate were not always time- and dose-dependent. At the 0.6 mmol/kg/day dose, serum total CH and α-LP-CH levels were equal to control values at day +7, but were significantly reduced at day +4 (Fig. 2, Panels A and C). This refractoriness to the hypocholesterolemic activity of clofibrate might be related to a progressive reduction in serum circulating levels of clofibric acid, the major active metabolite of clofibrate (30–32). In a recent report, we (30) showed that [C-14]-clofibrate treatment (0.4 mmol/kg/day × 2) to rats for 14 days reduced the plasma level of [C-14]-clofibric acid by 30 and 50% at days +7 and +14, respectively. The concomitant reduction in plasma clofibric acid levels after chronic treatment (30,31) may be related to an increased distribution of drug into adipose tissue (30). In the present study, the liver/body weight ratios also showed a refractoriness to different doses of clofibrate after 7 days of treatment. Clofibrate-induced increases in these ratios were +18, +59, +39 and +39% at doses of 0.1, 0.2, 0.4 and 0.6 mmol/kg/day, respectively. Possibly this reversibility of the biological responses to clofibrate is related to a metabolic tolerance. Such a phenomenon has not been reported in man after chronic clofibrate therapy (33). Thus, differences in antihyperlipoproteinemic and metabolic effects to clofibrate and related analogs may be dependent upon the dosage regimen used in various species.

These and previous data (13,14) show that certain chroman analogs possess significant platelet antiaggregatory and hypolipidemic activities. Within this limited series, phenylchroman III and chlorochroman II were found to be most effective in these two pharmacological systems. The hypolipidemic and antiaggregatory properties of II were similar to those observed for clofibrate. However, the sensitivity of the response of II in sucrose-fed fasted rats was greater than that observed in chow-fed fasted rats. Altering the 6-substituent in the chroman nucleus provides a method of separating antiaggregatory from serum and liver lipid lowering actions. Chlorochroman II, exhibiting both pharmacological effects, remains a promising drug and warrants further preclinical studies in other animal species (34). The functional selectivity of III as an antiaggregatory agent also merits attention for the further development of antithrombotic drugs.

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Chemical Ionization Mass Spectrometry of Wax Esters

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ABSTRACT

Chemical ionization (CI) mass spectra of long-chain saturated, monenoic and dienoic wax ester analogues, using isobutane and methane as reagent gases, are reported and compared. Because less fragmentation occurs, CI spectra are simpler than the electron impact spectra. The quasi molecular ion (M + 1) is the base peak in the spectra. Saturated, monenoic and dienoic wax ester mixtures have been analyzed by this technique.

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INTRODUCTION

Wax esters occur widely in nature in a variety of plant and marine species. They are usually found as mixtures of esters consisting of saturated or monoenoic fatty acids and alcohols, with the acid and alcohol groups varying in chain length from C_6 to C_{34}. The electron impact (EI) mass spectra of wax esters have been studied in considerable detail (1-5). These spectra exhibit considerable fragmentation features characteristic of compounds with long hydrocarbon backbones. Diagnostically significant ions, such as molecular ions and cleavage ions indicative of the alcohol and acid moieties, are observed, but are a rather small portion of the total ions generated. When analyzing complex mixtures with many alcohol and acid combinations of a given chain length, the low abundances of these ions and the generation of many intense structurally undiagnostic "hydrocarbon" fragments complicate quantitation of the wax composition. Chemical ionization mass spectrometry (CI-MS) is a much gentler ionization process that produces much less fragmentation. We report here a systematic study of the CI-MS of wax esters using isobutane, ammonia and methane as the reagent gases. The spectra are much less complex than the conventional EI mass spectra, with structurally diagnostic ions accounting for most of the ions observed.

EXPERIMENTAL PROCEDURES

GC- and GC-MS

The mass spectra were obtained on a Kratos MS30 mass spectrometer equipped with a combined CI/EI source. Ions in the EI mode were produced at 70 eV with a source pressure of ca. 10^{-6} torr, whereas with CI they were produced at 100-200 eV with the source pressure estimated at ca. 1 torr. Wax esters, individually or in mixtures, were introduced by direct insertion probe or by GC inlet via a single-stage glass jet separator. Glass GC columns (1 m × 2 mm) packed with 3% OV-1 were temperature programmed from 230-300 C at 2 C/min. The injector, detector and jet separator were held at 270 C while the source of the mass spectrometer was 200 C. Sample sizes analyzed were of the order of 100 ng for pure compounds and as much as 5-10 μg for complex mixtures. Mass spectral data were acquired and analyzed using a DS-50-s data system (Kratos).

Preparation of Standard Wax Esters

Acid chlorides were prepared by adding 2 ml of oxalyl chloride (Aldrich Chemical Co., Milwaukee, WI) to 400 mg ofthe appropriate fatty acid at room temperature. When the reaction was complete (5 min), excess oxalyl chloride was removed under N_2. Two of the acid chlorides (16:0, Eastman, Rochester, NY and 16:1, Nu-Chek, Prep, Elysian, MN) and 4 alcohols (16:0, 16:1, 18:0, and 18:1, Nu-Chek Prep) were purchased. To prepare wax esters, equal molar amounts of the alcohol and acid chloride were mixed in benzene and pyridine (10 ml/0.5 ml) and allowed to stand 15 min with occasional swirling. The reaction mixture was then concentrated under N_2 and the wax esters were purified by preparative layer chromatography (PLC) on 2-mm layers of silica gel with hexane/ether (70:30) and HPLC on μ-Bondapack C-18 (Waters Associates) or ODS-2 (Whatman) reverse-phase semipreparative columns with acetone/acetonitrile (2:1).

Sample Preparation and Separation

Winterized sperm whale oil wax ester samples (0.5 g) were separated from triglycerides by column chromatography with High-Flosil (Applied Science Labs, State College, PA). PLC on 1-mm coated with 20% $AgNO_3$ in silica gel

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[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

separated the wax esters by degree of unsaturation (solvent: benzene/chloroform, 1:1). Bands located under ultraviolet (UV) light after spraying with a dichlorofluorescein solution were scraped from the plate. Wax esters were then recovered from the silica by ether extraction. Hydrogenations were carried out in toluene/ethanol (1:10) with 10% Pd on charcoal as the catalyst.

RESULTS

Isobutane, the most popular reagent gas for CI-MS, yielded intense quasi molecular ions (M+1) from protonation of the molecular species that are usually the base peaks. Smaller ions at M−1, M+43 and M+57 frequently occur due to the loss of a proton or the attachment of a propyl or butyl group to the molecule. Relatively little other fragmentation occurs. Ammonia is becoming more popular as a reagent gas because it usually gives even less fragmentation than isobutane, and frequently none occurs. In wax esters, ammonia does not protonate the molecule to give M+1 but, rather, attaches an ammonium ion (NH_4^+) to the molecule to give M+18 as an intense and sometimes only ion. Methane, the other common CI reagent gas, gives relatively more fragmentation than isobutane, although the spectra produced often appear quite similar. Because CI mass spectra produced often appear quite similar. Because CI mass spectra of homologous compounds show fragmentations that are characteristic within each group containing the same number of double bonds, only a few representative examples have been chosen for detailed discussion.

Saturated Esters

Figure 1, the isobutane CI mass spectrum of palmityl stearate (16:0-18:0), shows a protonated molecular ion m/z 509 as the base peak. Small ions at M+41, M+43 and M+57 (m/z 550, 552 and 566) arise by attachment of the reactant ions m/z 41, 43 and 57 from the reagent gas, isobutane. In EI-MS of wax esters, the double hydrogen rearrangement ion depicted in Scheme 1 (5) is predominant decomposition of the odd electron parent ion with rearrangement of two hydrogen atoms.

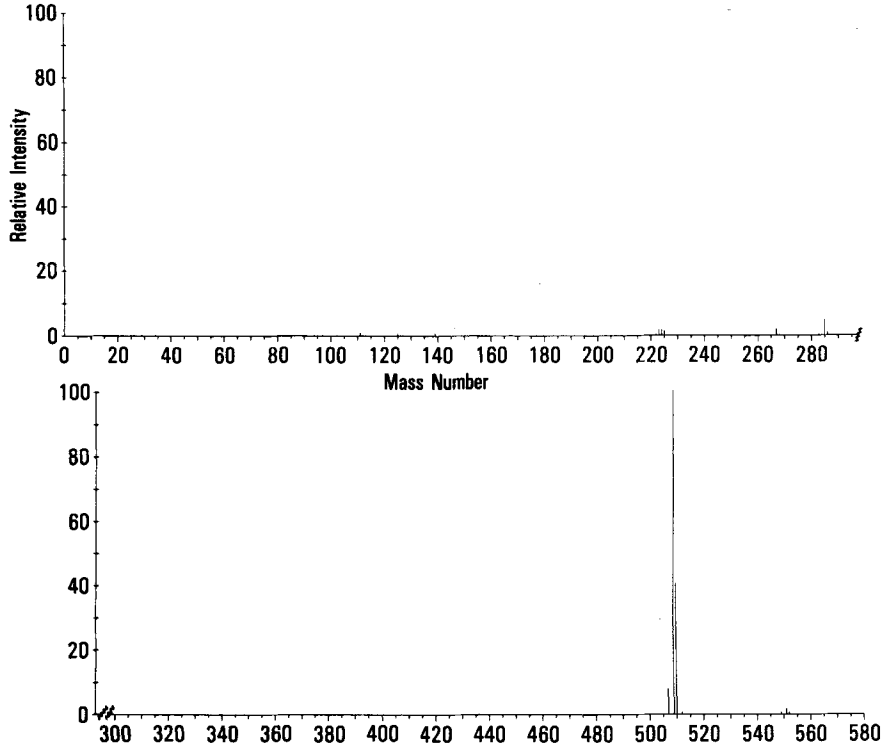
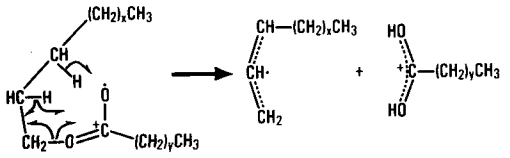


FIG. 1. Isobutane CI mass spectrum of palmityl stearate.



SCHEME 1

This ion is the base peak in EI mass spectra of wax esters. In isobutane CI, saturated esters show much less of this fragmentation. $RCO_2H_2^+$ at m/z 285 in palmityl stearate (16:0–18:0) is about 7% of the protonated molecular ion. The other significant fragmentations that are observed are the RCO^+ ion at m/z 267, the R'^+ ion at m/z 225, the $(R'-H)^+$ ions, which are not observed in the EI mass spectra of wax esters, arise by charge transfer during the double hydrogen rearrangement. Very little additional fragmentation is observed. In contrast, under EI-MS conditions, spectra show many nondiagnostic lower molecular weight fragmentations that account for most of the total ion current. Under ammonia CI conditions, the most prominent product ion is the adduct ion observed at M+18 arising from attachment of the ammonium ion (NH_4^+-m/z 18) from the reagent gas to the molecule. Small ions are sometimes observed in the region of the molecular ion at M^+ or $(M+H)^+$. Significant quantities of fragmentation ions are not observed. Methane CI mass spectra of saturated wax esters show considerable fragmentation. Figure 2 is the methane CI mass spectrum of palmityl stearate (16:0–18:0). The base peak in the spectrum is the double hydrogen rearrangement ion at m/z 285. Both protonated molecular ion (m/z 509) and hydride-extracted molecular ion (m/z 507) are observed in about equal intensities (~90% of base peak). Adduct ions formed by attachment of reactant gas ions are observed at M+17, M+29 and M+41. The RCO^+ ion at m/z 267 and the R'^+, $(R'-H)^+$ and $(R'-2H)$ at m/z 225, 224 and 223 are observed as strong signals (> 10% base peak). Considerable nonspecific fragmentation of the type observed in EI is observed in methane CI spectra.

Unsaturated Esters

In monoenoic wax esters, the location of the double bond in the acyl or alkoxyl moiety of

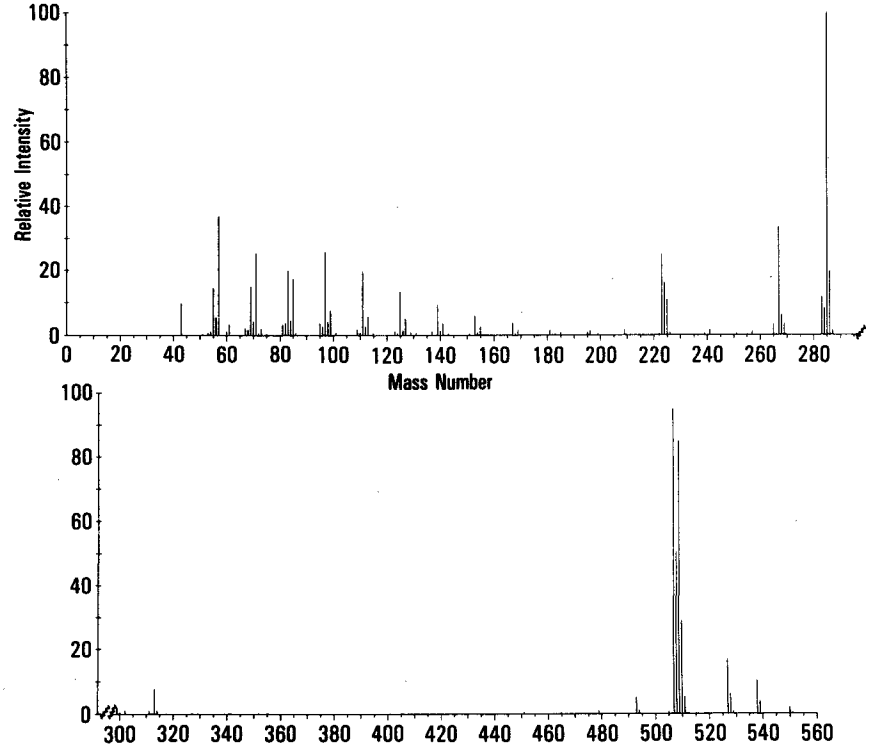


FIG. 2. Methane CI mass spectrum of palmityl stearate.

the molecule dramatically affects fragmentation. When the unsaturation is in the acyl end of the ester, the spectra are quite similar to those of the saturated wax esters. The only substantial difference in the isobutane species is the appearance of an ion from $[RCO-H]^+$ of about equal intensity to that for RCO^+ and a smaller ion from $[RCO-2H]^+$. When methane is the reagent gas, the spectra are again similar to those from saturated esters except that the $(RCO_2H_2)^+$ ion is only about half as intense as the M+H ion rather than appearing as the base peak. The $[RCO-H]^+$ and $[RCO-2H]^+$ ions are still intense. When the unsaturation is in the alkoxyl end of the wax ester, there is considerably more fragmentation in the isobutane CI spectra (see Fig. 3). The fragment ions observed are the same, but the $RCO_2H_2^+$ and RCO^+ ions (285 and 267) are about 5 times as intense as the ones for monoenes with the unsaturation in the acyl part of the ester. The $(R'-H')^+$ ion (223) is very much larger ($\sim$10 time as intense) as are the $(R')^+$ and $(R'-2H)^+$ ions. Also, some nondiagnostic "hydrocarbon" ions are observed at lower mass. Because the fragmentation of monoenes appears to depend on which side of the ester group the double bond is positioned, the method of Spencer (4) for quantitating acyl and alkoxyl group distributions using EI-MS fragmentation probably will not work in isobutane CI-MS.

Diunsaturated wax esters with one double bond on each side of the ester group give CI-mass spectra as would be predicted from the saturated and monoene ester spectra. Figure 4 shows the isobutane CI spectrum of palmitoeoyl oleate (16:1-18:1). The same fragments observed in the saturates and monoenes are observed. Again, most of the ion current is carried by the protonated molecular ion. Somewhat less fragmentation is observed than in the spectra of monoenoic waxes with the unsaturation in the alcohol moiety. Tables 1 and 2 list the isobutane and methane CI spectra of the synthetic eight wax ester standards.

DISCUSSION

To quantitatively analyze mixtures of wax esters, Aasen et al. (2) reported that the sum of ion intensities for 3 significant ions from each acid-alcohol combination could be used to

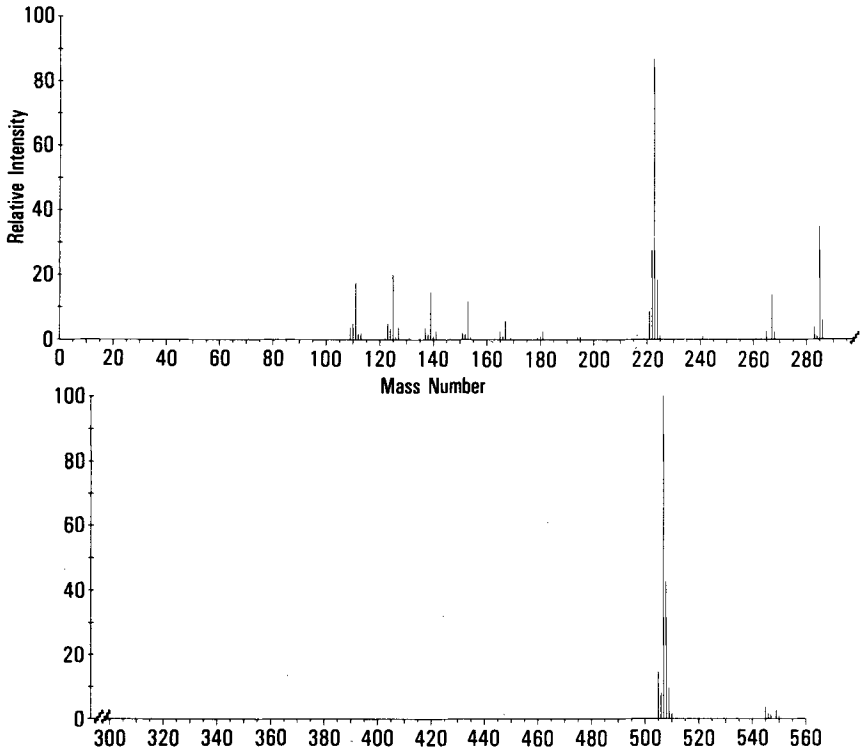


FIG. 3. Isobutane CI mass spectrum of palmityl stearate.

TABLE 1

Isobutane CI Mass Spectra of Wax Esters

Wax ester Alcohol-acid	M+57	M+43	M+1	$RCO_2H_2^+$	RCO^+	$RCO-H^+$	$RCO-2H^+$	R'^+	$R'-H^+$	$R'-2H^+$
16:0-18:0	566(0.5)	552(3)	509(100)	285(7.5)	267(2.1)			225(1.5)	224(1.8)	223(1.8)
18:0-16:0	566(0.3)	552(3)	509(100)	257(8.1)	239(3.3)			253(2.2)	252(2.5)	251(2.4)
16:1-18:0		548(1)	507(100)	285(35)	267(14)			223(8.7)	222(2.8)	221(0.9)
18:1-16:0		548(1)	507(100)	257(33)	239(17)			251(7.3)	250(2.5)	249(0.3)
16:0-18:1		548(0.5)	507(100)	283(7.4)	265(5.1)	264(4.7)	263(1.2)	225(0.6)	224(1.2)	223(0.5)
18:1-16:0		548(1)	507(100)	255(9.3)	237(5.6)	236(8.4)	235(1.3)	253(2.0)	252(0.6)	251(1.2)
16:1-18:1			505(100)	283(3.4)	265(2.6)	264(0.6)	263(0.7)	223(5.9)	222(4.0)	221(3.9)
18:1-16:1			505(100)	255(3.0)	237(2.4)	236(0.7)	235(0.6)	251(4.3)	250(3.1)	249(2.4)

TABLE 2

Methane CI Mass Spectra of Wax Esters

Wax ester Alcohol-acid	M+H	M-H	$RCO_2H_2^+$	RCO^+	$RCO-H^+$	RCO-2H	R'^+	R'-H	R'-2H
16:0-18:0	509(85)	507(95)	285(100)	267(33)			225(15)	224(18)	223(23)
18:0-16:0	509(97)	507(100)	257(99)	239(33)			253(10)	251(15)	251(18)
16:1-18:0	509(60)	509(90)	285(50)	267(43)			223(100)	222(68)	221(49)
18:1-16:0	507(85)	507(100)	257(73)	238(63)			251(96)	250(78)	249(49)
16:0-18:1	507(100)	505(53)	283(23)	265(18)	264(13)	263(5)	225(4)	224(4)	223(9)
18:0-16:1	507(100)	505(60)	255(33)	237(27)	236(23)	235(12)	253(7)	252(5)	251(6)
16:1-18:1	505(100)	503(40)	283(23)	265(26)	264(5)	263(9)	223(20)	222(13)	221(16)
18:1-16.1	505(100)	503(40)	255(30)	237(28)	236(5)	235(8)	251(15)	250(12)	249(14)

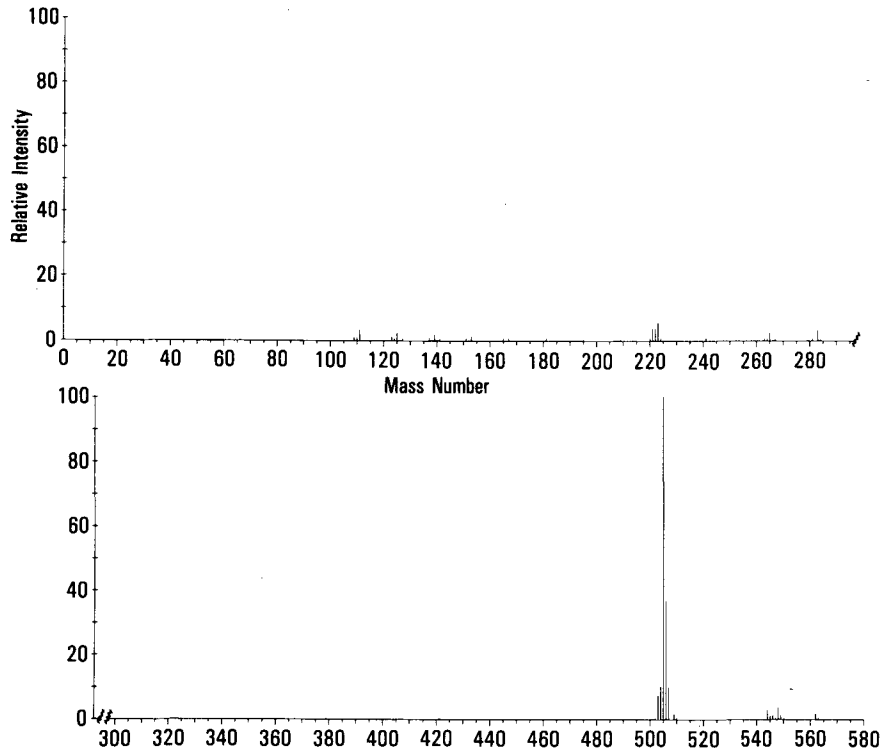


FIG. 4. Isobutane CI mass spectrum of palmitoleoyl oleate.

estimate the proportions of the different combinations. Spencer et al. (3,4) extended their technique to GC-MS analysis of jojoba oil and sperm whale oil wax esters using the $(RCO_2H)^+$, $(RCO_2H_2)^+$ and $(R'-1)^+$ for each combination in the saturated fraction and in the hydrogenated diene fraction. The greatly simplified fragmentation patterns observed in CI mass spectra make identification and summation of the selected ions for the various ester combinations much easier. We found excellent agreement with the published results (4) for sperm whale oil saturates and hydrogenated dienes. However, in monoenes, the location of the double bond in the alkoxyl or acyl end of the ester profoundly affects the fragmentation, and the proposed procedure did not work on monoene standard mixtures. Without standards of all possible combinations to calculate elaborate correction factors, it does not appear possible to quantitatively analyze monoenoic wax esters without hydrogenation. However, hydrogenation destroys information necessary to determine which moiety of the wax ester had been unsaturated. It appears likely that reduction to a mixture of long chain alcohols with deuterohydrazine followed by MS (2) should be applicable to solving this problem, because corrections for isotopic impurities in the samples should be much simpler in the CI mass spectra where fragmentation is so much less extensive.

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Complexities in Lipid Quantitation Using Thin Layer Chromatography for Separation and Flame Ionization for Detection[1]

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ABSTRACT

The use of thin layer chromatography (TLC) for separation (using silica gel coated quartz rods) and subsequent flame ionization for detection (FID) was examined to determine whether this method could be used for the quantitation of lipids. However, response factors (RF) for various lipids were different and depended upon several variables including the amount of material analyzed. For example, RF were 3-fold greater when 10 μg of tripalmitin was analyzed as compared to 1 μg of the same material. The amount of lipid detected by FID was also dependent upon the rate at which it passed through the flame. During analysis of methylpentadecanoate, detector response increased with scan speed, while at all speeds it was completely removed from the rod. On the other hand, depending upon the amount of cholesterol or phospholipid analyzed, the response either increased, remained unchanged or decreased with scan speed. During a fast scan, detector response was reduced because some material remained on the rod. Thus, the detector response is influenced by sample volatility. In conclusion, there appears to be a complex relationship between detector response and the amount of heat available per microgram of sample. Since we could not find a direct correlation between detector response and sample quantity, it would be difficult to use TLC-FID as a tool for quantitating the components of a lipid mixture.
Lipids 18:74-80, 1983.

INTRODUCTION

The Iatroscan (TH-10 TLC Analyser MK III, Iatron Laboratories, Tokyo, Japan) was designed to detect compounds using a flame ionization detector (FID) after being separated on silica gel coated quartz rods (chromarods) by thin layer chromatography (TLC). Although this technique had been used for qualitative analyses of plasma lipid composition (1,2), it has also been used for the quantitation of tissue lipids (3-6). Applications of TLC-FID have recently been reviewed by Ackman (7). In previous studies, response factors (RF) have been determined for several different lipids (3,4). However, the reported values are inconsistent. For example, in one study, the RF for cholesteryl esters was twice that of fatty acids (4), while in another study, they were nearly the same (3). Furthermore, the values for the RF of fatty acids differed by 50% in the two studies. These differences in RF indicate that some of the problems in using TLC-FID for sample quantitation remain unresolved. The present studies were designed to identify these problems and determine if this method can be used for the quantitative analyses of lipids. Several parameters are discussed that affect detector response in TLC-FID.

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MATERIALS AND METHODS

Materials

Authentic lipid standards were obtained from Applied Science Laboratories (State College, PA) and Supelco Inc. (Bellefonte, PA). Their purity was checked by TLC or gas liquid chromatography (GLC). Solvents were of GLC grade, obtained from J.T. Baker (Phillipsburg, NJ). Chromarods (Type S-II) and the Iatroscan were obtained from Iatron Laboratories, Inc. (Tokyo, Japan).

Methods

Prior to chromatographic procedures, rods were stored in deionized and distilled water, activated by heating at 100 C for 15 min, and then scanned through the hydrogen flame of the FID. The flow rates of hydrogen and air were 160 ml/min and 2000 ml/min, respectively. Lipids were dissolved in chloroform/methanol (2:1) at a concentration of 1-10 mg/ml. Generally, 1 μl of the lipid solution was spotted on the chromarod using a micropipette. Undeveloped rods containing samples were scanned (4.2 mm/sec) in an Iatroscan equipped with a FID. Peak areas were measured by a

Hewlett-Packard 3390 A Integrator (Palo Alto, CA). Response factors were calculated as (peak area)/(amount analyzed) and were normalized to that of 5 μg of methylpentadecanoate.

Response factors of fatty acid methyl esters were determined using a Varian Aerograph (Model 2740, Walnut Creek, CA) equipped with a FID and a 6 ft × 1/8 in. stainless steel column packed with 5% DEGS on HP Chromosorb G. Fatty acid methyl esters (ca. 5 μg) were injected into the column at 180 C and areas were computed using a Varian Chromatography Data System (CDS-111).

RESULTS

Comparison of GLC-FID with TLC-FID

Flame ionization detection as used in GLC gives accurate and reproducible results such that volatile organic compounds can be quantitated. When fatty acid methyl esters of various chain lengths or degree of unsaturation were analyzed by GLC, RF deviated only slightly (<10%) from unity (8). We compared the RF of methylpentadecanoate (C_{15}), methylpalmitate (C_{16}) and methylbehenate (C_{22}) both using GLC and TLC-FID (Table 1). Values obtained using GLC did not deviate more than 3% from unity. In contrast, RF obtained from the TLC-FID method deviated more than 70% from one. Also from Table 1, response factors appear to increase with hydrocarbon chain length when the TLC-FID technique is used. This is surprising, since both GLC and TLC-FID use FID for detection and quantitation. Reasons for this discrepancy will be discussed.

TABLE 1

Response Factors of Fatty Acid Methyl Esters Using GLC and TLC-FID

Methyl esters	GLC	TLC-FID
C_{15}	1.00 (7)	1.00 ± 0.18 (10)
C_{16}	1.02 ± 0.02 (7)	1.32 ± 0.12 (10)
C_{22}	1.03 ± 0.06 (7)	1.73 ± 0.26 (39)

Response factors (RF) of methylpentadecanoate (C_{15}), methylpalmitate (C_{16}) and methylbehenate (C_{22}) were determined using GLC or TLC-FID. A mixture containing 5 μg of each of the methyl esters of C_{15}, C_{16} and C_{22} was analyzed by GLC using C_{15} as the internal standard. With TLC-FID, methyl esters were separately analyzed. Values represent the mean of separate analyses ± SD and are normalized to C_{15}. The number of analyses are given in parenthesis.

Response Factors of Lipids

When free fatty acids were analyzed by TLC-FID, although RF deviated from unity (1.84–2.35), the values were similar (Table 2). This was also true of triglycerides (TG) with RF ranging from 1.15 to 1.31. Generally, cholesteryl palmitate and cholesterol had higher RF than other lipids that were examined (Table 2). These results confirm those of others in which the RF of cholesterol was high compared to that of glycerolipids (4,6). The RF for cholesterol was about the same as that of cholesteryl palmitate but ca. 3 times that of TG (Table 2). Also, the RF of phospholipids (phosphatidylserine and phosphatidylcholine) were similar to those of fatty acids (Table 2), as observed previously (4).

There are several differences between RF obtained in this study and those reported earlier (Table 3). While the absolute RF may differ between studies depending upon the standard chosen (we used methylpentadecanoate) and the conditions of analysis, the ratio of any two of these factors should be the same in all studies. However, these ratios are not constant and sometimes vary as much as 4-fold (Table 3). These large differences in RF ratios cannot be due to the presence of TG or fatty acids with varying acyl chain lengths with each study since the chain length of TG or fatty acids does not influence RF appreciably (Table 2).

TABLE 2

Response Factors of Lipids Using TLC-FID

Lipids	Response factor[a]
Cholesterol[b]	3.35 ± 0.59 (4)
Cholesteryl palmitate	3.76 ± 0.31 (16)
Tripalmitin	1.31 ± 0.15 (5)
Tristearin	1.15 ± 0.36 (16)
Triarachidin	1.20 ± 0.10 (10)
Palmitic acid	1.94 ± 0.30 (45)
Stearic acid	2.35 ± 0.19 (30)
Arąchidonic acid	1.84 ± 0.14 (10)
Phosphatidylcholine[b]	2.44 ± 0.23 (5)
Phosphatidylserine	2.09 ± 0.27 (15)

[a]Values given are mean ± SD. The number of determinations is given in parenthesis. Analysis was carried out with 8 μg of cholesterol and 10 μg of cholesteryl palmitate. Other compounds were analyzed using 5 μg.

[b]Chromarods spotted with phosphatidylcholine were developed using chloroform/methanol/water (80:35:3.5, V/V). Those spotted with cholesterol were developed using petroleum ether/diethylether/acetic acid (97:3:1, V/V). Rods were scanned (4.2 mm/sec) after drying at 100 C for 5 min.

TABLE 3

Inconsistency in Relative Response Factors Using TLC-FID

Reference	Lipid class[a]				Ratios of response factors[b]			
	CE	CH	TG	FFA	CE/CH	CE/TG	CE/FFA	CH/TG
(3)	1.19	0.69	1.03	1.17	1.73	1.16	1.02	0.67
(4)	1.57	1.21	0.66	0.84	1.30	2.38	1.87	1.83
[c]	3.76	3.35	1.31	1.94	1.12	2.87	1.94	2.56

[a]CE = cholesteryl esters, CH = cholesterol, TG = triglycerides, FFA = free fatty acids.

[b]Values were calculated from corresponding response factors.

[c]Data was obtained from Table 2 of the present study.

In this comparison of response factor ratios (Table 3), in each case, the numerator is the RF for either cholesterol or cholesterol esters. There is no significance to these ratios except that they demonstrate large variations and deviations from one study to the next. More consistent ratios could be calculated from, e.g., TG/FFA. However, even these values vary from 0.68 (Table 3) to 0.88 (3). More work in this area would help clarify why some ratios vary more than others.

Variations in RF were seen in fatty acid methyl esters of different chain length (Table 1), but not of TG with different acyl groups (Table 2). This may be because the methyl esters are more volatile than TG. The effect of volatility on RF in TLC-FID will be discussed.

Increase in Response Factor with Sample Quantity

During determinations of FID response using different amounts of lipids, we observed a more likely cause for the variations in RF ratios. We observed that, instead of being constant, RF increased with the quantity of lipid analyzed (Fig. 1). It is due to this phenomenon that Martin-Ponthieu et al. were unable to obtain a direct relation between weight of phospholipid spotted on chromarods and the corresponding peak area (5).

It has been recommended that 1–10 μg is a reasonable working range for sample analysis using the Iatroscan (3) However, within this range, RF for cholesterol, TG, fatty acid methyl esters and phospholipids increased 2- to 3-fold as the amount of sample was increased (Fig. 1). Similar correlations between response and sample quantity have been reported for neutral lipids (9). Hence, the RF of a lipid is not constant over a small range of substrate concentrations. Unfortunately, limiting the sample size would not help improve the usefulness of TLC-FID for quantitation since the working range is already very narrow (1–10 μg).

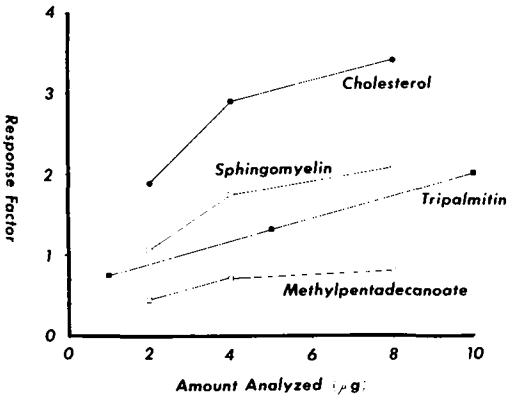

FIG. 1. The influence of sample quantity on response factor. Samples were spotted on chromarods and analyzed using the Iatroscan TH-10. Tripalmitin was spotted and analyzed undeveloped. Rods spotted with cholesterol or methylpentadecanoate were developed using petroleum either/diethyl ether/acetic acid (97:3:1, V/V). Chromarods spotted with sphingomyelin were developed with chloroform/methanol/water (80:35:3.5, V/V).

The RF values for methylpentadecanoate are less than 1.0 in this figure since they were based on the results given in Table 1. RF values for all compounds varied significantly between experiments. This could have been due to different characteristics of the chromarods that were used. For these reasons, data for each table or figure were collected together as an independent experiment. Therefore, values may be more easily reproduced when all conditions including rod and rod history are similar.

Rod Uniformity

Although RF are not constant in TLC-FID as opposed to RF from GLC, another concern is whether this method generates reproducible data. Previously, a standard lipid mixture containing cholesterol, tristearin, stearic acid and cholesteryl palmitate was developed on chromarods and analyzed 30 times to determine the reproducibility of the TLC-FID technique (3). While the percentage of cholesteryl palmitate remained similar between selected rods, that of tristearate varied as much as 2-fold (12.5 vs 22.4). In addition, the proportion of cholesterol was found to be similar in some rods (26.8 vs 26.0), while that of cholesteryl palmitate varied as much as 50% (14.6 vs 20.8) (3). We suspected that these discrepancies could be due to the nonuniformity of chromarods. If the rods were not isotropic along their length, response would be a function of the position of the sample. Therefore, we examined the uniformity in the RF of tripalmitin at various positions on chromarods. For this purpose, 5 μg of tripalmitin was spotted every 1.5 cm along several chromarods and analyzed by FID. Along some rods, peak areas differed by as much as 35%. This nonuniformity of chromarods could partially explain some of the previously reported variations in the areas detected using the TLC-FID system. While not all rods demonstrated this variability, every position on a rod cannot be analyzed. Instead, this represents the presence of heterogenieties which influence FID response in the commerically available chromarods.

Optical glass chambers are recommended to visualize the solvent front during sample development (10). However, we as well as others have observed that it is often difficult to visualize the solvent front even through these chambers. Therefore, some investigators recommend that rods be developed for constant time periods rather than relying on the height of the solvent front (3,4). We have also observed that the rate of mobility of solvents on one chromarod is often different from that on another. As a result of these variables, lipid samples may not chromatograph in an identical manner when analyzed consecutively. Therefore, the response of the FID to a sample may not be consistent since: (a) the distance traveled by a particular lipid could vary after repeated rod development, and (b) RF are influenced by sample position.

Variation in Response Factors with Scan Speed

We have demonstrated that RF are influenced by the quantity, composition, and position of the lipid on the rod. In addition to these variables, others have also reported large variations in response with scan speed (4, 11). Bradley et al. demonstrated variations in RF of phosphatidylcholine, free fatty acids, TG, cholesterol and cholesteryl esters with scan speed (4). However, their study showed that only the RF for phosphatidylcholine varied greatly. They found the RF for phosphatidylcholine decreased with scan speed (4). In contrast, other investigators have reported that the detector response from octadecanoic acid increased with scan speed (11). Thus, the manner in which RF is influenced by scan speed appears to be dependent upon the material.

To understand in more detail how scan speed influences RF of other lipids, we measured these effects using methylpentadecanoate, cholesterol and phosphatidylcholine (Fig. 2). The RF of methylpentadecanoate increased with scan speed when analyzing two quantities within the normal working range of the instrument (Fig. 2). The increase in RF was less when larger amounts were analyzed (0.75 vs 1.20). The RF increased with scan speed when either 5 or 10 μg of cholesterol was analyzed. However, the increase was much greater with 5 μg (1.1) than with 10 μg (0.2). In contrast, the RF of phosphatidylcholine decreased with scan speed for 10 and 5 μg but was unchanged for 1 μg. In these cases, the RF decreased 0.8, 0.8 and increased 0.02, respectively (Fig. 2). Differ-

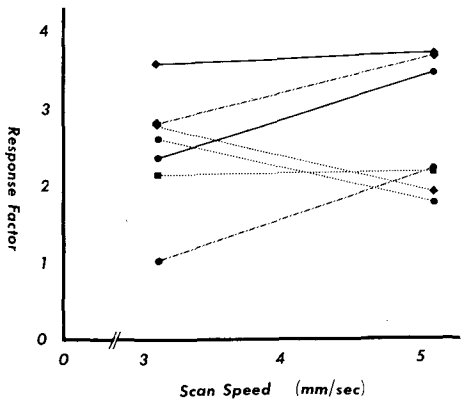

FIG. 2. The combined influence of sample quantity and scan speed on response factors. Either 1 μg (■), 5 μg (●) or 10 μg (♦) of methylpentadecanoate (—·—·), cholesterol (—) or phosphatidylcholine (-----) were spotted on a chromarod and analyzed undeveloped. Each data point represents the mean of 4-7 analyses. Scan speeds were 3.1 and 5.1 mm/sec.

ences between the effect of scan speed on RF of phosphatidylcholine reported by Bradley et al. (4) and those reported in Figure 2 could, therefore, be due to the differences in the amount of material analyzed.

Incomplete Pyrolysis

After 10 μg of phosphatidylcholine was passed through the hydrogen flame at a scan rate of 5.1 mm/sec, ca. 10% of the sample remained. But, when the same amount of phosphatidylcholine was analyzed at a scan rate of 3.1 mm/sec, all material was removed. Furthermore, when 5 μg of phosphatidylcholine was analyzed at the 5.1 mm/sec rate, ca. 10% of the sample remained while all material was removed at the slower (3.1 mm/sec) scan rate. After 1 μg of phosphatidylcholine was analyzed, no residual material was detected at either scan rate. For all 4 conditions in Figure 2, all methylpentadecanoate was liberated from the rod after analysis. However, a small amount (ca. 1%) of material remained after analyzing 10 μg of cholesterol at a scan rate of 5.1 mm/sec. No residual material was detected when slower scan rates or less cholesterol were analyzed. From these results, it appears that when the RF for phosphatidylcholine decreased in Figure 2 for 10 μg and 5 μg this was at least partially due to incomplete removal of the sample at fast scan rates. Furthermore, the smaller increase in RF with scan speed for 10 μg as opposed to 5 μg of cholesterol (Fig. 2) may be at least partially due to this phenomenon.

The slowest scan speed should, therefore, be ideal for lipid quantitation to insure complete pyrolysis of a lipid mixture containing phospholipids. However, when slow scan speeds are used, rod life is reduced (3-5,7,10,11), the coefficient of variation in RF is elevated (4,11), and sensitivity is reduced for several lipid classes (Fig. 2) (11). The effective life of a chromarod is only 10–25 scans (3,7) although some attempts have been made to increase rod life for special application to more than 50 analyses (12). Since slow scan speeds reduce rod life, the use of such speeds would diminish the utility of TLC-FID.

Premature Loss of Sample

One explanation for the increase in RF with the quantity of some materials on a rod is it evaporates before reaching the FID (7). This hypothesis, for example, could explain the increase in the RF of methylpentadecanoate (Fig. 2) or octadecanoic acid (11) with scan speed. To test this, we scanned some rods, each containing several spots of methylpentadecanoate. Each of these had been burned for 15 sec adjacent to the first sample (arrow, Fig. 3). Although only 1 μg was spotted in each position, no significant loss of material was observed. Therefore, it is unlikely that samples are lost by vaporization immediately before detection in the FID.

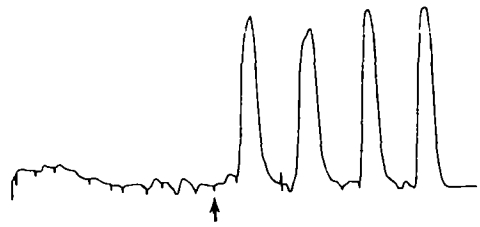

FIG. 3. Absence of premature loss of lipid before detection. Methylpentadecanoate (1 μg) was spotted at 4 positions 1.5 cm apart on a chromarod. The rod was burned for 15 sec at one position near the first of the spots as indicated by an arrow. The figure shows a chart recording from the subsequent scan of the rod without interruption. No premature loss of material was observed.

An Alternate Explanation

Since material is not lost prematurely as the rod scans through the FID, the dependence of RF on sample quantity is probably due to some variability in products formed by reactions which take place between the sample and the top of the collector. When the scan rate and gas flow rates are fixed, there is a fixed amount of heat available to each point on a chromarod. However, different types of quantities of substances may require different amounts of heat for optimal ionization (and optimal response). To determine whether the amount of heat influences FID response, we disaligned several rods, each containing a series of identical samples at one end relative to the flame. Each rod was spotted in 7 equally spaced positions with methylpentadecanoate (1 μg) and one end was displaced a few millimeters (< 2) from the normal scanning position. While the pyrolysis product from all the samples were easily within the range of the FID (detector id = 13 mm), the samples furthest from the normal scanning position gave the lowest response (Fig. 4). No material was detected in a subsequent scan of the same rod using normal alignment (Fig. 4). Therefore, when less heat is available to methylpentadecanoate, the RF is reduced even though all the material is removed from the rod. This shows that the response of the FID is, in some

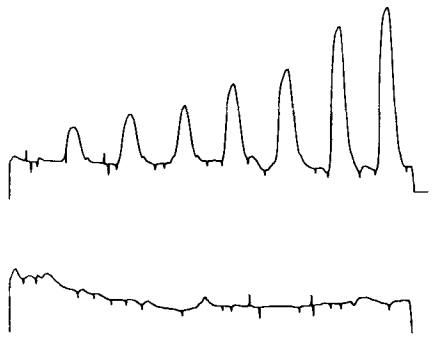

FIG. 4. The influence of rod alignment on response factor. A chromarod was spotted in 7 equally spaced positions with 1 μg of methylpentadecanoate at each location. One end of the rod (left) was displaced a few millimeters so that it was just outside the groove in which it normally rests. The rod was scanned and the response was recorded (top). On a subsequent scan, the rod was returned to its normal position and no residual material was detected (bottom).

cases, strongly dependent upon the amount of heat available to the sample. Such variations in response may be due, e.g., to alterations in the ratio of vaporized to pyrolized material.

DISCUSSION

TLC-FID is a rapid and simple method for separation and analysis of lipid samples. Small quantities of lipid mixtures (1–10 μg) are adequate for analysis. Each sample can be analyzed in less than 30 min and the components of up to 10 lipid mixtures can be analyzed at one time. This method has also been adapted to analysis of isomeric or unsaturated lipids by impregnating chromarods with boric acid (13, 14) or silver nitrate (14–16), respectively. These advantages make the TLC-FID a good tool for screening samples to determine qualitative differences (1,17). However, in the present study, we have demonstrated that TLC-FID cannot be easily adapted to quantitating the components of many complex lipid mixtures.

In the present study, we have demonstrated that there is a strong dependence of RF on several variables in TLC-FID. These include the type and amount of a compound and its position on a chromarod during detection. The RF varies with the position of a compound due to the nonuniformity of rods. This could be overcome by using rods which are better designed for sample quantitation. Furthermore, the strong dependence of RF on the type as well as the amount of material prevents the use of this method for sample quantitation without developing complex mathematical relationships between RF and these variables.

When FID is used in GLC, many of the compounds that have been analyzed have response factors which remain constant over a wide range of sample quantities (18). However, we have demonstrated that this simple relationship does not hold for the TLC-FID method. There are several differences between GLC and TLC-FID. In a lipid mixture, each component has a different melting and boiling temperature. Some compounds (such as phospholipids) have several phase transition temperatures in the range between room temperature (25 C) and the temperature of the hydrogen flame (ca. 3000 C). Before a sample is pyrolized, it goes through each of the phase transitions that exist between the starting temperature and the flame temperature. In GLC, these transitions are minimized or eliminated by starting at a high enough temperature for the sample to be in the gas phase. In TLC-FID, the samples are pyrolized from room temperature, leaving a number of phase transitions in some samples to be surpassed before pyrolysis. The complexity of the kinetics of the ionization of each of these lipid compounds in a hydrogen flame using TLC-FID can be significantly different from one another. Since samples have a relatively short time to be pyrolized at standard scan rates (3–5 mm/sec), it is not surprising that some compounds may have RF which vary with sample quantity, scan speed, etc. Therefore, each compound should have a unique relationship between RF and the relative amount of heat available to each sample.

If the response of the FID is dependent upon sample quantity, then it also depends on sample distribution. Generally, samples that are developed along most of the rod length will have a broader sample distribution than those which remain near the origin. The magnitude of the influence of sample distribution on RF is unknown. However, since the maximum distance that a sample can chromatograph along a rod is small (< 10 cm), variations in RF could be much smaller from sample distribution after rod development than from sample quantity.

In this study, some samples were developed before analysis while others were analyzed undeveloped. These are clearly identified in the figure and table legends. Development is likely to influence RF values due to the dependence of RF on sample distribution. However, rod development is not likely to influence the manner in which RF varies with sample concentration, scan speed or rod alignment since both developed and undeveloped samples behave similarly (Fig. 1). Therefore, our conclusions

about the usefulness of TLC-FID for sample quantitation is valid both for developed or undeveloped samples.

One aspect that has recently been emphasized with regards to the TLC-FID and lipid quantitation is that the relative accuracy of this technique is much greater than that of other methods (7). Our experience has been that TLC-FID is not nearly as quantitative as we expected. Instead, the RF of each lipid appears to behave differently with many of the variables that we tested. Therefore, we need a much better understanding of the manner in which all tested compounds behave under analysis conditions before quantitation can become feasible.

ACKNOWLEDGMENTS

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Electron Spin Resonance Spectra of the Chromanoxyl Radicals Derived from Tocopherols (Vitamin E) and Their Related Compounds[1]

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ABSTRACT

The well resolved electron spin resonance (ESR) of the tocopheroxyl and chromanoxyl radicals derived from α-, β-, γ- and δ-tocopherols (vitamin E), 5,7-dimethyltocol, tocol and their model compounds in degassed toluene by treatment with 2,2-diphenyl-1-picrylhydrazyl were recorded. Their hyperfine coupling constants were determined and assigned using spectrum simulation. Their g-factors were also measured. On the basis of these parameters, the α-tocopheroxyl radical is similar to the 2,2,5,7,8-pentamethylchroman-6-oxyl, 5,7-dimethyltocoxyl and 2,2,5,7-tetramethylchroman-6-oxyl radicals. This suggests that the presence of methyl groups at C-5 and C-7 in tocopherols and chroman-6-ols is of great importance to their antioxidant action. The ESR parameters obtained here are very useful for the identification and quantification of a variety of tocopheroxyl radicals.
Lipids 18:81-86, 1983.

INTRODUCTION

Vitamin E (mainly α-tocopherol) has been used as one of the safest antioxidants for the prevention of lipid deterioration in foodstuffs (1) and further, suggested to play an important role in the inhibition of lipid peroxidation in vivo (2,3). It is known that the effectiveness of various tocopherols in vitro depends on the reactivity of their phenolic groups (4), and that the first step in the tocopherol-induced chain-breaking process of autoxidation is the formation of tocopheroxyl radicals by abstraction of the phenolic hydrogens (5). So it requires the knowledge of the properties of tocopheroxyl radicals to understand the action of vitamin E.

Although the ESR spectra of tocopheroxyl radicals have been studied (6,7), their resolution was rather poor. Previously we recorded the considerably resolved ESR spectra of the radicals derived from α-tocopherol and its model compound with superoxide ion (8). However, we had difficulty in analyzing them completely because of their insufficient resolution. Recently, Mukai and the others observed the well resolved ESR spectrum of the α-tocopheroxyl radical generated by lead dioxide oxidation of α-tocopherol and determined its hyperfine coupling constants (9).

Now we wish to report the ESR spectra of the tocopheroxyl and chromanoxyl radicals formed by hydrogren abstraction of α-, β-, γ- and δ-tocopherols, 5,7-dimethyltocol, tocol and their model compounds with DPPH, and their hyperfine coupling constants and g-factors.

MATERIALS AND METHODS

Materials

d-α-, d-γ- and d-δ-Tocopherols were obtained from Eisai Research Laboratories, Tsukuba, Japan. dl-β-Tocopherol, dl-5,7-dimethyltocol, dl-tocol, and 2,2,5,7,8-pentamethyl-, 2,2,5,7-tetramethyl-, 2,2,5,8-tetramethyl-, 2,2,7,8-tetramethyl- and 2,2-dimethylchroman-6-ols were synthesized in our laboratory as described previously (10). Potassium nitrosodisulfonate, so-called Fremy's salt, was prepared by a known method (11). Toluene (Dotite Spectrosol) was purchased from Dojin Chemical Laboratory, Kumamoto, Japan, and DPPH from Wako Pure Chemical Industries, Osaka, Japan.

Procedure

Under nitrogen, 1 ml of a 2 mM tocopherol or chromanol solution in toluene was placed in a 5-mm quartz sample tube equipped with both an adjustable Teflon plunge and a side arm containing 1 μmol DPPH; for δ-tocopherol, tocol and 2,2-dimethylchroman-6-ol, however, their 12 mM solutions were used. The toluene solution was degassed under 10^{-2} torr by a freezing-thawing procedure. Immediately after mixing the DPPH in the degassed solution under the reduced pressure, we set the sample tube in an ESR cavity.

[1] TMIG-I No. 43

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Abbreviations: ESR = electron spin resonance; DPPH = 2,2-diphenyl-1-picrylhydrazyl.

Spectroscopic Measurements

ESR spectra were recorded on a Varian E-109 spectrometer (X-band) with an E-233 large access cylindrical cavity. All spectra were taken at room temperature under the following settings: modulation frequency 100 kHz, microwave power 10 mW, modulation amplitude 0.0125 or 0.025 mT, time constant 0.128 sec, scan time 8 min. Magnet fields were calibrated by the use of a Fremy's salt standard (a_N = 1.3091 ± 0.0004 mT, g = 2.0054) (12, 13).

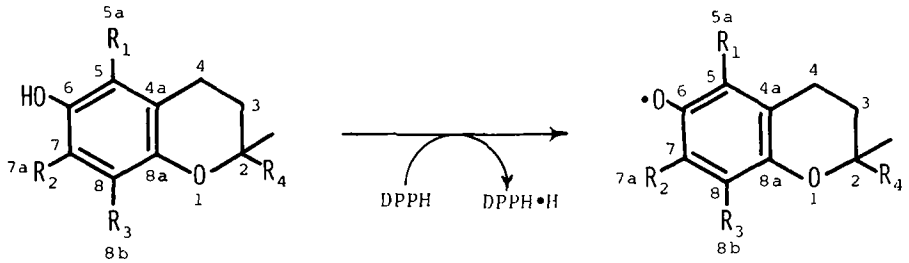
Calculations

Spectrum simulations were carried out employing a Varian E-900 data aquisition system. For computer-simulated spectra, line shape is Lorentzian and line widths are 0.025 (the γ-tocopheroxyl and model radicals), 0.028 (the α-tocopheroxyl and model radicals), 0.030 (the δ-tocopheroxyl radical) and 0.035 mT (the other radicals).

RESULTS AND DISCUSSION

We observed the well resolved ESR spectra of the radicals derived from tocopherols and their related compounds in degassed toluene by treatment with DPPH. The structures of tocopherols, their related compounds and the tocopheroxyl and chromanoxyl radicals together with the numbering of their atoms are given in Figure 1.

Figure 2 shows the ESR spectra of the α-tocopheroxyl (a) and model (b) radicals and the computer-simulated spectrum (c). The α-tocopheroxyl and model radicals gave the same spectrum, having four different hyperfine coupling constants: a_H's = 0.607 (5a-CH_3),



Parent compounds	Radicals	R_1	R_2	R_3	R_4
α-Tocopherol	α-Tocopheroxyl	CH_3	CH_3	CH_3	$C_{16}H_{33}$
α-Toc. model, 2,2,5,7,8-pentamethylchroman-6-ol	2,2,5,7,8-Pentamethylchroman-6-oxyl	CH_3	CH_3	CH_3	CH_3
5,7-Dimethyltocol	5,7-Dimethyltocoxyl	CH_3	CH_3	H	$C_{16}H_{33}$
2,2,5,7-Tetramethylchroman-6-ol	2,2,5,7-Tetramethylchroman-6-oxyl	CH_3	CH_3	H	CH_3
β-Tocopherol	β-Tocopheroxyl	CH_3	H	CH_3	$C_{16}H_{33}$
β-Toc. model, 2,2,5,8-tetramethylchroman-6-ol	2,2,5,8-Tetramethylchroman-6-oxyl	CH_3	H	CH_3	CH_3
γ-Tocopherol	γ-Tocopheroxyl	H	CH_3	CH_3	$C_{16}H_{33}$
γ-Toc. model, 2,2,7,8-tetramethylchroman-6-ol	2,2,7,8-Tetramethylchroman-6-oxyl	H	CH_3	CH_3	CH_3
δ-Tocopherol	δ-Tocopheroxyl	H	H	CH_3	$C_{16}H_{33}$
Tocol	Tocoxyl	H	H	H	$C_{16}H_{33}$
2,2-Dimethylchroman-6-ol	2,2-Dimethylchroman-6-oxyl	H	H	H	CH_3

FIG. 1. The structures of tocopherols, the related compounds and the tocopheroxyl and related radicals together with the numbering of their atoms.

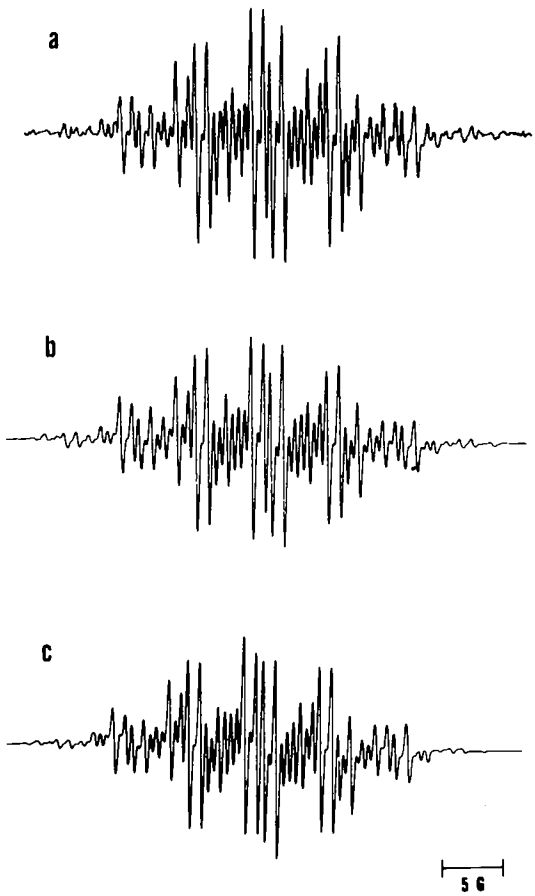


FIG. 2. The ESR spectra of the α-tocopheroxyl (a) and 2,2,5,7,8-pentamethylchroman-6-oxyl (b) radicals and the computer-simulated spectrum (c).

0.455 (7a-CH_3), 0.098 (8b-CH_3) and 0.152 mT (4-CH_2). The assignment of the hyperfine coupling constants has been confirmed by the analysis of spectra of the 5a-, 7a- and 8b-CD_3-isotopomers of the model radical (14). The values of the hyperfine coupling constants were very close to those reported by Mukai and the others (9).

Figures 3, 4, 5 and 6 show the ESR spectra (a) of the 5,7-dimethyltocoxyl, β- and γ-tocopheroxyl and tocoxyl radicals, respectively, with the spectra (b) of their model radicals and the computer-simulated spectra (c). There is no difference between the spectra of each of the 5,7-dimethyltocoxyl, β- and γ-tocopheroxyl and tocoxyl radicals and the corresponding model radical. The spectra of the 5,7-dimethyltocoxyl, β- and γ-tocopheroxyl radicals were reconstructed provided that an unpaired electron interacted with the four groups of inequivalent protons, each of which had one, two, three or three equivalent protons. The hyperfine coupling constants of the 5,7-dimethyltocoxyl and model radicals are a_H's = 0.591 (5a-CH_3), 0.464 (7a-CH_3), 0.090 (8b-H) and 0.140 mT (4-CH_2), of the β-tocopheroxyl and model radicals a_H's = 0.640 (5a-CH_3), 0.460 (7a-H), 0.091 (8b-CH_3) and 0.174 mT (4-CH_2), and of the γ-tocopheroxyl and model radicals a_H's = 0.603 (5a-H), 0.482 (7a-CH_3), 0.112 (8b-CH_3) and 0.130 mT (4-CH_2); they were assigned on the basis of the hyperfine coupling constants of the α-tocopheroxyl radical. The spectra of the tocoxyl and model radicals were also simulated by the use of the following hyperfine coupling constants: a_H's = 0.592 (5a-H), 0.513 (7a-H), 0.079 (8b-H) and 0.138 mT (4-CH_2).

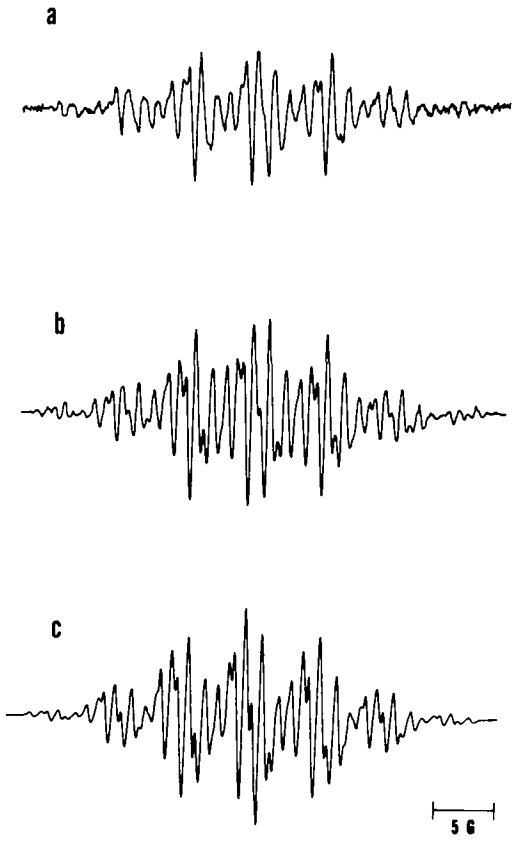


FIG. 3. The ESR spectra of the 5,7-dimethyltocoxyl (a) and 2,2,5,7-tetramethylchroman-6-oxyl (b) radicals and the computer-simulated spectrum (c).

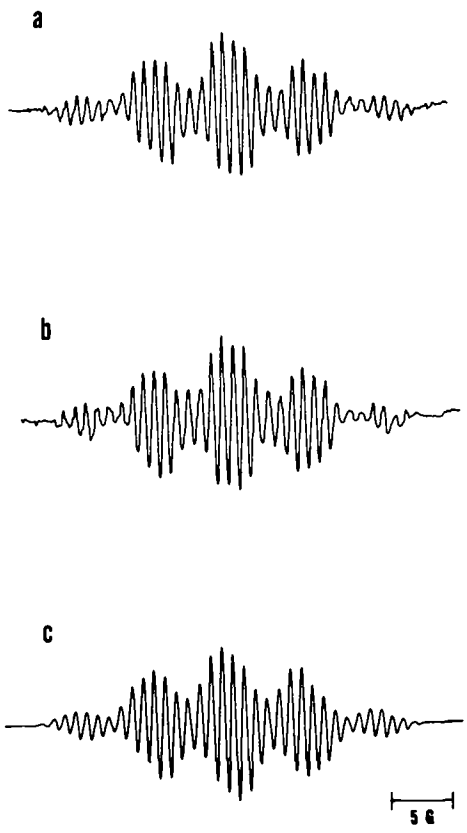


FIG. 4. The ESR spectra of the β-tocopheroxyl (a) and 2,2,5,8-tetramethylchroman-6-oxyl (b) radicals and the computer-simulated spectrum (c).

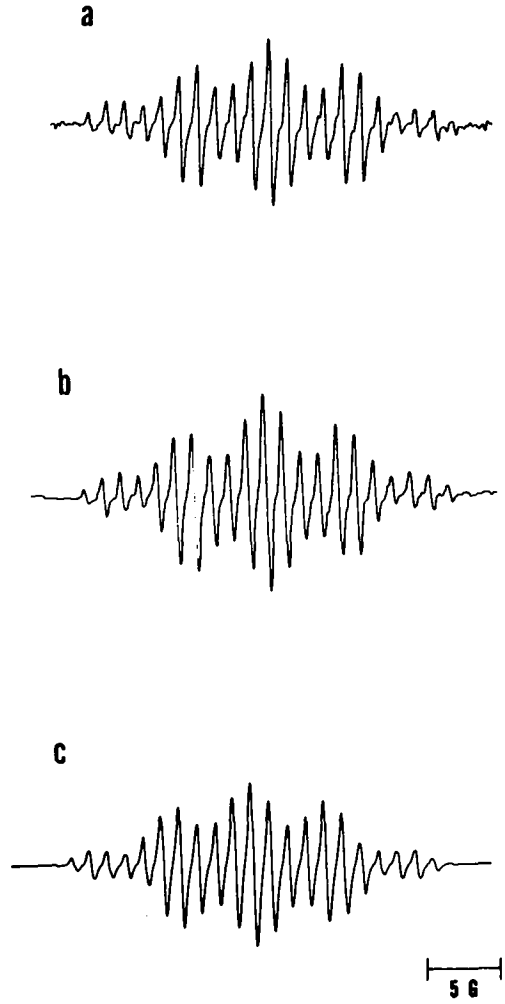


FIG. 5. The ESR spectra of the γ-tocopheroxyl (a) and 2,2,7,8-tetramethylchroman-6-oxyl (b) radicals and the computer-simulated spectrum (c).

The ESR and computer-simulated spectra of the δ-tocopheroxyl radical are shown in Figure 7. The hyperfine coupling constants of the δ-tocopheroxyl radical were estimated to be a_H's = 0.619 (5a-H), 0.482 (7a-H), 0.103 (8b-CH_3) and 0.145 mT (4-CH_2).

All the hyperfine coupling constants of the tocopheroxyl and chromanoxyl radicals are listed in Table 1. The magnitude of hyperfine coupling constants due to aromatic protons in the radicals is similar to that due to the protons of a methyl group substituted for the aromatic proton. The magnitude of the hyperfine coupling constants due to the aromatic and methyl protons at the α- and β-positions of C-5, C-7 and C-8 in the radicals decreases in that order of the aromatic carbon atoms; theoretically, it is proportional to the spin density at the aromatic carbon atoms (15). The values of the hyperfine coupling constants due to the methylene protons at C-4 in the radicals drop characteristically into the narrow range from 0.130 to 0.174 mT. For the γ-tocopheroxyl and model radicals, the hyperfine coupling constants (both, 0.112 mT) due to the protons of a methyl group at C-8 may be somewhat overestimated and those (both, 0.130 mT) due to the methylene protons at C-4 somewhat underestimated, because their values appear to be deviated from the values of the corresponding hyperfine coupling constants of the other radicals.

As given in Table 1, the g-factors of the radicals were measured on the basis of the g-factor of Fremy's salt. The g-factors of the tocopheroxyl radicals agree closely with those

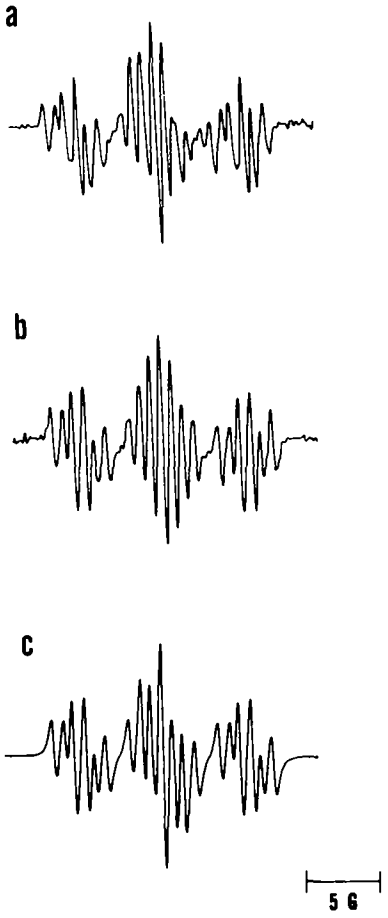


FIG. 6. The ESR spectra of the tocoxyl (a) and 2,2-dimethylchroman-6-oxyl (b) radicals and the computer-simulated spectrum (c).

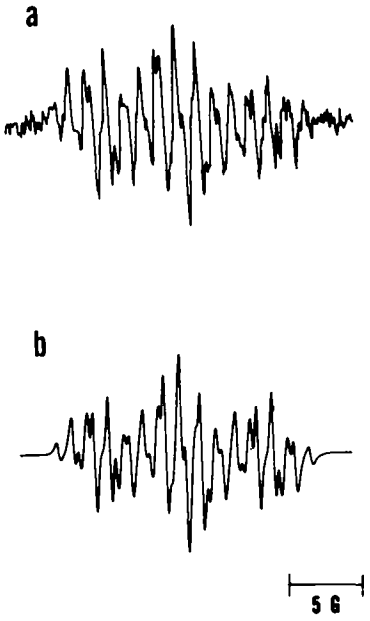


FIG. 7. The ESR spectrum of the δ-tocopheroxyl radical (a) and the computer-simulated spectrum (b).

TABLE 1

ESR Parameters of the Radicals Derived from Tocopherols and their Related Compounds

Radicals	Hyperfine coupling constants (mT)				g-Factors
	R_1[a]	R_2[a]	R_3[a]	4-CH_2	
α-Tocopheroxyl and 2,2,5,7,8-pentamethylchroman-6-oxyl	0.607	0.455	0.098	0.152	2.0046
5,7-Dimethyltocoxyl and 2,2,5,7-tetramethylchroman-6-oxyl	0.591	0.464	0.090	0.140	2.0046
β-Tocopheroxyl and 2,2,5,8-tetramethylchroman-6-oxyl	0.640	0.460	0.091	0.174	2.0047
γ-Tocopheroxyl and 2,2,7,8-tetramethylchroman-6-oxyl	0.603	0.482	0.112	0.130	2.0047
δ-Tocopheroxyl	0.619	0.482	0.103	0.145	2.0049
Tocoxyl and 2,2-dimethylchroman-6-oxyl	0.592	0.513	0.079	0.138	2.0049

[a]R_1, R_2 and R_3 = H or CH_3. See Figure 1.

of the corresponding model radicals. The magnitude of the g-factors is in the following order: the α-tocopheroxyl and 2,2,5,7,8-pentamethylchroman-6-oxyl radicals = the 5,7-dimethyltocoxyl and 2,2,5,7-tetramethylchroman-6-oxyl radicals > the β-tocopheroxyl and 2,2,5,8-tetramethylchroman-6-oxyl radicals = the γ-tocopheroxyl and 2,2,7,8-tetramethylchroman-6-oxyl radicals > the δ-tocopheroxyl radical = the tocoxyl and 2,2-dimethylchroman-6-oxyl radicals.

In regard to the magnitude of hyperfine coupling constants and g-factors, the α-tocopheroxyl radical is very similar to the 2,2,5,7,8-pentamethylchroman-6-oxyl, 5,7-dimethyltocoxyl and 2,2,5,7-tetramethylchroman-6-oxyl radicals. We have already found that the radical scavenging ability of α-tocopherol and 5,7-dimethyltocol is much higher than that of the other tocopherols (16). These findings make us attach importance to the presence of methyl groups at C-5 and C-7 in tocopherols and chroman-6-ols functioning as antioxidants.

On the basis of the ESR parameters obtained here, a variety of tocopheroxyl radicals can be identified and quantified easily. This is very useful for the analysis of the antioxidant action of tocopherols in various systems.

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METHODS

Reversed-Phase Thin Layer Chromatography of Some Common Sterols

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ABSTRACT

The chromatographic mobilities of 17 sterols and squalene on reversed-phase thin layer plates with four nonaqueous solvent systems is described. A degree of separation adequate to identify several of the sterols was obtained. It was possible to separate the pairs: cholestanol, epicholestanol; coprostanol, epicoprostanol; 5β-cholestan-3α and 3β-ol and lanosterol, dihydrolanosterol.
Lipids 18:87-89, 1983.

Investigations of sterol metabolism in the oomycetes necessitated a rapid screening method to confirm the metabolism of sterols and to indicate whether a homogeneous product existed prior to analysis by gas liquid chromatography (GLC). A simple method was also needed that would demonstrate successful enzymatic conversion of radiolabeled precursors. Although many excellent liquid chromatographic procedures, recently reviewed (1), are available for the separation of sterols, they do not offer the option of simultaneous analysis of multiple samples inherent in thin layer chromatography (TLC). No satisfactory thin layer procedure was available for this purpose, because normal phase systems poorly separate individual sterols and reverse-phase systems. At least those previously described (2), require impregnation of various supports with stationary phases obviously unsuitable for contact with the films used for autoradiography. The latter systems, although yielding reasonable separations, were thus considered unsatisfactory by us for routine, large-scale screening studies. This suggested the examination of the chromatographic mobility of a number of sterols on octadecyl bonded-phase thin layer plates. The results of this investigation are reported in this communication.

EXPERIMENTAL

Bonded-phase thin layer plates ($KC_{18}F$, 50 X 200 mm, 0.2 mm thickness) were purchased from Whatman, Inc., Clifton, NJ. The plates were prewashed in $CHCl_3$ followed by hexane/ethyl acetate (9:1) which removed an objectionable iodine-intensified yellow color that interfered with visualization of the sterols. Reagent grade alcohols were purchased from Fisher Scientific Co., Raleigh, NC. Other solvents were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI—all were used without further purification. Brassicasterol, campesterol, lophenol, 5β-cholestan-3β-ol, 5β-cholestan-3α-ol, 5-cholesten-3α-ol, 5α-cholestan-3α-ol, 5α-cholestan-3β-ol, 7-cholesten-3β-ol, 7-ketocholesterol, and fucosterol were purchased from Steraloids, Inc., Wilton, NH. Desmosterol and lanosterol were obtained from Applied Science Division, State College, PA. Ergosterol and squalene were obtained from Sigma Chemical Co., St. Louis, MO. Sitosterol, stigmasterol and 5-cholestan-3β-ol were obtained from Aldrich Chemical Co., Milwaukee, WI. Cycloartenol was prepared from jack-fruit by the method of Nath (3). GLC on 3% SE-30 and 1% QF-1 (4) indicated that all compounds were >99.9% pure except for lanosterol, which contained 35% dihydrolanosterol, and sitosterol, which contained 40% campesterol.

All compounds were dissolved in hexane (1 mg/ml) and 20 μl of each solution were applied to the plates 2 cm from the edge. Absolute and 95% aqueous methanol, ethanol, *n*-propanol and *n*-butanol, and 4 different mixed solvent systems (Table 1) were tested. Development was typically for 17 cm, which required about 90 min. All runs were performed in glass tanks lined with Whatman No. 3 filter paper at room temperature. Three different agents were used to visualize the sterols: (1) 10% phosphomolybdic acid in 95% ethanol followed by heat-

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TABLE 1

Reversed-Phase Thin Layer Chromatography of Some Sterols

Compound	R_{CH} Value[a]			
	I	II	III	IV
5α-Cholestan-3β-ol	1.00	1.00	0.83	0.81
5α-Cholestan-3α-ol	1.34	1.27	1.11	1.12
5β-Cholestan-3β-ol	1.30	1.13	1.07	1.06
5β-Cholestan-3α-ol	1.28	1.12	1.27	1.25
5-Cholesten-3β-ol	1.00	1.00	1.00[b] (1.17)	1.00[b] (1.09)
5-Cholesten-3α-ol	–	1.25	1.11	1.12
7-Cholesten-3β-ol	0.91	1.05	0.83	0.93
7-Ketocholesterol	–	0.64	–	–
Lophenol	1.27	1.03	1.00	1.08
Campesterol	1.06	1.03	0.90	0.94
Brassicasterol	1.02	1.00	0.95	1.02
Sitosterol	1.07	1.04	0.90	0.94
Fucosterol	1.00[b] (0.30,0.90)	1.00	0.96	1.25
Stigmasterol	1.02	1.00	0.88	0.96
Lanosterol	1.50	1.20	1.11	1.12
Dihydrolanosterol	1.42	1.20	1.11	1.12
Cycloartenol	(1.16) 1.55[b]	1.20	(1.09) 1.25[b]	(1.25) 1.43[b]
Squalene	0.96	1.04	(0.80) 1.38[b,c]	6.88, 1.35[c]

I: Hexane/ethyl acetate (9:1, v/v).
II: Petroleum ether/diethyl ether/acetic acid (90:10:1, v/v/v).
III: Acetonitrile/chloroform (40:35, v/v) (4).
IV: Acetonitrile/chloroform/ethyl acetate (55:23:15) (4).

[a]± 1%.

[b]Major spot.

[c]Considerable trailing.

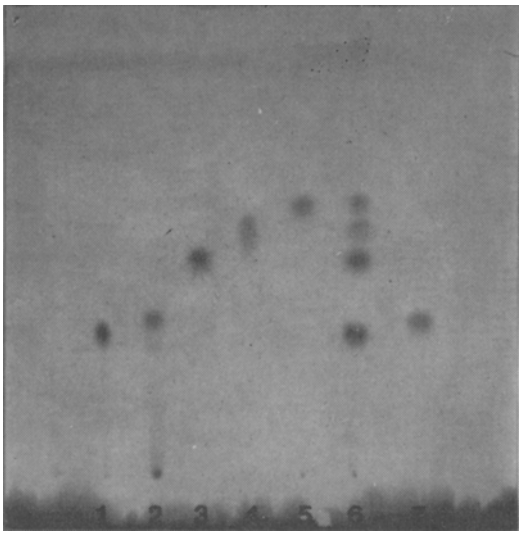

FIG. 1. Chromatogram of sterols run in solvent I (Table 1). From left to right: (1) 7-cholesten-3β-ol, (2) fucosterol, (3) lophenol, (4) 5α-cholestan-3α-ol, (5) lanosterol, (6) mixture of 1,3,4,5, (7) cholesterol.

ing at 100 C (5), (2) iodine vapor, and (3) 10% sulphuric acid in 50% MeOH followed by heating at 100 C (6).

RESULTS AND DISCUSSION

All components remained at the origin with the alcoholic solvent systems. The nonaqueous solvent systems given in Table 1 yielded adequate separations and demonstrate the utility of reversed-phase TLC for sterol separations.

Solvent I was quite effective in separating the C30 sterols cycloartenol and lanosterol from the C27 sterols such as cholesterol. The breakdown products of ergosterol were clearly shown with this system and, surprisingly, fucosterol was also shown to be impure. The impurities of the latter, possibly epimeric sterols, were not detectable by GLC. This system may thus represent a valuable clean-up step prior to GLC which sometimes separates sterol epimers with difficulty (7) or not at all (4).

Solvent II was effective in separating 7-ketocholesterol, a very common oxidation product of cholesterol (8). This system may prove valuable for the rapid verification of the purity

of commercial radiolabeled cholesterol preparations prior to their use.

Solvent III was preferred for separating the pairs cholestanol, epicholestanol; coprostanol, epicoprostanol; 5β-cholestan-3α-ol and 3β-ol. This solvent also separated lanosterol-dihydrolanosterol and revealed a small, unidentified impurity present in cholesterol. Squalene showed up as two distinct spots with a series of faint spots of greater mobility. Solvent IV exhibited a slightly altered selectivity from solvent III.

We have preferred to use solvents I and III for demonstrating conversion of C30 compounds to C27. It is our opinion that visualization agent 3, which does not char the background, is by far the most effective as it will react equally with nearly all substances, whereas 1 and 2 were less effective for visualizing the saturated sterols. Agent 3 permitted us to detect 0.1 μg sterol per spot.

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Analysis of Conjugated Bile Acids by High Performance Liquid Chromatography and Mass Spectrometry

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ABSTRACT

Because of the known advantages of coupling high performance liquid chromatography with mass spectrometry (HPLC-MS) in biological fluids, studies on the reversed-phase HPLC-MS system for direct analysis of conjugated bile acids in human bile samples are described. Ten samples of gallbladder bile of apparently healthy subjects were examined. The amounts of each tauro- and glycoconjugated bile acid as trifluoracetate were determined by mass fragmentography. Quantitation of at least 1 ng of each bile acid was possible.
Lipids 18:90-95, 1983.

INTRODUCTION

Bile acids are usually available in biological fluids in conjugation with glycine or taurine. The classical technique for the analysis of these conjugates involves vigorous alkaline or acidic hydrolysis, or enzymatic hydrolysis with clostridial cholylglycine hydrolase and derivatization for analysis by gas liquid chromatography (GLC).

However, each method fails to identify the nature of the conjugate. In recent years, a great deal of attention has been focused on high performance liquid chromatography (HPLC) methods for the separation and detection of conjugated and free bile acids (1-8).

Recently, we have proposed (8) a sensitive and accurate method for separation and quantification of bile acids by using an RP-18 column. Carboxylic acids (free and glycine-conjugated bile acids) as parabromophenacyl esters were determined by absorbance measurement at 254 nm; simultaneous quantitation of less than 20 ng of each bile acid was possible. Taurine- and glycine-conjugated bile acids were separated and quantified by ultraviolet (UV) absorbance at 200 nm. Simultaneous quantitation of at least 100 ng of each conjugated bile acid was possible. In the last case, acidification of the mobile phase (methanol/H_2O, 70:30, v/v) to pH 3.1 with phosphoric acid produced deleterious effects on the life and performance of the bonded reversed-phase column used. Because the advantages of coupling HPLC with mass spectrometry (MS) in biological fluid analysis, a systematic examination of the mass spectra of conjugated bile acid is desirable.

This paper describes the detection and quantification of conjugated bile acids in gallbladder bile samples of 10 healthy subjects by a double system HPLC-MS.

Each conjugated bile acid was separated by reversed-phase (RP) HPLC, collected during the chromatographic run and derivatized for mass fragmentography (MF).

MATERIALS AND METHODS

Reagents and Chemicals

Acetonitrile lichrosolv, ethanol lichrosolv, diethyl ether, phosphoric acid, acetic acid and trifluoracetic anhydride were all from Merck (Darmstadt, W. Germany) and were used as received. The diazomethane was prepared by KOH saponification of N-nitrosomethylurea in ether according to Arndt (9).

Taurocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid, taurolithocholic acid, glycholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid and glycolithocholic acid were obtained, all as the sodium salt, from Calbiochem (Lucerne, Switzerland). Their purity was checked by thin layer chromatography (TLC) prior to use as described by Cass et al. (10). All were found to be 96-98% pure. ^{14}C-Taurocholic acid and ^{14}C-glycholic acid were from Amersham Radiochemical Centre (Amersham, England).

Samples

Ten subjects without any gallbladder damage

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Abbreviations: TUDCA = tauroursodeoxycholic acid; TCA = taurocholic acid; TCDCA = taurochenodeoxycholic acid; TDCA = taurodeoxycholic acid; TLCA = taurolithocholic acid; GCA = glycocholic acid; GCDCA = glycochenodeoxycholic acid; GDCA = glycodeoxycholic acid; GLCA = glycolithocholic acid; TBA = total bile acids.

or dyslipidemia were included in this study. They were all males and ranged in age from 30 to 44 years. Moreover, liver function tests in all were normal.

The bile samples were aspirated, directly, from the gallbladder by needle puncture during gastrectomy performed in 10 patients with uncomplicated duodenal peptic ulcer. No complication from this procedure was encountered postoperatively. This procedure is absolutely safe. However, the patients were previously informed about the aim of our research and were consenting.

Conjugated bile acids were extracted from bile according to Mingrone et al. (8) and the samples directly injected into the HPLC column.

High Performance Liquid Chromatography

Analyses were conducted using the Hewlett-Packard 1084 B liquid chromatograph equipped with a scanning spectrophotometer with a wavelength range from 190 to 540 nm. The liquid chromatograph includes an integrator giving areas and times for each peak in the chromatogram. A reversed-phase (RP-18) Spheri 5,5 μm, 25 cm x 4 mm, column Brownlee Labs (Santa Clara, CA) was used for analyzing all bile acids.

Chromatographic column and solvents were operated at 40 ± 1 C. The conditions were as follows: mobile phase starting with an isocratic elution for 20 min, 30% CH_3CN in water at pH 3.10 with H_3PO_4, then gradient elution to 80% CH_3CN in 120 min; detector: (UV) 200 nm; flow rate: 1 ml/min; sensitivity: au from 64.0 to 128.0 x 10^{-4}/cm (depending on the amount of injected substance); chart speed: 0.25 cm/min.

Radioactivity Count by Liquid Scintillation Spectrometer

Quantities of 0.01, 0.05, 0.1, 1 and 2 μCi of ^{14}C-GCA and ^{14}C-TCA, respectively, were added to 10 μl of gallbladder bile sample diluted to a volume of 1 ml with NaCl 0.9%. The recovery was evaluated through the various steps of extraction and derivatization of bile acids which were present in HPLC eluates before the introduction in MS via direct probe. Each determination was made twice. The radioactivity was counted with a liquid scintillation spectrometer (Packard-Tricarb model 3385). Quench correction was made utilizing the external standard method (ca. 96% efficiency).

Preparation of Samples for Mass Spectrometry

Methyl esters of standard glycoconjugated bile acids were prepared by adding diazomethane in ether to the solution of bile acids in ether/ethanol (5:1, v/v).

Trifluoracetate derivatives (11) of standard tauroconjugated and methylated glycoconjugated bile acids were prepared by suspending 1 mg of each bile acid in 0.2 ml of methylene dichloride, adding 0.2 ml of trifluoracetic anhydride, heating for 20-30 min at 30 C and evaporating under vacuum the excess of reagents.

In bile samples, each glyco- and tauroconjugated bile acid eluted during the chromatographic run was dried under vacuum and derivatized as described above. Bile acid quantification was performed by MF.

Mass Spectrometry

Instrument. Mass spectrometric analyses were performed by a low resolution mass spectrometer and mass spectral data system LKB Model 2091/2130. Ionization was obtained by electron impact.

Qualitative analysis. The mass spectra of each derivatized conjufated bile acid standard was recorded at 20 eV and at a source temperature of 250 C by direct inlet probe. The probe temperature was raised from 25 C to 250 C in ca. 30 min. A repetitive scan, from mass 1 to 800, with a scan time of 2 sec was used.

Quantitative analysis. For quantitative determination of each conjufated bile acid, a calibration curve was carried out using ethanolic solutions containing 1, 5, 10, 20, 50 and 100 ng/μl, respectively. For each solution, 1 μl was pipetted directly in the sample holder, vacuum evaporated, injected into the source and recorded by the computer.

RESULTS

Linearity, sensitivity and reproducibility of HPLC methodology strongly resembled those previously reported, utilizing a different mobile phase (8). The total recovery of ^{14}C-GCA and ^{14}C-TCA was ca. 90%.

Figure 1 shows the separation of a synthetic mixture of tauro- and glycoconjugated bile acids.

Figure 2 demonstrates the chromatogram of conjugated bile acids of one gallbladder bile sample.

Figures 3A and 3B show, respectively, the mass spectrum of trifluoracetate taurocholic acid and the mass spectrum of methyl ester-trifluoracetate glycocholic acid.

Table 1 shows the difference in abundance of fragment ions derived from individual trifluoracetate (TFA) derivatized tauroconjugated bile acids.

Table 2 provides similar data for methyl esters of TFA glyco-conjugates.

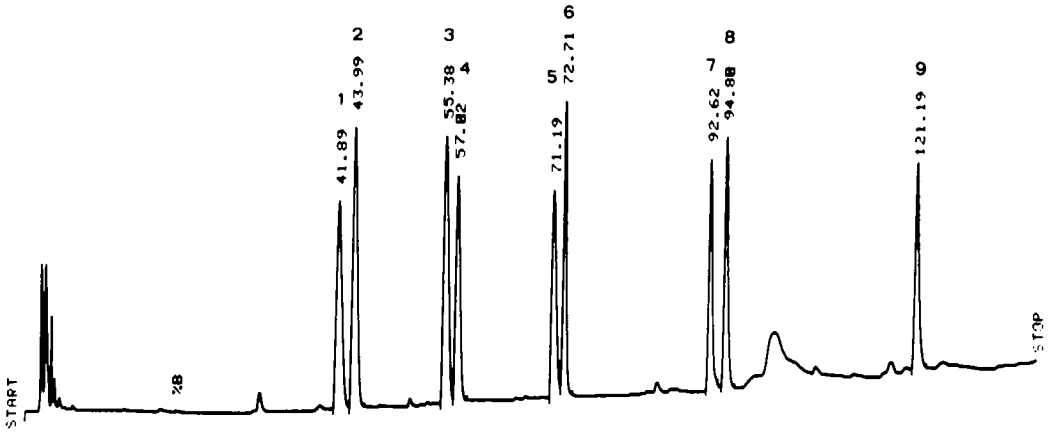
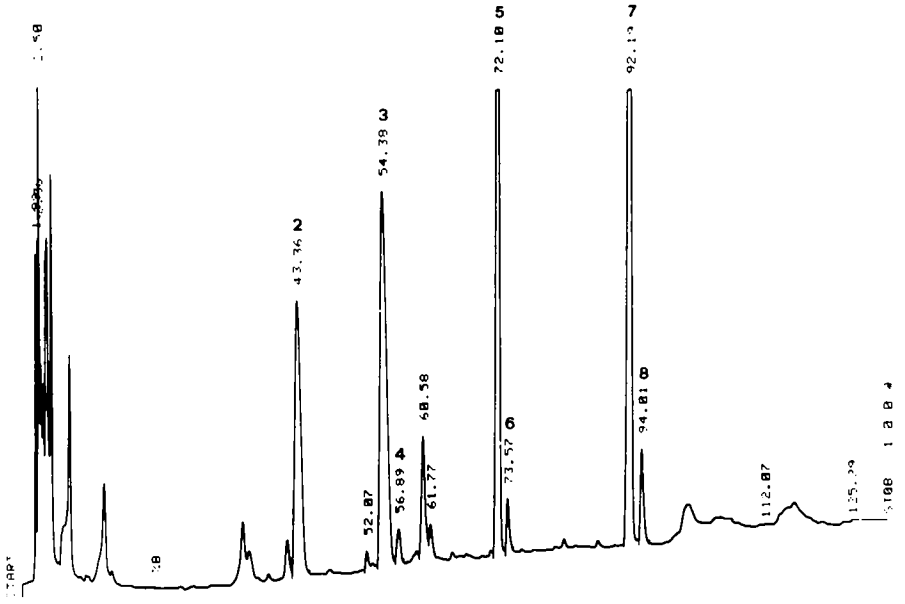


FIG. 1. Separation of a synthetic mixture of conjugated bile acids. Conditions are described under Methods. Peak identification: 1, TUDCA; 2, TCA; 3, TCDCA; 4, TDCA; 5, TLCA; 6, GCA; 7, GCDCA; 8, GDCA; 9, GLCA. The peak corresponding to RT of TUDCA was not further investigated by MS.



FIG, 2. Separation of conjugated bile acids from one sample of human gallbladder bile. For further details and peak identification, see Figure 1 and the text.

Table 3 shows for each derivatized conjugated bile acid the ions monitored for mass fragmentography. The calibration curve for each derivatized conjugate standard, monitored by different ions (see Table 3), showed a linear plot in the range of amounts examined.

In Table 4 are reported the amount (μg/ml) of individual bile acids found in 10 gallbladder bile samples of healthy subjects. Statistical analysis was evaluated by Student's t-test.

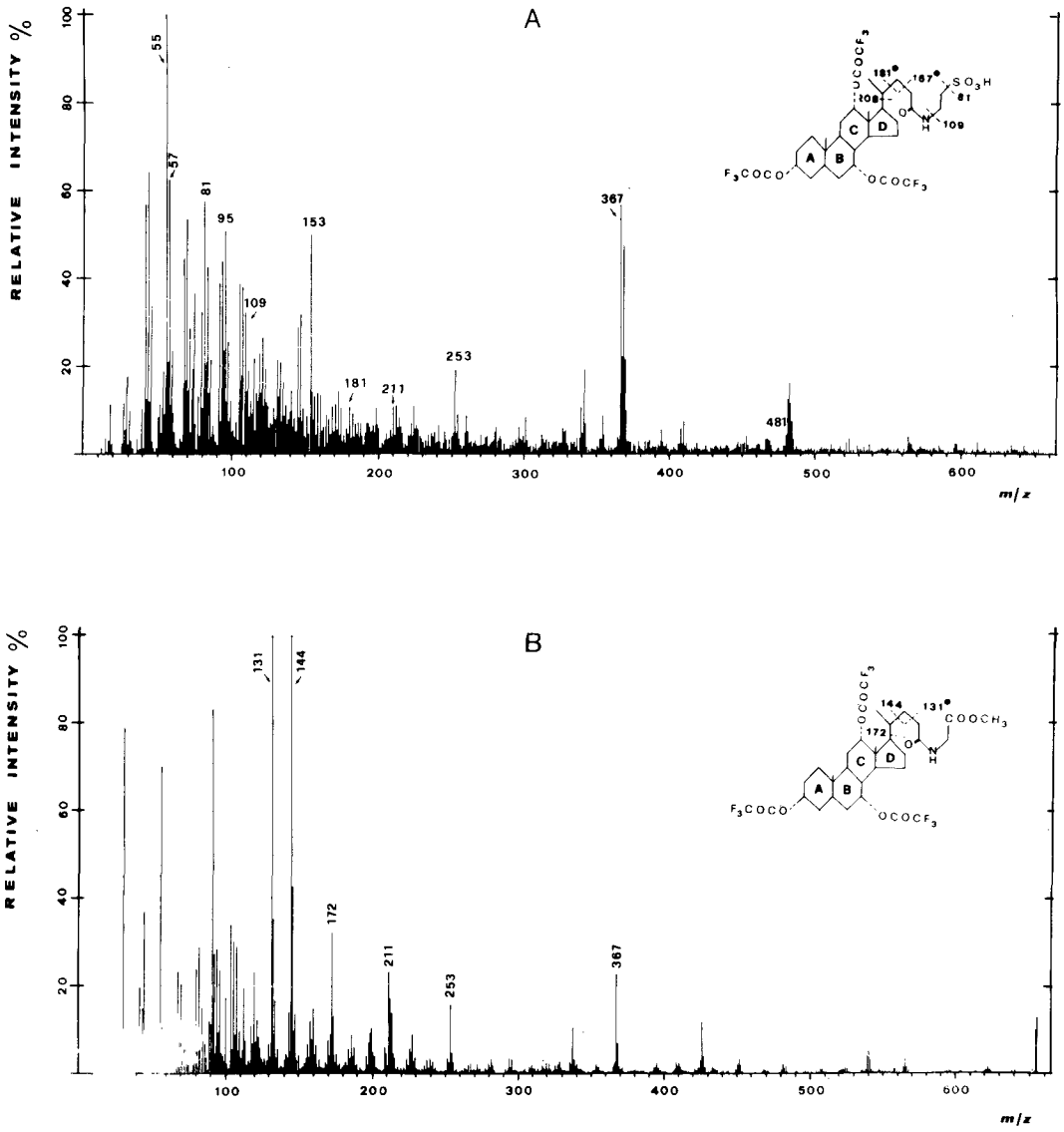


FIG. 3. Mass spectrum of (A) trifluoracetate-TCA and (B) methyl ester-trifluoracetate-GCA.

DISCUSSION

The use of a slow gradient elution with an acidic mobile phase did not appear to reduce the performance of the RP-18 column during the investigation, which took place over a period of about 6 months. Although HPLC analysis is highly sensitive and allows very good separation, statements concerning the quantitation of bile acids, which had similar retention time (RT) as standards, must be more conservative.

In fact, due to the presence of a large number of interfering substances in samples, many of the peaks assigned to known conjugated bile acids might contain more than one component. Therefore, definitive identification and quantitation of substances eluted in HPLC awaited completion of MS studies. In the gallbladder bile, no significant differences ($t<0.05$) were found between the values obtained by HPLC and MF (see Table 4).

TABLE 1

Fragment Ions and Relative Intensities of Individual Trifluoracetate Derivative Tauroconjugated Bile Acids

	TCA		TCDCA		TDCA	TLCA	
	m/z	ri	m/z	ri	ri	m/z	ri
	55	100	55	83.5	100	55	14.8
	57	62.6	57	100	28.7	57	12
	64	1.6	64	3.8	3.7	64	100
SO_3H	81	57.9	81	50.4	80.6	81	5.8
CH_2-SO_3H	95	50.9	95	42	61.6	95	25.9
CH_2-CH_2-SO_3H	109	32.3	109	21.2	38	109	3
NH-CH_2-CH_2-SO_3H	124	11.1	123	8.1	10.6	124	1.5
HCO-NH-CH_2-CH_2-SO_3H	153	50.1	153	37.7	60	153	1.3
CH_2 | COH-NH-CH_2-CH_2-SO_3H	167•	8.1	167	4.2	3.6	167	0.9
CH_3 | CH_2 | CO-NH-CH_2-CH_2-SO_3H	181•	10.8	181	5.4	7.2	181	1
CO-NH-CH_2-CH_2-SO_3H	208	3.9	208	5.8	3.2	208	0.8
M-(208+(114 X 3)+42	211	10.7	–	–	–	–	–
208 + 42	250	1.2	250	3	3.6	250	–
M-(208+(114 X 3))	253	19.4	–		–	–	–
M-(208+(114 X 2)+42)	325	3.1	214	24.6	22.6	–	–
M-(208+(114 X 2))	367	56.8	255	36.1	18.5	–	–
M-(208 + 114 + 42)	439	1.5	535	1.7	–	215	0.7
M-(208 + 114)	481	12.9	369	51.9	99.3	257	56
M-(208)	595	0.6	483	3.4	32.5	371	0.8
Molecular ion	803	–	691	–	–	579	–

Fragments derived from McLafferty rearrangement of the sidechain are marked by •. m/z 114 corresponds to CF_3-COOH group and m/z 42 to ring D.

TABLE 2

Fragment Ions and Relative Intensities of Glycoconjugated Bile Acids as Methyl Ester-Trifluoracetate Derivatives

	GCA		GCDCA		GDCA	GLCA	
	m/z	ri	m/z	ri	ri	m/z	ri
CH_2 | COH-NH-CH_2-CO-O-CH_3	131•	100	131	100	100	131	100
CH_2 | CH_2 | CO-NH-CH_2-CO-O-CH_3	144	100	144	100	83.5	144	51.3
CO-NH-CH_2-CO-O-CH_3	172	32.3	172	5.4	18.6	172	4.1
M-(172+(114 X 3)+42)	211	23.3	–	–	–	–	–
172 + 42	214	3.7	214	4.3	3.2	214	1.8
M-(172 + (114 X 3))	253	15.7	–	–	–	–	–
M-(172+(114 X 2)+42)	325	0.6	213	2.9	7.8	–	–
M-(172+(114 X 2))	367	22.8	255	5	15.7	–	–
M-(114 X 3)	425	1.4	–	–	–	–	–
M-(114 + 172)	481	1	369	2.3	17	257	2.9
M-(114 X 2)	539	1	427	3	4.8	–	–
M-(172 + 42)	553	0.2	441	0.2	0.1	329	0.3
M-172	595	0.5	483	0.2	0.6	371	1.1
M-114	653	1.2	541	1	10	429	3.8
Molecular ion	767	–	655	0.4	0.9	543	2.1

For significance of •, m/z 114 and m/z 42, see Table 1.

TABLE 3

Ions Monitored for mass fragmentography

TCA	81	253	367	481	
TCDCA	81	255	369	483	
TDCA	81	255	369	483	
TLCA	64	95	257	371	
GCA	131	144	211	253	367
GCDCA	131	144	–	255	369
GDCA	131	144	–	255	369
GLCA	131	144	–	257	429

TABLE 4

HPLC and MF Determination of Conjugated Bile Acids in 10 Human Gallbladder Bile Samples

	HPLC	MF
TCA	2760 ± 1620	2457 ± 1295
TCDCA	3480 ± 2940	2874 ± 2119
TDCA	1380 ± 720	1268 ± 645
TLCA	55 ± 65	53 ± 60
GCA	2400 ± 1860	2328 ± 1793
GCDCA	2880 ± 1500	2815 ± 1436
GDCA	1620 ± 840	1541 ± 738
GLCA	30 ± 31	27 ± 30
TBA	14700 ± 5160	13370 ± 3974

Mean ± SD is reported for each bile acid.

The relevant amounts of conjugated bile acids make ineffective the interference of possible substances present in bile extract and UV absorbance at a wavelength of 200 nm. In this connection, the HPLC method would appear a suitable method for assaying conjugated bile acids in bile. On the contrary, the use of MF becomes imperative in the case of some biological fluids like serum, because of the complexity in composition and the low quantity of bile acids below the sensitivity limits (100 ng) of the HPLC method (8). Spectra derived from fragmentation by electron impact were reported by Shaw and Elliot for a series of naturally occurring conjugated bile acids, their 5-alpha-analogues and methyl esters of glycoconjugates (12-14).

Mass spectrometry of nonderivatized standards of conjugated bile acids determined directly by injection into the ion source (our unpublished data) did not provide satisfactory results, especially as regards quantitative analysis of bile acids in biological samples, because of either low sensitivity of total ion current, or the reproducibility of relative intensities of fragment ions. Both the occurrences can be ascribed to a decomposition phenomenon during probe heating.

The problem was overcome by the use of TFA derivatives of conjugated bile acids. In this way, in fact, detectability limit was at least 1 ng for each examined bile acid.

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COMMUNICATIONS

Alteration in Membrane Permeability by Diacylglycerol and Phosphatidylcholine Containing Arachidonic Acid

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ABSTRACT

The phospholipid metabolites, stearoylarachidonylglycerol and diarachidonylglycerol, stimulate transepithelial sodium transport in frog skin epithelium. The increase in Na transport is due to an increase in the unidirectional influx of sodium, is amiloride sensitive and is prevented with pretreatment with indomethacin, mefanamic acid and phospholipase inhibitor, mepacrine. The data suggest a possible role of phospholipid metabolism and prostaglandin biosynthesis in the regulation of transepithelial ion transport.
Lipids 18:96-99, 1983.

INTRODUCTION

The primary function of cellular membranes is the selective control of the movements of ions and other solutes and to serve as messengers for the transfer of information from extracellular to intracellular compartments (1). Phospholipids have been suggested as being involved in the transport of ions across membrane lipid barriers (2) and contributing to the cell permeability properties (3). Previously, we have shown that phospholipase C stimulates sodium transport across frog skin in vitro (4) and across the crystalline lens of the toad (5). The increase in sodium transport was associated with an increase in the unidirectional sodium influx which was inhibited by amiloride (4), a diuretic which specifically blocks Na transport in epithelial membranes (6). It was suggested that such an enzyme may function in controlling membrane permeability and provide a 'model' for hormonal effects. Recently, specific increases in the metabolism of phospholipids by phospholipases have been suggested as being the result of a widespread cellular response to activation of certain types of cell-surface receptors for hormones and neurotransmitters (7). Furthermore, phospholipid metabolites, such as phosphatidic acid, have been suggested as being intracellular ionophores (8) and may play a significant role in the regulation of cellular calcium concentrations. Alterations in membrane permeability to ions may be the consequence of membrane phospholipid breakdown.

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Presently, we report on a series of phospholipids and their metabolites on transepithelial sodium transport in frog skin epithelium, a tissue often used as a model for the mammalian collecting duct.

MATERIALS AND METHODS

Frogs, *Rana pipiens*, were obtained from a commercial supply house (Lake Champlain Frog Farm, Vermont) and kept in the laboratory without feeding at 23 C.

ELECTRICAL MEASUREMENTS

Ventral abdominal skins of frogs were mounted as diaphragms in Ussing-type divided chambers (surface area, π cm^2) for recording PD (potential difference), Isc (short-circuit) and unidirectional fluxes of sodium as has been previously described (9). Transepithelial potential difference was measured and current was sent across the membranes through Ringer-agar bridges. The PD bridges were connected through calomel cells to a potentiometric recorder and the Isc (the amount of current necessary to bring the PD to zero) was obtained and recorded with an automatic voltage-clamp apparatus connected to a potentiometric recorder. Unidirectional fluxes of sodium were measured using ^{22}Na as the radioisotope (New England Nuclear, Boston, MA). The ^{22}Na was added to one chamber compartment and periodic samples were taken from the opposite compartment. To establish the stability of sodium fluxes across the skin, four 30-min

periods were measured before the addition of the experimental agent. The agent was then added and fluxes were measured for four more 30-min periods. The results presented represent the mean flux values of two 30-min periods immediately prior to adding the agent and the mean of the next two periods after agent administrations. A stable flow of isotope was an indication of isotope equilibration.

Fluxes were calculated from the specific activity of the "hot" solutions and samples were counted in a liquid scintillation counter (Beckman).

Phospholipids and diglycerides were obtained from Sedary Research Laboratories, Inc. (London, Ontario). All phospholipids were dissolved in chloroform/methanol (2:1) and added at the appropriate concentrations to tubes, dried under nitrogen and resuspended in Ringer's solution by sonication.

The statistical analysis was performed using Student's t-test.

RESULTS AND DISCUSSION

When the phospholipid metabolite diacylglycerol, either diarachidonyl (DAG) or steroylarachidonyl (SAG), was placed in the solution bathing the corium side of the frog skin, it produced a sustained increase in the short-circuit current Isc) and potential difference (Fig. 1 and Table 1). There was no apparent change in transepithelial resistance. The effect was prolonged and the Isc returned to the initial baseline values when the lipid was removed from the bathing solution. When DAG or SAG was added to the epidermal surface, only minimal effects on Isc were observed. The Isc in these epithelia is normally an indication of active transmural sodium transport. The diuretic amiloride blocked the increment in Isc induced by DAG (Fig. 1); a similar response was also observed with SAG. This observation is consistent with an effect of these diacylglycerols in stimulating transmural sodium transport, similar to that observed previously with phospholipase C (4).

Transepithelial sodium transport can be stimulated by antidiuretic hormone and aldosterone, and this increase is reflected in an increase in the influx of sodium with no change in the efflux. The increase in Isc produced by DAG could be accounted for by an increase in the unidirectional influx of sodium (from pond to blood side) with no appreciable effect on the backflux (blood side to pond). The sodium influx was 1.2 ± .05 μ eq/cm^2/hr before the addition of DAG and increased to 2.1 ± .09 μ eq/cm^2/hr 30 min after DAG (mean ± SE of 9 experiments; $p < .05$ using a paired Student's t-test). The sodium backflux was 0.68 ± .05 μ eq/cm^2/hr before and 0.89 ± .10 μ eq/cm^2/hr

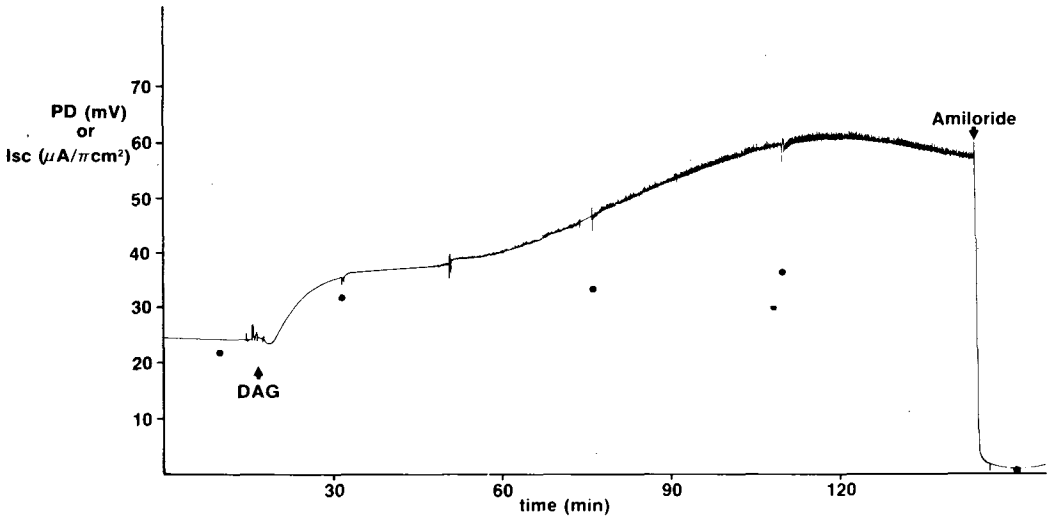

FIG. 1. The effects of diarachidonylglycerol (DAG) (50 μg/ml on the corium side) on the PD (mV) and Isc (μA/π cm^2) across the frog skin in vitro. The skins were mounted as described in the text. Amiloride (10^{-5} M) was added to the epidermal side of the frog skin. The Isc is represented as the continuous line and PD as the filled circles.

TABLE 1

Effects of Phospholipid and Phospholipid Metabolites on Potential Differences (PD) and Short-Circuit Current (Isc) across Isolated Frog Skin

	Initial	+ Drug
A. Stearoylarachidonyl-glycerol (SAG) (7)		
PD (mV)	51 ± 5	77 ± 7[a]
Isc ($\mu A/cm^2$)	21 ± 2	30 ± 3[a]
B. Diarachidonylglycerol (DAG) (7)		
PD (mV)	28 ± 5	43 ± 7[a]
Isc ($\mu A/cm^2$)	12 ± 2	22 ± 4[a]
C. Diarachidonyl phosphatidyl-choline (7)		
PD (mV)	20 ± 6	41 ± 10[a]
Isc ($\mu A/cm^2$)	10 ± 2	21 ± 3[a]
D. Phosphatidic acid (8)		
PD (mV)	63 ± 19	46 ± 13
Isc ($\mu A/cm^2$)	28 ± 6	20 ± 8
E. Cardiolipin (8)		
PD (mV)	29 ± 3	29 ± 3
Isc ($\mu A/cm^2$)	16 ± 2	14 ± 2
F. 1,2, Diolein (8)		
PD (mV)	43 ± 8	40 ± 8
Isc ($\mu A/cm^2$)	27 ± 4	24 ± 3
G. Dipalmitoyl-glycerol (6)		
PD (mV)	36 ± 3	35 ± 3
Isc ($\mu A/cm^2$)	23 ± 3	24 ± 2
H. Dipalmitoyl phosphatidyl choline (8)		
PD (mV)	22 ± 8	23 ± 8
Isc $\mu A/cm^2$)	22 ± 2	22 ± 1
I. Dioleoyl phosphatidyl-choline (8)		
PD (mV)	23 ± 2	22 ± 2
Isc ($\mu A/cm^2$)	12 ± 3	14 ± 3
J. Stearoyl-α-lysophospha-tidylcholine (8)		
PD (mV)	46 ± 9	39 ± 13
Isc ($\mu A/cm^2$)	33 ± 7	29 ± 8

Drugs were added to the serosal (blood side) solution at concentrations of 50 μg/ml (A-C) or 100 μg/ml (D-J). The values presented represent the mean ± SE of 20 min readings before and after administration of the drug. The number of experiments are indicated within the parenthesis. The composition of the bathing solutions was: 111 mM NaCl, 4 mM $NaHCO_3$, 2.54 mM $CaCl_2$, 3.35 and 5 mM glucose, final pH, 8.0.

[a] $p < .05$ for difference from initial period, using a paired Student's t-test.

30 min after the addition of DAG (mean ± SE of 9 experiments; not statistically significant, $p < .05$ using a paired Student's t-test). It is apparent that DAG specifically stimulates the active transmural transfer of sodium through amiloride-sensitive channels mimicking that produced by the endogenous hormones and to what was previously reported for phospholipase C.

Diacylglycerol is a product of phospholipase C action on phospholipids, specifically phosphatidylinositol (PI). The fatty acid composition of PI is relatively important as the predominant form appears to be steroyl-arachidonyl phosphatidylinositol (10). It is apparent that DAG or SAG could alter membrane permeability in frog skin preparations and that the common fatty acid is arachidonic. We therefore investigated several substituted phospholipid compounds on their ability to alter the Isc in frog skin. Dioleoylglycerol was without an effect as was dipalmitoylglycerol (Table 1). Phosphatidic acid and cardiolipin, which have been suggested as being a calcium ionophores (8), had no effect on the electrical properties of the frog skin (Table 1). Diarachidonyl phosphatidylcholine, similarly to the arachidonic substituted diglycerides, also stimulated transmural PD and Isc (Table 1), whereas dioleoyl phosphatidylcholine, dipalmitoyl phosophatidylcholine and stearoyllyso-phosphatidylcholine had no effect.

It has been suggested that phospholipase activation and the release of arachidonic acid is essential for the synthesis of prostaglandins (11). The role of prostaglandins in modulating membrane permeability has been under investigation for several years (12). The administration of arachidonic acid or prostaglandins (PGE_2) increases transepithelial sodium transport in these epithelia (13,14). Pretreatment of frog skins with indomethacin, a cyclooxygenase inhibitor, prevented the increase in Isc produced by DAG. The Isc in the presence of indomethacin (10^{-5} M) was 10 ± 2 $\mu A/cm^2$ before the addition of DAG and 11 ± 3 $\mu A/cm^2$ 20 min after the addition of DAG (mean ± SE of 6 experiments). Similar results were obtained using SAG. Mefanamic acid, another prostaglandin synthesis inhibitor, also prevented the increase in transepithelial sodium transport.

The results suggest that the changes in membrane permeability produced by these diglycerides may be mediated through the production of prostaglandins. This further implies that arachidonic acid (AA) is being released from these diglycerides by the action of endogenous phospholipases. Mepacrine, a phospholipase inhibitor (15), prevented the increase in sodium transport produced by SAG, suggesting that phospholipase activity is essential to the actions of these diglycerides.

We have previously identified phospholipase in frog skin epithelium and that this enzymes activity was enhanced in the presence of the mineralocorticoid hormone aldosterone (15). It was also reported that the increase in sodium transport incuded by aldosterone could be pre-

vented by the prostaglandin synthetase inhibitors indomethacin and mefanamic acid.

The role of phospholipase in prostaglandin synthesis is generally related to the availability and regulation of the prostaglandin precursor arachidonic acid (12). There is some controversy concerning the release of arachidonic acid by phospholipases. In platelets, it has been suggested that AA is released from diacylglycerol by a diglyceride lipase following a phosphatidylinositol specific phospoholipase C effect (16). Others have suggested that, following a stimuli, a stepwise release of arachidonic acid occurs with first the actions of phospholipase C on phosphatidylinositol to form diacylglycerol, which is quickly phosphorylated by diglycerol kinase to phosphatidic acid (PA) with a subsequent release of AA (17). Recently, a phospholipase A_2 specific for phosphatidic acid has been reported in platelets and that phosphatidic acid precedes the release of AA (18). The present data suggests that a phospholipase C metabolite, diacylglycerol, containing an arachidonic acid in position 2, can alter the permeability of an epithelial membrane to sodium and that this response is mediated through the production of prostaglandins. This also suggests that an endogenous phospholipase mobilizes arachidonic acid from diacylglycerol; perhaps a diglyceride lipase as has been previously described for platelets (16). Alternatively, a phospholipase A_2 acting on diacylglycerol or a phosphorylated metabolite could be contributing the free-arachidonic acid. The latter suggestion appears more likely in light of our observations with mepacrine. These findings are consistent with our previous report on the mechanism of action of aldosterone (15).

The control of electrolyte transport by hormones and second messengers is complex and incompletely understood. The present data suggest that phospholipid metabolites can alter membrane permeability and that the increase in sodium transport observed is mediated through the release of arachidonic acid and the synthesis of prostaglandins. Increases in phospholipid breakdown have been implicated in a variety of cellular responses mediated through hormone receptor activation and it is possible that such effects may be occurring in the regulation of transepithelial ion transport.

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Sterol Composition of Two Freshwater Molluscs of Genus *Diplodom*

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ABSTRACT

The sterol composition of two taxonomically related freshwater species, *Diplodom patagonicus* and *Diplodom variabilis*, respectively, from Lake Nahuel Huapi and the Río de la Plata river were studied by gas liquid chromatography and mass spectrometry. Cholesterol was the main sterol in both species and it was followed by 24-methylcholesta-5,22-dien-3β-ol, 24-methylcholest-5-en-3β-ol, 24-ethylcholesta-5,22-dien-3β-ol and 24-ethylcholest-5-en-3β-ol. The river species collected within the proximity of marine influence showed less cholesterol and more 24-methylcholesta-5,22-dienol, 24-methylcholest-5-enol and 24-ethylcholesta-5,22-dienol than the lake species.
Lipids 18:100-102, 1983.

The sterol composition of bivalve molluscs has been studied in depth only in marine molluscs (1) and very limited information exists about freshwater species (2-4). The compositions are complex and, although Δ5 sterols are considered typical of molluscs, cholesterol is the predominant sterol in both types of molluscs. The presence of sterols of the 28 and 29 carbons (24-alkyl substituted) usually found in molluscs is generally related to the diet, since these sterols are considered to be of vegetable origin. However, it has been shown (4,5) that the assignment of 24-alkyl and 24-dealkyl structures to vegetable and animal origins is not reliable.

In a previous study (6), free and esterified sterols were detected in the freshwater bivalve mollusc, *Diplodom patagonicus*, from Lake Nahuel Huapi, but they were not studied in detail. In this report, the sterol compositions of the lake species, *D. patagonicus*, and of a taxonomically related estuarine river species, *D. variabilis*, were determined by gas liquid chromatography (GLC) and mass spectrometry (MS).

MATERIALS AND METHODS

Specimens of *D. patagonicus* and *D. variabilis* from Lake Nahuel Huapi and the Río de la Plata river, Argentina, were collected during the same season (December) and under the conditions already described (7).

Lipids were extracted from total soft tissues with chloroform/methanol (2:1, v/v) (8). Total lipid extracts were saponified at 80 C for 1 hr with 10% KOH in ethanol and the unsaponifiables were recovered by extraction with petroleum ether. These were separated by thin layer chromatography (TLC) using Absorbosil-5 plates (Applied Science Labs.) and hexane/ethyl ether (80:20, v/v). Sterols were localized on the plates by spraying either with H_2SO_4/AcH (50:50, v/v) or phosphomolibdic acid (9). Areas corresponding to the sterols, but not sprayed, were separated from the plates and eluted with chloroform/ethyl ether (1:1, v/v).

GLC separation of free sterols was performed in a Hewlett-Packard apparatus model 5840, equipped with a dual-flame ionization detector, using a flexible silica capillary column (methyl silicone fluid, 12 m × 0.2 mm id). The column temperature was programmed from 240 to 280 C at a rate of 10 C/min. GLC-MS analysis was performed with a Hewlett-Packard apparatus model 5995 GC-MS coupled to a Hewlett-Packard model 9885 computer. Gas chromatography (GC) was conducted with the same capillary column but under isothermal conditions (244 C). The conditions were: source temperature 215 C, ionization potential 70 eV, and scan speed 380 amu/sec. Helium was used as the carrier gas at a flow rate of 1 ml/min. The sterols separated by GLC and the mass spectra were identified by comparison with authentic standards provided by Sigma Chemical Co., and Makor Chem. Co., processed in the same way.

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TABLE 1

Sterol Composition (%) of *Diplodom patagonicus* and *Diplodom variabilis*

Sterol	RRT	*D. patagonicus* (lake)	*D. variabilis* (river)
I Cholesterol	1.00	80.9	48.3
II 24-Methylcholesta-5,22-dienol	1.05	3.0	23.7
III 24-Methylcholest-5-enol	1.10	6.1	10.5
IV 24-Ethylcholesta-5,22-dienol	1.17	4.3	11.3
V 24-Ethylcholest-5-enol	1.25	5.7	6.2

RESULTS AND DISCUSSION

Table 1 shows the sterols isolated from the unsaponifiable materials of *D. patagonicus* and *D. variabilis*, and identified by GLC-MS. The total ion current chromatogram presented the same five signals detected in the GLC analysis. All sterols were identified on the basis of GLC retention times and mass spectral data compared with known standards. Cholesterol was the main sterol in both cases. The remaining four sterols were assigned the structures: 24-methylcholesta-5,22-dien-3β-ol, 24-methylcholest-5-en-3β-ol, 24-ethylcholesta-5,22-dien-3β-ol and 24-ethylcholest-5-en-3β-ol, respectively.

Table 1 shows that the sterol composition of the two species of freshwater molluscs is consistent with the composition found in other freshwater species (4) and simpler than that of marine bivalves (1). However, Gordon and Collins (10) have recently found only 8 sterols in *Crassostrea gigas*.

It has been recognized, but not definitely stated, that cholesterol is higher in marine bivalve molluscs than in freshwater bivalves (4). Table 1 shows that cholesterol is 80% of the sterols found in *D. patagonicus* (lake) and less than 50% of sterols of *D. variabilis* (river). In a previous work (7) which was performed with the same animals, it was shown that the river specimens were strongly influenced by the proximity of the ocean and occasional intrusions evoked by the wind. In the *D. patagonicus* from the lake, the fatty acid composition was of the "land" type with the predominance of ω6 unsaturated acids. The same species, acclimatized after transplanting into the Río de la Plata river, as well as the native *D. variabilis* from the river, showed a marine influence in the form of predominance of ω3 unsaturated fatty acids. Therefore, it was assumed that the specific food composition provided by the environment was the main factor responsible for this composition modification. The same may be presumed with the sterol composition.

Table 1 also shows that, in addition to cholesterol percentage, the main difference between *D. patagonicus* and *D. variabilis* was a high ratio of 24-methylcholesta-5,22-dienol (sterol II) found in *D. variabilis*. Although it was not possible to determine the 24-C configuration by the analytical methods employed, the structure of sterol II (Table 1) may be assigned to brassicasterol or to the isomer, crinosterol. Both, but especially the brassicasterol, are found in large quantities in diatoms (1). Therefore, it may be speculated that the significantly higher ratio of this sterol in river molluscs than in Lake Nahuel Huapi specimens is due to the phytoplankton composition of the Río de la Plata river. There is an abundance of diatoms in the river in contrast with the very poor planktonic population of Lake Nahuel Huapi (S. Guarrera, personal communication).

The relation between sterol composition and diet is necessarily limited by the possible capacity of the mollusc to synthesize sterols. Indications of sterol biosynthesis in *D. patagonicus* have been found since specimens incubated with [1-^{14}C] fatty acid incorporated radioactivity in free sterols (7). This labeling may be attributed to de novo synthesis of sterol from the labeled acetate produced by β-oxidation of [1-^{14}C] acids or to the incorporation of labeled acetate during alkylation of sterols (3).

Two reactions, the dealkylation of 24-substituted sterols to produce 27-C sterols or the alkylation of cholesterol, have been recognized in bivalve molluscs (11). Both would involve demosterol formation as an intermediate. Demosterol has been found in marine molluscs (1) but not in the animals studied here, or in freshwater molluscs in general. Therefore, the absence of this metabolite in *D. patagonicus* and *D. variabilis* suggests for sterols the origin may be mainly trophic. However, de novo biosynthesis or the conversion of cholesterol to 24-substituted sterols, leaving aside the demosterol route (4), cannot as yet be discarded as a possibility.

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Fatty Acid Composition of *Ixiolaena brevicompta* F. Muell. Seed Oil

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ABSTRACT

The seeds of *Ixiolaena brevicompta* F. Muell. (family Compositae) contained an oil (12%) in which the major fatty acids present are palmitic (7%), stearic (5%), oleic (8%), linoleic (51%) and crepenynic (octadec-*cis*-9-en-12-ynoic) acid (25%). The structure of the acetylenic acid was established by chemical and physical methods. The bioactivity of crepenynic acid is discussed with respect to extensive mortalities of sheep grazing on mature seeded *I. brevicompta*.
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Ixiolaena brevicompta F. Muell. (Compositae) is a native annual or biennial herb, 15-50 cm high, which is distributed widely on the riverine flood plains of western New South Wales and Queensland. The mature seeded plant is considered responsible for causing muscular degeneration in sheep in these areas, where it can form the predominant vegetation, and extensive mortalities have been recorded (1). During our investigation of the possible toxic components of *I. brevicompta*, we examined the fatty acid composition of the seed oil. This communication presents evidence for the presence of crepenynic (octadec-*cis*-9-en-12-ynoic) acid as a major component of the oil. This acetylenic acid was first isolated in 1964 from the seed oil of *Crepis foetida* L. (2) and more recently from several other plant species (3,4). The bioactivity of this unusual fatty acid is discussed.

MATERIALS AND METHODS

The mature seeds of *I. brevicompta* were crushed and repeatedly extracted with hexane using a Soxhlet extraction apparatus. After the removal of solvent, a sample of the extracted oil was transmethylated (5) and the crude fatty acid methyl esters were fractionated by column chromatography on Florisil (6). The purified ester fraction was examined by gas liquid chromatography (GLC) using a glass column (4 m, 2 mm id) packed with 10% Silar 10C on Gas-Chrom Q. The column was heated at 200 C and the flow rate of the carrier gas (nitrogen) was 40 ml/min. The methyl esters, with the exception of methyl crepenynate, were identified by the comparison of their GLC retention times with those of a standard ester mixture. Methyl crepenynate was observed at a retention time of 2.6 relative to methyl stearate 1.0. A Hewlett Packard 3390A Integrator was used to measure peak areas.

Methyl crepenynate was isolated from the total ester fraction by low temperature argentation thin layer chromatography (TLC) using Kieselgel GF_{254}, type 60 (layer thickness 0.5 mm) containing 30% silver nitrate (7). Development of the ester mixture was carried out twice in the same direction at -20 C using toluene/hexane (90:10, v/v) as solvent. Visualization of the components was achieved by spraying with a 0.2% w/v solution of 2′,7′-dichlorofluorescein in methanol followed by examination under ultraviolet (UV) light. The acetylenic ester was recovered by diethyl ether (2x 10 cm^3) extraction of the band which ran midway between methyl linoleate and methyl oleate on the TLC plate. The UV spectrum of the ester (in hexane) was recorded on a Gilford 2600 spectrophotometer, the infrared (IR) spectrum (as a thin film) on a Perkin Elmer 521 spectrometer, and the ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra (as C_6D_6 solutions) on a Bruker CXP100 spectrometer. The low-resolution and high-resolution mass spectra were recorded using a Varian MAT-311A mass spectrometer. The products obtained from the hydrazine reduction (8) of methyl crepenynate were identified by GLC and mass spectrometry. Products obtained from the oxidative cleavage (9) of methyl crepenynate and of the unsaturated esters derived from the partial reduction of the acetylenic ester were identified by GLC using the column and conditions described above.

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RESULTS AND DISCUSSION

The mature seeds of *I. brevicompta* contained an oil (12%) in which the main fatty acids were: palmitic, stearic, oleic, linoleic and the acetylenic acid, crepenynic, present at 7%, 5%, 8%, 51% and 25%, respectively.

The structure of the methyl ester of the acetylenic acid was established by the combination of a number of chemical and physical techniques. The GLC-pure methyl ester gave a low-resolution mass spectrum with prominent and diagnostic ions as follows: M/Z 292 M^+ (5), 94(57), 93(92), 91(55), 81(65), 80(79), 79 (100), 67(75), 55(89), 43(53), 41(94). The high-resolution mass spectral examination gave a molecular weight of 292.2406 which was consistent only with the molecular formula $C_{19}H_{32}O_2$ (calc., 292.2402). Partial hydrogenation using hydrazine (8) gave a complex mixture consisting of methyl stearate (7%), oleate (5%), octadec-*cis*-12-enoate (12%), linoleate (8%), the $C_{19}H_{32}O_2$ ester (27%) and the major product, a new unsaturated ester of molecular formula $C_{19}H_{34}O_2$ (39%). Oxidative cleavage (9) of the latter esters indicated that the original compound had two sites of unsaturation at C9 and C12 and the major hydrogenation product only one at C12. The UV and IR spectra of the original ester showed the absence of conjugated unsaturation and *trans*-olefinic groups. The ^{1}H-NMR spectrum showed a complex multiplet at δ5.51 (2H), assigned to the two olefinic protons at C9 and C10, and two multiplets at δ2.11 (4H) and δ2.95 (2H) assigned to the methylene groups at C8 and C14, and C11, respectively (10). Independent irradiation of the compound at δ2.11 and 2.95 led to the collapse of the multiplet at δ5.51 to two doublets at δ5.37 (J 11 H_Z) and 5.63 (J 11 H_Z) indicative of a *cis*-configuration. This evidence indicated that the original ester was methyl octadec-*cis*-9-en-12-ynoate and that the major partial hydrogenation product was methyl octadec-12-ynoate (11). Subsequently, an authentic sample of methyl crepenynate, prepared from the seed oil of *Afzelia bella* (3), was shown to be identical with our acetylenic ester.

Several long-chain acetylenic fatty acids have been shown to inhibit enzymes involved in some important biological pathways (12,13). For example, acetylenic fatty acids are known to inhibit soybean lipoxidase and prostaglandin synthetase but only when the acetylenic acid has a structure analogous to the polyunsaturated fatty acid substrate for these enzymes (14). Of the four isomeric octadecenynoic acids having one acetylenic bond and either a *cis*- or *trans*-ethylenic bond in the C9 or C12 positions, all except octadec-*cis*-9-en-12-ynoic (crepenynic) acid, strongly inhibited prostaglandin synthetase in vitro (14). None of these acids, however, significantly inhibited soybean lipoxidase (14). Acetylenic fatty acids have also been the subject of toxicity tests in various animal feeding trials. However, when rats were fed a ration containing crepenynic acid in the form of the natural triglyceride extracted from *Crepis rubra* seed, no ill effects were observed (15).

The components in mature seeds of *I. brevicompta* which cause muscular degeneration and extensive mortalities in sheep have yet to be determined. While crepenynic acid is an unusual seed oil component, the evidence above suggests that additional factors may be responsible for the syndrome. Some species of Compositae contain highly toxic sesquiterpene lactones which have been implicated in stock poisoning (16). The mature seeds of *I. brevicompta* are currently being investigated for the presence of such a toxin.

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17Z-Tetracosenyl 1-Glycerol Ether from the Sponges *Cinachyra alloclada* and *Ulosa ruetzleri*[1]

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ABSTRACT

The 1-glycerol ether of 17Z-tetracosenol has been identified as a major constituent of the dichloromethane-soluble extracts of two marine sponges, *Cinachyra alloclada* and *Ulosa ruetzleri*. Spectral analysis and chemical degradations led to the assigned structure. Batyl alcohol was also found, in lesser quantities, in *U. ruetzleri*, but not in *C. alloclada*.
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INTRODUCTION

In our continuing study of the chemistry of sponges from Bermuda waters (1) we undertook an investigation of *Cinachyra alloclada*, because it exhibited antimicrobial activity (against *Staphylococcus aureus, Bacillus subtilis*, and *Pseudomonas aeruginosa*) in qualitative field screens, and *Ulosa ruetzleri* (formerly *Dysidea crawshayii* (2)) because it has been reported to be a nickel concentrator (2).

In the course of resolving the components of the dichloromethane-soluble extracts of these sponges, we found that both organisms produced an unusually long-chain monoalkenyl glycerol ether as a major constituent. In this report, we describe the isolation and characterization of that glycerol ether, as well as the identification of some companion metabolites.

EXPERIMENTAL

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 250 MHz multinuclear Fourier transform spectrometer, using $CDCl_3$ as solvent and internal standard; chemical shifts are reported in ppm relative to tetramethylsilane ($\delta = 0$). Mass spectra (MS) were obtained with either a Kratos MS-50 or Varian MAT CH-5 mass spectrometer, operating at 70 eV in the electron ionization mode. Melting points were determined on a Mel-Temp apparatus and are uncorrected.

Collection and Extraction of Sponges

Specimens of *U. ruetzleri* and *C. alloclada* were collected from Church Bay and Shark's Cove in Harrington Sound, Bermuda, in October, 1979 at depths of 2-5 m. The sponges were stored in acetone at −5C prior to extraction. In both cases, the acetone was decanted and filtered. The sponges were ground in a Waring blender with fresh acetone; the acetone was removed by suction filtration and the sponge residues were steeped twice in dichloromethane for 24 hr. The combined acetone extracts were reduced in vacuo to an aqueous suspension. The dichloromethane extracts were then equilibrated with the aqueous suspension; subsequent evaporation of the dichloromethane phases gave the crude extracts: 36.0 g from *U. ruetzleri* (213 g dry wt), and 20.06 g from *C. alloclada* (327.2 g dry wt).

Isolation of Glycerol Ethers

From *C. alloclada*. A portion (9.20 g) of the dichloromethane-soluble extract was chromatographed on Florisil (283 g) using, as eluent, combinations of hexane, ethyl acetate and methanol of gradually increasing polarity; 13 fractions were collected. Fractions 9 and 10 were combined (640 mg) and permeated through Sephadex LH-20 with CH_2Cl_2-MeOH (1:1). Six fractions were collected; a portion of Fraction 3 (323 of 366 mg) was dissolved in hot ethanol and stirred with activated charcoal. Filtration and evaporation of the filtrate gave 197 mg white solid. Crystallization from acetone-hexane gave **I** as amorphous crystals, mp 52-53.5 C; IR $\nu_{max}(CS_2)$ 3600, 3400, 2940, 2860, 1115, 1055, 720 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 5.33 (2H, m), 3.85 (1H, m), 3.70 1H, dd, J = 12,4), 3.64 1H, dd, J = 12,6), 3.45 (4H, overlapping m), 2.60 (OH, br), 2.25 (OH, br), 2.03 (4H, m) 1.58 (2H, m), 1.25 (34H, br), 0.88 (3H, br t, J ~ 6.5); ^{13}C-NMR ($CDCl_3$): δ 129.74 (d, 2C), 72.25 (t), 71.70 (t), 70.50 (d), 64.08 (t), 31.76 (t), 31.64 (t), 29.53 (t, 8C), 29.17 (t, 3C), 28.82 (t), 27.06 (t, 3C), 25.94 (t, 2C), 22.50 (t), 13.91 (q); MS: m/z 426.4085 (calculated for $C_{27}H_{54}O_3$—426.4073; 25%), 377 (33), 351.3608 (calculated for $C_{24}H_{47}O$—351.3627; 10%), 334.3599 (calculated for $C_{24}H_{46}$—334.3599; 100%), 283.2985 (calculated for $C_{19}H_{39}O$—283.3001; 18%), 253 (18), 250 (12), 97 (14), 96 (13), 95 (12), 83 (50), 71 (9), 69 (40).

From U. ruetzleri. A portion of the dichloromethane-soluble extracts (26.4 g) was chromatographed on Florisil (150 g); elution commenced with hexane and proceeded through a series of

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combinations of hexane, ethyl acetate and methanol of gradually increasing polarity. Fifteen fractions were collected. Fraction 8, 656 mg, was permeated through Sephadex LH-20 with CH_2Cl_2/MeOH (1:1); eight fractions were collected. Fraction 3, 213 mg, was subjected to low pressure chromatography on reverse phase gel (Baker C_{18}). Gradient elution [CH_3CN/H_2O (3:2) to CH_3CN] eluted 15 mg of impurities; continued elution with $MeOH/CH_3CN/THF$ (3:1:1) gave 182 mg of an off-white solid. Crystallization from hexane-EtOH gave the glycerol ether **1**, mp 50-51.5 C; MS: m/z 426.4138 (calculated for $C_{27}H_{54}O_3$—426.4073; 0.2%), 391 (0.2), 377 (0.5), 351.3595 (calculated for $C_{24}H_{47}O$—351.3627; 0.3%), 334.3612 (calculated for $C_{24}H_{46}$—334.3599; 3%), 180 (2), 166 (3), 165 (3), 153 (2), 152 (4), 151 (5), 139 (5), 138 (8), 137 (11), 135 (5), 131 (3), 125.1328 calculated for C_9H_{17}—125.1330 14), 124 (14), 123 (22), 111 (34), 110 (26), 109 (39), 97 (75), 96 (69), 95 (63), 85 (24), 83 (100), 82 (74), 81 (59), 75 (32), 71.0862 (calculated for C_5H_{11}—71.0861; 31%), 69 (86), 68 (30), 67 (47).

Isolation of 5 from *U. ruetzleri*

Fraction 4, 285 mg, from the Sephadex LH-20 chromatography was permeated through Bio-Beads SX-8 with CH_2Cl_2/cyclohexane (3:2); 4 fractions were collected. Fraction 3, 98 mg, was subjected to low pressure chromatography on reverse-phase gel (Baker C_{18}); elution with MeOH-THF (1:1) gave 66 mg of batyl alcohol, **5**, mp 67.5-68 C (Lit (3), mp 70.5-1 C); MS: m/z 344 (M^+, 2), 326 (1), 313 (8), 283, (24), 253 (25), 252, (16), 127 (11), 125 (11), 113 (13), 111 (17), 97 (29), 93 (33), 85 (50), 83 (33), 71 (67), 57 (100), ^{1}H-NMR ($CDCl_3$): δ 3.82 (1H, m), 3.67 (1H, dd, J = 12,4), 3.59 (1H, dd, J = 12,6), 3.45 (4H, overlapping m), 1.95 (2 OH, br), 1.53 (2H, m), 1.23 (30 H, br), 0.86 (3H, br t, J ~ 6.5).

Isolation of 3 from *U. ruetzleri*

Fraction 7 from the Sephadex LH-20 chromatography was permeated through Bio-Beads SX-8 with CH_2Cl_2/cyclohexane (3:2). The fifth, and largest, fraction obtained yielded **3**, *E*-1-(4-hydroxyphenyl)-buten-3-one, 6 mg tan solid, $\nu_{max}^{CHCl_3}$ 3600 (sh), 3300 (br), 2930, 2860, 1670, 1590, 1500, 1420, 1360, 1325, 1165, 1100, 970, 845 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 7.47 (1H, d, J = 16), 7.43 (2H, d, J = 8.2), 6.87 (2H, d, J = 8.2), 6.58 (1H, d, J = 16), 2.36 (3H, s).

Acetylation of 1

A solution of 36 mg of **1** in 1 ml dry pyridine and 0.5 ml acetic anhydride was sealed under nitrogen for 2.5 hr. Solvent and excess reagent were evaporated in vacuo and the residue was permeated through Sephadex LH-20 with CH_2Cl_2/MeOH (1:1) to yield 36 mg of the diacetate **2**; ^{1}H-NMR ($CDCl_3$): δ 5.32 (2H, br t), 4.87 (1H, m), 4.32 (1H, dd, J = 12.5,4), 4.16 (1H, dd, J = 12.5,6.2), 3.52 (2H, br d, J = 6), 3.40 (2H, m), 2.07 (3H, s), 2.05 (3H, s), 2.00 (4H, m), 1.52 (2H, m), 1.25 (34H, br), 0.88 (3H, br t).

Oxidation of 1

The glycerol ether **1** (69 mg) was suspended in 15 ml of an H_2O solution of 2 mmol $NaIO_4$ and 0.2 mmol $KMnO_4$ (4). The mixture was stirred at room temperature for 4.5 hr. The aqueous suspension was extracted with CH_2Cl_2 (3 × 10 ml). The combined CH_2Cl_2 extracts were reduced to a thick oil which was dissolved in 5 ml acetone. This solution was then treated with Jones reagent (5). Reaction was complete in 20 min, whereupon the excess oxidants were destroyed by addition of isopropanol. The solvents were evaporated in vacuo and the residue was suspended in H_2O and extracted with Et_2O (5 × 10 ml). The combined Et_2O phase was dried over Na_2SO_4 and evaporated to give an oily white solid, which was permeated through Sephadex LH-20 with CH_2Cl_2/MeOH (1:1) to give, in separate fractions, heptadecanedioic acid [^{1}H-NMR ($CDCl_3$): δ 2.33 (4H, t, J = 7), 1.59 (4H, m), 1.25 (22H, br); MS: m/z 300 (M^+, 2%), 299 (4), 265, ($M - H_2O$, OH, 5) 224 ($M - OH$, CH_2CO_2H, 6), 140 (11), 126 (15), 125 (20), 112 (44), 111 (40), 98 (100), 97 (53), 83 (30), 82 (58), 81 (57), 73 (36), 71 (48), 69 (70), 43 (72)], and heptanoic acid [MS: m/z 130 (M^+, 1.5%), 129 (7), 115 (10), 101 (15), 98 (9), 87 (30), 83 (10), 74 (15), 73 (50), 69 (20), 61 (28), 60 (100), 57 (50), 55 (67), 45 (45), 43 (84).

RESULTS

Florisil chromatography of the dichloromethane soluble extracts of both *C. alloclada* and *U. ruetzleri* gave large, moderately polar fractions whose ^{1}H-NMR spectra indicated the presence of multiply oxygenated aliphatic compounds. A sequence of gel permeation chromatography, reverse-phase low pressure chromatography and decolorization with charcoal yielded **1**, an amorphous white solid. The ^{13}C-NMR spectrum of **1** indicated the presence of four carbons bearing heteroatoms, three methylenes and one methine, and the infrared (IR) spectrum revealed the existence of a hydroxyl group or groups (3600, 3400 cm^{-1}). ^{1}H-NMR decoupling experiments failed to disclose conclusively the disposition of the functional groups, because of the close proximity of the signals for seven hydrogens between δ 3.45 and 3.85. Acetylation of the natural product gave the diacetate **2**, which established the presence of two hydroxyls in **1** and dispersed the proton signals sufficiently for

definitive structural assignment by means of decoupling experiments. Irradiation of the one proton multiplet at δ 4.87 resolved the doublets of doublets at δ 4.32 and 4.16 to a pair of mutually coupled doublets, assigned to a methylene bearing an acetate. This irradiation also reduced the broad two proton doublet at δ 3.52 to a singlet. Irradiation of the signal at δ 3.45 simplified the multiplet at δ 1.52, which was, in turn, shown to be coupled to protons in the large signal at δ 1.25. Since the multiplets at δ 3.45 and 3.52 were virtually unchanged from the corresponding signals in **1**, those methylenes were assigned to an ether linkage. These data, then, established a glycerol unit with an ether linkage at a primary carbon.

The presence of two olefinic protons in the ^{1}H-NMR spectrum, the absence of IR absorptions near 970 cm^{-1} and the ^{13}C-NMR chemical shift of the olefinic carbons (6) indicated that a *cis*-monoalkenyl group comprised the remainder of the molecule. High resolution mass spectroscopy provided the molecular formula, $C_{27}H_{54}O_3$, and the fragmentation pattern suggested that the olefinic linkage resided between C-17 and C-18 (m/z 125, C_9H_{17}, and m/z 71, C_5H_{11}: allylic cleavages).

To determine unequivocably the position of the double bond, the glycerol ether **1** was submitted to consecutive Lemieux (4) and Jones (5) oxidations. Not only was the olefinic bond cleaved, but the glycerol moiety was degraded as well, resulting in formation of heptadecanedioic acid and heptanoic acid. These products confirmed the structure assignment of **1** as 17*Z*-tetracosenyl glycerol-l-yl ether.

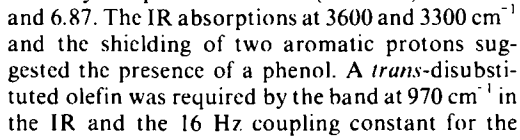
Batyl alcohol, the l-glycerol ether of octadecanol, **5**, was isolated in a similar manner from the *U. ruetzleri* extracts; its identity was established by examination of the ^{1}H-NMR and MS data. The quantity obtained was roughly 36% that of **1**. No batyl alcohol was detected in the *C. alloclada* extracts.

The minor constituent **3** was obtained during the purification of **1** and **5** from *U. reutzleri* and was readily identified from the ^{1}H-NMR and IR spectra. The *para*-disubstituted benzene ring was indicated by the pair of doublets ($J \sim 8$ Hz) at δ 7.43 and 6.87. The IR absorptions at 3600 and 3300 cm^{-1} and the shielding of two aromatic protons suggested the presence of a phenol. A *trans*-disubstituted olefin was required by the band at 970 cm^{-1} in the IR and the 16 Hz coupling constant for the olefinic protons. The highly conjugated ketone carbonyl (1670 cm^{-1}) and the deshielded methyl group completed the proposed structure. We suspect that **3** may be an artifact derived from the aldol condensation of the acetone used for extraction with *p*-hydroxybenzaldehyde, a common naturally occurring compound; we recently found the related compound **4** in the tunicate *Eudistoma olivaceum* (7).

DISCUSSION

While batyl, chimyl (the glycerol ether of hexadecanol) and related unsaturated alcohols are widely distributed in nature, having been found in elasmobranch fish oils (8), human milk and bone marrow (9), atherosclerotic human aortas (10), pig spleens (11), the fat of neonatal calves (12), and, most recently, in the colostrum and milk of cows, goats, pigs and sheep (13), the isolation of **1** as the principal glycerol ether from two quite different sponges represents an intriguing departure from the ethers found in vertebrates. These results provide corroboration of the concept set forth by Litchfield (14) that at least some sponges display unusual lipid metabolism. Morales and Litchfield (15) have found, for example, that 48% of the fatty acids in *Microciona prolifera* consist of C_{24} to C_{28} chain lengths, including 2.2 weight percent of 17-tetracosenoic acid. They suggested that these long-chain fatty acids would generate unusual physical parameters in the lipid bilayer. It seems likely, then, that **1** would also contribute to the unique character of sponge membranes.

Hallgren and Larsson (9) have suggested that the discoveries of glycerol ethers in milk, fetal and neonatal tissues, and rapidly reproducing bone marrow indicate probable growth stimulant activity in these compounds. This premise has been buttressed by the isolation of glycerol ethers in colostrum, which is values for its beneficial effects on the newborn. Selachyl alcohol has been shown to stimulate the growth of *Lactobacillus lactis* (16). Noteworthy as well are the observations that the effectiveness of radiation treatments in cancer therapy was enhanced (17,18) and that some amelioration of radiation sickness was achieved (19) by administration of glycerol ethers. It is possible that these compounds serve a similar function as growth factors in sponge tissue; additional investigation of these compounds and their function in sponges is clearly warranted. Glycerol



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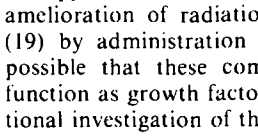
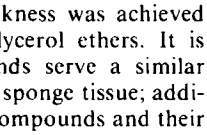
2 R = $COCH_3$



3



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ethers may be widely distributed in sponges; *Spongia* sp. was recently reported to contain uncharacterized glycerol ethers as 0.4-3.7% of total lipids (20).

The alcohol portion of **1**, 17*Z*-tetracosenol, while relatively uncommon, has been found in such diverse sources as the Harderian glands of rats (21), as wax esters, and as a major volatile in the scent mark of the Marmoset monkey, *Saguinus fuscicollis* (22), as the butyrate ester.

The antimicrobial activity exhibited by *C. allo-clada* is not due to **1**. Since there are numerous reports in the literature on the formation of glycerol complexes with metals, including nickel (23-25), the possible role of glycerol ethers in the chelation and concentration of nickel in *U. ruetzleri* is now under investigation in this laboratory.

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Complex Formation in Sonicated Mixtures of β-Lactoglobulin and Phosphatidylcholine[1]

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ABSTRACT

β-Lactoglobulin, the major whey protein of bovine milk, is secreted via the endomembrane system of the mammary gland. The primary structure of β-lactoglobulin shares certain characteristics with membrane proteins, although the soluble protein assumes a globular conformation. We have prepared complexes of β-lactoglobulin and phosphatidylcholines by dissolving both in a helix-forming solvent (chloroform methanol). The complex is stable when transferred to aqueous solutions and sonicated to form vesicles. Both ionic and hydrophobic interactions appear to be involved in complex formation. We have used spectroscopy (circular dichroism, fluorescence, and nuclear magnetic resonance) and electron microscopy to study these complexes. At pH 3.7, the small, single bilayer vesicles produced by sonication are protected against aggregation by the presence of the protein. As determined by circular dichroism, the proportion of α-helix in β-lactoglobulin is increased by complexation with phosphatidylcholine. Circular dichroism and fluorescence spectra show the involvement of at least 1 tryptophan residue in the conformational change. At pH 7.2, β-lactoglobulin-phosphatidylcholine vesicles form aggregates as observed by electron microscopy and ^{31}P nuclear magnetic resonance spectroscopy. These aggregated vesicles could be resuspended by raising the pH. The ability of the partially unfolded β-lactoglobulin to interact with lipids is believed to be important to its transport through the endomembrane system.
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The secretion of proteins involves their transport across membranes. The milk system, involving several classes of proteins and membrane complexes, is particularly difficult to study (1). The use of a model system allows us to focus on the interactions of one protein with a specific lipid membrane component. Our model consists of β-lactoglobulin, a secreted protein for which detailed primary (2) and secondary (3-5) structural information is available, and phosphatidylcholine, a typical membrane lipid. β-Lactoglobulin, the major whey protein of bovine milk, has been studied extensively because of its ability to form complexes with itself (6) and other proteins (7-9). As a secretory protein, β-lactoglobulin is known to pass through the membranes of the endoplasmic reticulum. Mercier and Gaye (10) have shown that a conformational change is associated with the transport of β-lactoglobulin across this membrane. These authors also found that, when β-lactoglobulin is synthesized in vitro in the presence of microsomes, its signal peptide is cleaved while the protein is passing through the membrane, and the protein then assumes its native conformation. In a microsome-free system, the signal peptide is not cleaved and is not accessible to enzymatic attack after the protein folds (10). Although its physiological function has not yet been determined, β-lactoglobulin does share certain structural characteristics with membrane proteins. The average hydrophobicity of β-lactoglobulin is 1230 on the Bigelow scale (11), higher than most soluble proteins. The ratio of charged to nonpolar residues as calculated by the method of Barrantes (12) is 1.13, placing β-lactoglobulin between the integral (average value 0.59) and the peripheral membrane proteins (average value 1.37) in this characteristic. Segrest and Feldman (13) have suggested that residues 130-143 of β-lactoglobulin (2) may form an amphipathic helix, similar to those found in lipoproteins and some membrane proteins.

Protein-detergent models have been used to study membrane-like behavior. The interactions of β-lactoglobulin with sodium dodecyl sulfate (SDS) have been extensively studied (4,14-16). The choice of dipalmitoyl phosphatidylcholine (DPPC) as the major lipid of interest here is based on the occurrence of 16:0 as one of the major acyl chains in bovine milk phospholipids (17). Additionally, studies of free fatty acid binding (18) and polymer bound alkane binding (19) showed β-lactoglobulin to have a strong preference for palmitate. Interactions of β-lactoglobulin with phosphatidylcholines were first reported by ourselves (20), more recently others have demonstrated similar interactions for another whey protein, α-lactalbumin (21,22). The results reported herein describe the properties of the sonicated complex formed between solvent-treated β-lactoglobulin and phosphatidyl-

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cholines as studied by optical spectroscopy (ultraviolet (UV) absorption, circular dichroism (CD), fluorescence), ^{31}P nuclear magnetic resonance (NMR), and electron microscopy.

MATERIALS AND METHODS

β-Lactoglobulin was isolated from bovine milk and purified by the method of Aschaffenburg and Drewry (23); homogeneity was established by SDS-polyacrylamide gel electrophoresis. DPPC and dimyristoyl phosphatidylcholine (DMPC) were purchased from Sigma* and used without further purification. Each lipid gave a single spot when chromatographed on silica gel plates using $CHCl_3/CH_3OH/H_2O$ (65:25:4). All other chemicals were of reagent grade.

Sample Preparation

Homogeneous lipid-protein solutions were prepared by diluting 1 vol of β-lactoglobulin (≤10 mg/ml) in 0.14 M KCl with 5 vol of $CHCl_3/CH_3OH$ (2:1). The precipitated protein was then redissolved by dropwise addition of 3% HCl in CH_3OH. Dry lipid was added and the mixture vortexed to give a clear solution. Solvent was removed under a stream of dry N_2, leaving a lipid-protein film. Final traces of solvent were removed by storing the sample overnight under high vacuum. The films were dispersed in 0.02 M Tris in 0.14 M KCl, pH 7.2, or 0.02 M acetate in 0.14 M KCl, pH 3.7, and adjusted to the buffer pH with 0.1 M KOH. Values for pH were chosen to be above and below pH 5.2, the isoionic point of β-lactoglobulin. Small unilamellar vesicles were formed by sonicating the aqueous mixtures at 50 C under N_2 for 30-60 min with a Heat Systems W185 sonifier equipped with a microtip (24). Centrifugation at 100,000 g for 1 hr removed titanium particles and multilamellar or aggregated vesicles. Both DPPC and DMPC, gave similar results in these experiments when the sonication temperature was above the lipid transition temperature. All illustrations used here were obtained with DPPC.

Analytical

The concentration of β-lactoglobulin in suspensions at pH 3.7 was determined from the absorbance at 278 nm ($E^{1\ mg/ml}_{1\ cm} = 0.96$) (3) after correction for light scattering by the extrapolation of log A vs log λ between 400 and 320 nm, where the protein does not absorb; such corrections were typically ca. 0.01 A. At pH 7.2, the protein-lipid complexes aggregated, and Peterson's modification (25) of the Lowry method (26) was used to determine protein content. Lipid concentrations were determined by the method of Ames and Dubin (27) for total phosphate. Sucrose density gradient (10-25%) centrifugation (16 hr at 200,000 g) was used to estimate the ratio of lipid to protein in the vesicle complex.

*Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

Optical Spectra

A Cary (Model 14) spectrophotometer was used to record absorption spectra. A Jasco 41-C spectropolarimeter calibrated by the 2-point method of Chen and Yang (28) was used for CD measurements. The solutions used were visually clear, and the extrapolated baseline (log A vs log λ) was not greater than 0.01 A at 200 nm in the 0.05-cm cell used for far-UV CD spectra. Fluorescence measurements were made with an Aminco Bowman spectrophotofluorimeter operated in the ratio mode. Inner filter effects were minimized by using a 2.9-mm pathlength cell and diluting samples having $A^{1\ cm}_{280\ nm} > 0.15$. All reported spectra are the average results of at least 5 determinations for which the variability was not greater than 10% at 210 nm.

Nuclear Magnetic Resonance

The ^{31}P resonance decoupled spectra were obtained at 24.15 MHz with ca. 15W decoupling power and a 0.5 KHz bandwidth. The proton decoupler was centered at 47.80 kHz, corresponding to a δ of 5.0 ppm on the proton resonance scale relative to external TMS. A JEOL FX 60Q spectrometer equipped with temperature control and field stabilization via a deuterium lock was operated in the Fourier transform mode. Free induction decays were obtained from 10,000 to 100,000 transients with a delay time of 0.48 sec and a pulse angle of 90°.

Electron Microscopy

Vesicles were deposited on 200 mesh copper grids, negatively stained with phosphotungstic acid, at the appropriate pH, and viewed in a Zeiss 10-B electron microscope operating at 60 kV.

RESULTS

Sonicated vesicles provide a well characterized system (29) suitable for optical and magnetic resonance spectroscopy. These techniques allow us to observe both the effect of the lipid on the protein and of the protein on the lipid. Measurements were made at pH 3.7 and 7.2 to allow variation in the charge distribution on β-lactoglobulin while maintaining the pH-dependent monomer-dimer equilibrium of the protein (30).

Centrifugation of mixtures of native β-lactoglobulin and phosphatidylcholine resulted in separation of a multilamellar lipid pellet and a lipid-free protein solution. The behavior of sonicated mix-

tures of DPPC with native β-lactoglobulin was pH-dependent, but did not appear to involve lipid-protein interactions. At pH 7.2, the supernatant resulting from sonication and centrifugation of a mixture containing 4 mg/ml DPPC and 1 mg/ml β-lactoglobulin contained all of the protein, and 90-95% of the lipid. The supernatant was clear and the UV and CD spectra were identical with those of β-lactoglobulin in the absence of lipid. At pH 3.7, the initial supernatant contained all of the protein and 55-60% of the lipid. The turbidity of this supernatant increased with time so that when the sample was recentrifuged 16 hr later, only 20% of the lipid remained suspended. Essentially all of the protein remained in the supernatant and had the spectral properties of native β-lactoglobulin. These data show that native lactoglobulin did not interact with DPPC. On the other hand, when the lipid was added to β-lactoglobulin dissolved in the H_2O/$CHCl_3$/CH_3OH/HCl system, interactions did occur. The physical behavior of such complexes after removal of the solvent, resuspension in buffer, sonication, and centrifugation depended on whether the pH was above or below the isoionic point (pH 5.2) of the protein. Immediately after centrifugation, all samples were essentially clear, indicating that the lipid was present primarily as a suspension of single bilayer vesicles. Except in the case of the lipid-protein mixture at pH 7.2, most of the lipid was suspended and only very small pellets, consisting primarily of titanium from the sonication probe, were formed. At pH 3.7, the turbidity of a DPPC vesicle suspension without protein increased more rapidly than did that of an equal concentration of DPPC sonicated with the solvent-treated protein (Fig. 1). At pH 7.2, the DPPC vesicle suspension without protein was stable, but the inclusion of solvent-treated β-lactoglobulin led to pelleting of both lipid and protein. From sucrose density gradient centrifugation, the lipid-protein ratio for sedimenting complexes was determined to be 20 ± 5, in good agreement with the binding capacity of β-lactoglobulin for SDS (15). Significant changes in salt concentration interfered with formation of these complexes, in that at low ionic strength, stable vesicles were not formed, while at higher ionic strength the protein did not bind the lipid.

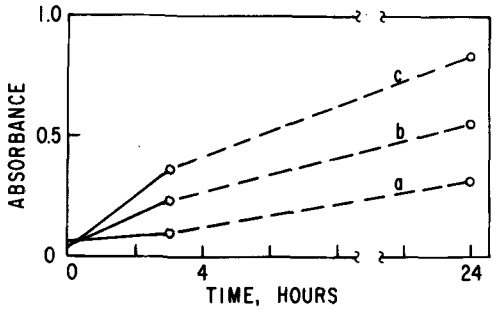

FIG. 1. Time-dependence of the turbidity of the supernatant from sonicated, centrifuged lipid, and lipid-protein samples; (a) 4 mg/ml DPPC, 1 mg/ml β-lactoglobulin, pH 3.7; (b) 4 mg/ml DPPC, pH 7.2; and (c) 4 mg/ml DPPC, pH 3.7. Sonication and centrifugation of 4 mg/ml DPPC with 1 mg/ml β-lactoglobulin at pH 7.2 left little protein or lipid in the supernatant.

Absorption and CD Spectra

Sonication did not significantly affect the spectral properties of native β-lactoglobulin. The shape of the UV-absorption band (Fig. 2) and the correlation between absorptivity and protein concentration were maintained. Solvent-treated β-lactoglobulin is relatively insoluble; it tends to adhere to the glass and is not easily resuspended in aqueous buffer at pH 3.7 or 7.2. That portion which does dissolve tends to renature, as can be seen in the absorption spectrum (Fig. 2) and the near-UV CD spectrum (Fig. 3). When sonicated and centrifuged in the presence of DPPC (2:1,w/w) at pH 3.7, virtually all of the solvent-treated protein was in the supernatant, along with most of the lipid and the suspension formed was reasonably clear (Fig. 2).

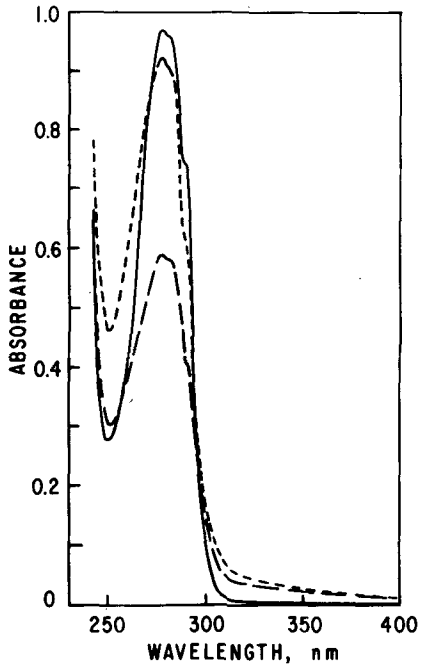

FIG 2. Absorption spectra of sonicated, centrifuged samples of β-lactoglobulin in 0.02 M acetate, 0.14 M KCl, pH 3.7; (——) native protein 1 mg/ml, (—— ——) an equivalent aliquot denatured as in the text, and (----) with 2.5 mg/ml DPPC included.

The CD spectrum of native β-lactoglobulin was in agreement with published spectra (3,4,14). The far-UV CD spectrum of native β-lactoglobulin (Fig. 3a) shows a negative dichroic peak in the 215-218 nm region typical of β-structures (3,4). The conformation of native β-lactoglobulin as determined by CD (3,4,14) and infrared (5) consists of about 10% α-helix with the remainder equally divided between aperiodic and β-structures. The shoulder at 230 nm in the spectrum of the resuspended solvent-treated protein indicates an increase in aperiodic structure and a decrease in β-structure (31). Evidence for ca. 10% α-helix (32) can be found in spectra of both native and solvent-treated β-lactoglobulin when dissolved in aqueous buffer. The double minimum at 222 and 208 nm in the spectrum is characteristic of α-helical structures and is seen only for proteins with at least 25% helix (32). The far-UV CD spectrum of β-lactoglobulin in the lipid-protein complex thus clearly indicates an increase in α-helical structures to at least 25-30%. If, despite our precautions, there are minor light-scattering effects, they would tend to reduce the apparent intensity of the CD signal (33) so that this estimate of helical structure may be considered as a minimum value.

The near-UV CD spectrum (Fig. 3b) of native β-lactoglobulin exhibits negative peaks at 293 and 285 nm, and a shoulder at 266 nm. Both the 293 and 285 nm peaks have been attributed to tryptophan (3,4). The near-UV CD spectrum of β-lactoglobulin in the lipid-protein complex consists of a broad, negative peak centered at 285 nm with a shoulder at 266 nm. The disappearance or at least diminution and nonresolvability of the 293 nm peak combined with the 285 nm minimum suggest that 1 of the 2 tryptophan residues per monomer is affected by the formation of the lipid-protein complex.

Fluorescence

The intrinsic fluorescence of β-lactoglobulin in the DPPC vesicle complex at pH 3.7 is 10% greater than that of the native protein. The emission maximum occurs at 333 nm for the native protein, as it does for the complex (Fig. 4).

NMR

Nonsonicated DPPC liposomes exhibit an axially symmetric proton decoupled ^{31}P NMR spectrum characterized by a broad peak with a shoulder ca. 40 ppm downfield, due to the chemical shift anisotropy of phosphate phosphorus in an environment with restrictive motion (34). Sonicated DPPC vesicles (Fig. 5a) exhibit only a narrow line 14 ppm downfield from the high-field maximum of the liposome pattern. The spectra of liposomes and vesicles are not appreciably affected by changes in pH nor by the presence of native β-lactoglobulin. The ^{31}P NMR spectrum of sonicated β-lacto-

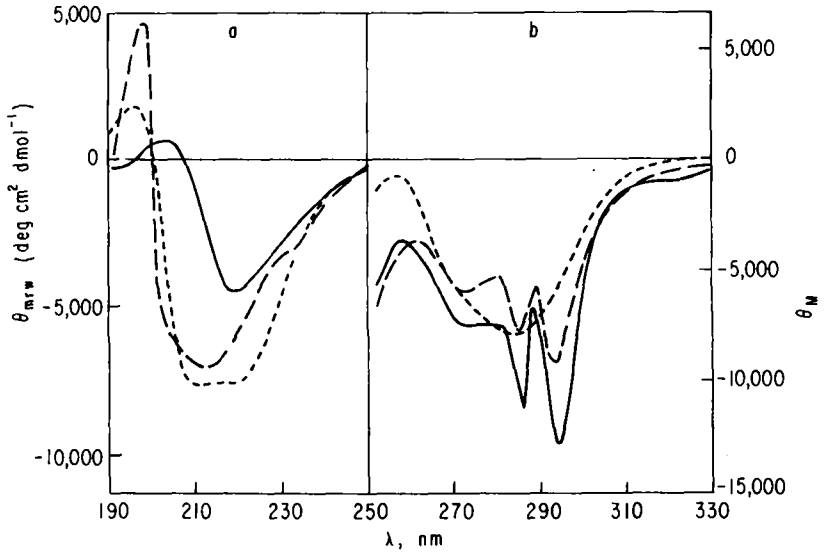

FIG. 3. The far-UV (a) and near-UV (b) CD spectra of (——) native β-lactoblobulin, (— — —) denatured, redissolved β-lactoglobulin, and (----) complex of denatured β-lactoglobulin with DPPC vesicles. All samples in 0.02 M acetate, 0.14 M KCl, pH 3.7. θ_M based on molecular weight of 18,000, θ_{MRW} based on a mean residue weight of 113. All spectra shown are averages of at least 3 separate determinations. Pathlengths of 1.0 cm in the near-UV and 0.05 cm in the far-UV were chosen to assure $A < 2.0$ at the λ recorded.

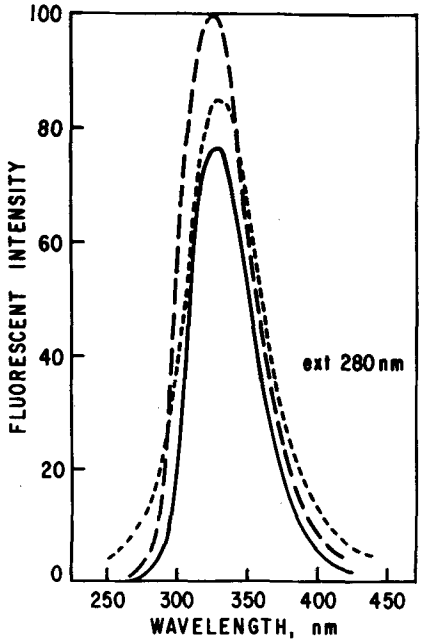

FIG. 4. Fluorescence emission spectra of sonicated, centrifuged samples of β-lactoglobulin in 0.02 M acetate, 0.14 M KCl, pH 3.7; (——) native protein, (----) denatured protein, and (— — —) protein-DPPC vesicle complex. Protein concentration 0.15 mg/ml.

globulin-DPPC complex at pH 3.7 closely resembled that of DPPC vesicles without protein; at pH 7.2 the ^{31}P NMR spectrum of the sonicated complex (Fig. 5b) contained elements of both vesicle and liposome spectra, but was not simply the sum of these. The restricted motion of the phosphorous seen here could be attributed to either fusion or aggregation of vesicles. When titrated to pH 11 where the solvent-treated protein becomes more soluble, the sample became less turbid and the ^{31}P NMR spectrum (Fig. 5c) of sonicated vesicles appeared, indicating that the earlier spectrum was due to the aggregation of vesicles, not fusion.

Electron Microscopy

Electron microscopy of the sonicated lipid-protein complex at pH 3.7 (Fig. 6) shows vesicles in the 200-600 Å range; while a few clusters are seen, they are generally not aggregated. Vesicles of DPPC at this pH appear to fuse so that only a few, much larger (1000-1500 Å) vesicles are seen in the micrographs (Fig. 6b). At pH 7.2, all of the vesicles were in the 200-600 Å range. Those containing lipid-protein complexes are flattened and aggregate to form stacks (Fig. 6c). DPPC vesicles at this pH appear as a uniform dispersion (Fig. 6d). β-Lacto-

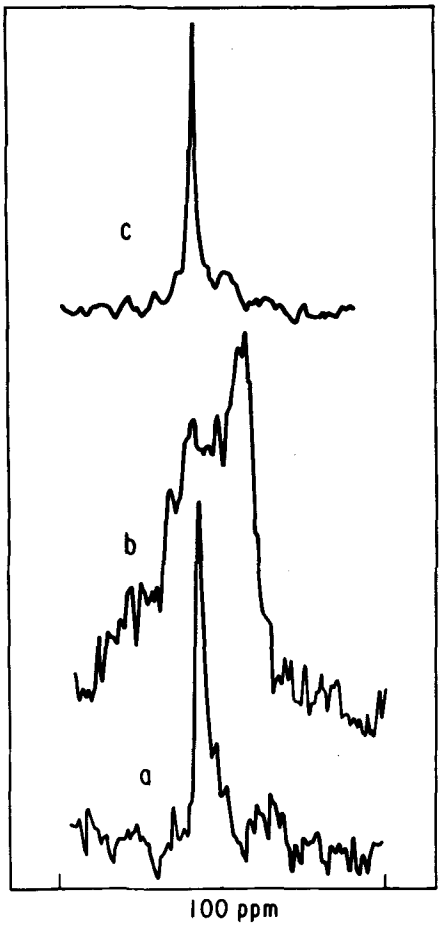

FIG. 5. 24 MHz proton decoupled ^{31}P NMR spectra obtained from sonicated samples of (a) DPPC at pH 7.2; (b) 1:25 (w/w) mixture of β-lactoglobulin with DPPC at pH 7.2; and (c) after titration of (b) to pH 11.

globulin which has been solvent-treated and sonicated at pH 3.7 appears as 42 Å particles, suggesting that little aggregation occurs. This dimension is in close agreement with the reported size of the β-lactoglobulin monomer (30)

DISCUSSION

The results presented here show that sonicated vesicles containing a complex of β-lactoglobulin and DPPC can be formed. Treatment of the protein with a helix-forming solvent is necessary as the native protein does not complex with the lipid. While this may appear to be drastic treatment for a water-soluble protein, it is similar to the conditions used in the isolation of some membrane proteins (35). Additionally, the lipid must be mixed with the protein while it is in the helical conformation because of β-lactoglobulin's tendency to renature

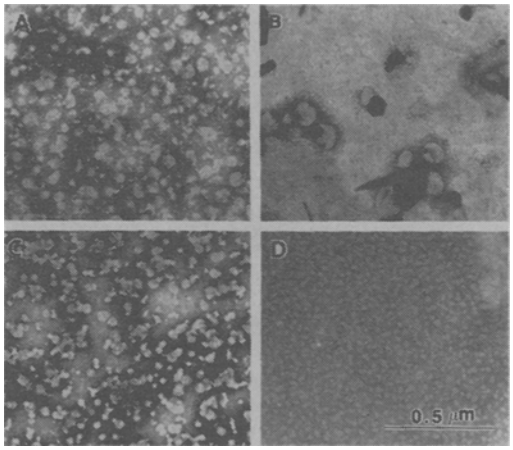

FIG. 6. Electron micrographs of phosphotungstic acid-stained vesicles: (A) DPPC with β-lactoglobulin, pH 3.7; (B) DPPC, pH 3.7; (C) DPPC with β-lactoglobulin, pH 7.2; (d) DPPC, pH 7.2. The lipid concentration is 0.1 mg/ml.

when transferred to an aqueous medium. This ability of the protein to renature when returned to a more native environment suggests that other proteins treated similarly may also be studied in their native conformations. Sonication must be done at a temperature above the lipid transition point, but the resulting complex can then be studied at room temperature.

Cosonication of native protein with phospholipid, a less drastic procedure than that used here, was found to bind immunoglobulin G to phosphatidylcholines (36). However, we have shown that sonication does not alter the conformation of β-lactoglobulin and that DPPC vesicles formed in the presence of native β-lactoglobulin behave as though there was no protein present. Additionally, cosonication of native β-lactoglobulin with DPPC does not lead to any significant encapsulation. However, the small capture volume of phosphatidylcholine vesicles (0.1 μl/μM) (37), and the low concentration of β-lactoglobulin (<0.06 mM) used here may account for this.

Increases in α-helix are a common indication of lipid complexation by serum lipoproteins (38) or lipid-binding polypeptides (33). The helical content of β-lactoglobulin increases from 10% to ca. 45% in 0.02 M SDS (14) and to nearly 60% in acidic CH_3OH (3). Our $H_2O/CHCl_3/CH_3OH/HCl$ solvent system should induce at least 50% helix; unfortunately, the high concentration of $CHCl_3$ prevents the measurement of the far-UV CD spectrum in this solvent system. The 25-30% helical content observed for a β-lactoglobulin-phosphatidylcholine complex is less than the solvent-induced helix, but is more than twice that of the native protein. Application of the Chou-Fasman (39) procedure to the amino acid sequence of β-lactoglobulin (2) results in an overestimation of helical content, that is, the potential for forming more helix than can be seen in the CD spectrum of the native protein. Some of these potentially helical regions are buried in the interior of the native protein so that the addition of lipid does not by itself cause their exposure and subsequent binding. However, once these regions are exposed by the organic solvent, they can participate in lipid binding which then stabilizes their helical conformation. The CD spectrum of β-lactoglobulin-DPPC vesicle complex shows a helical content consistent with the predictions of Chou and Fasman (39).

The emission maximum for either native β-lactoglobulin, or the β-lactoglobulin-DPPC vesicle complex occurs at 333 nm; however, the latter occurs with a 10% increase in intensity. In systems where the protein-phospholipid interaction increases the hydrophobicity of the environment of 1 or more tryptophan residues, a blue shift in the intrinsic fluorescence is likely to be seen; for mellitin, the shift is from 352 to 333 nm (40), for glucagon it is from 350 to 338 nm (35), and for very low-density lipoprotein it is from 350 to 345 nm (38). Precedent does exist in hydrophobic myelin protein where the maximum is at 329 nm with or without lipid (41). The binding of long-chain fatty acids to β-lactoglobulin increases the fluorescence intensity by 8% but does not shift the emission maximum (18). The formation of a β-lactoglobulin-DPPC vesicle complex apparently does not increase the hydrophobicity of the environments of tryptophans 19 and 61. However, an emission maximum of 333 nm suggests relatively hydrophobic environments for these residues in the native protein. The increased fluorescence yield is believed to be an effect of the conformational change seen in the CD data. While this conformational change does not affect the average hydrophobicity of the tryptophan environment, it may increase the distance between them and a carboxyl or other charged residue, thus decreasing fluorescence quenching.

At pH 3.7, observations obtained by both electron microscopy and ^{31}P NMR confirmed the presence of small, uniform vesicles in sonicated mixtures of denatured β-lactoglobulin and DPPC. The protein conformation as determined by CD in this system shows that β-lactoglobulin is bound to the lipid vesicles. At pH 7.2, evidence for aggregated protein-lipid vesicles is seen in both electron microscopy and ^{31}P NMR data. Titration to a higher pH disaggregates the vesicles possibly with the dissolution of denatured β-lactoglobulin from the outer surface, similar to the observations of Koter et al. (42) on the removal of calcium from calcium-aggregated vesicles.

In view of the CD data, the amphipathic helix model (13) of protein-lipid interaction seems reasonable. According to this model, the initial interaction is between charged amino acid residues in the protein and the polar head group of the lipid, after which the hydrophobic side of the helix is buried in the hydrocarbon chains of the lipid. Among the charged amino acid residues, glutamic acid is an excellent helix initiator (39), and the helix forming potential of positively charged lysine and arginine is enhanced by partial submersion in the lipid (13). Ionizable amino acids comprise 29% of the residues in β-lactoglobulin. Although the net charge per dimer of β-lactoglobulin can vary between +20 at pH 3.7 and −10 at pH 7.2 (43), the degree of lipid (19) and detergent (15) binding is similar at these pH values, indicating that hydrophobic interactions are the more important. The lipid-protein ratio of 20 ± 5 would be sufficient to coat a lipid vesicle (400 Å diameter) with a protein shell if the protein were completely extended. While complete unfolding of the protein is unlikely, a significant portion of the vesicle surface must be covered, or more protein would be expected to bind to the exposed lipid. The variation with pH of the net charge remaining on the protein after interaction with the lipid may account for the aggregation behavior of the complexes. At pH 3.7, one would expect all of the unused charges to be positive and for the protein coated vesicles to repel each other. At pH 7.2, both positive and negative charges should be available, if these are spread over the surface of the vesicles they may neutralize each other by attracting other such vesicles. At pH 11, the excess would be negative charge, only arginine (6 residues per dimer of β-lactoglobulin) would remain positively charged, leading to repulsion and possibly even removal of the protein from the lipid which would no longer be zwitterionic.

Initial ionic attraction to position lipid and protein molecules followed by hydrophobic interactions to stabilize a complex have been proposed to explain lipid-protein binding in other systems (13,18,35,40). Our results are consistent with this model in that the optical spectra, particularly CD, show hydrophobic interactions in the helix formation, while the ionic strength effects imply electrostatic interactions between the lipid and β-lactoglobulin (44).

Secretory proteins, in their native conformations, may not in general form lipid-protein complexes. However, with some degree of disruption, one should be able to obtain a form of the protein which will interact with lipids. Hydrophobicity and helix formation are important factors in the interactions of most membrane proteins and serum lipoproteins with lipids. For secretory proteins, the occurrence of helical structure in the final protein is less important than the potential for forming a transitory helix while transversing the membrane. There is no extended portion, 10 residues or more, of β-lactoglobulin for which the helical potential (39) is less than 1.03. Thus, this is a good illustration for the Engelman and Steitz theory (45) of helical hairpin insertion. According to their theory, the signal peptide orients the process and assures that the peptide will not come out of the membrane on the side of synthesis. Secretion is then driven either by a monatonic decrease in the polar character of the peptide chain from the amino to the carboxy end, or by energy derived from folding the peptide on the exterior side of the membrane. In β-lactoglobulin, residues 60 to 100 (the peptide chain is 162 residues) form the most hydrophobic portion, suggesting that the first pathway is probably not operational. The leader sequence of β-lactoglobulin is cleaved before secretion is complete (10) and the protein starts to fold into its native conformation which has ca. 10% helical structure. This dynamic folding would provide energy to complete the export process. The native structure is then stabilized by the formation of disulfide bonds making the secretory process irreversible.

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The Influence of Chenodeoxycholic Acid on Cholesterol and Bile Acid Turnover in Humans with Cholelithiasis

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ABSTRACT

The in vivo dynamics of cholesterol were determined in 8 individuals who were part of a national double-blind study testing the efficacy of chenodeoxycholic acid ingestion on the dissolution of gallstones. Despite the ingestion of this bile acid in amounts in excess of its normal endogenous flux, the conversion of neutral sterol to chenodeoxycholic acid continued. The flux of neutral sterol to endogenous chenodeoxycholate was lower for the patients ingesting bile acid than for one of the patients on placebo, but was similar to that of the other control and similar to previously published chenodeoxycholate flux in patients with cholesterol cholelithiasis. The remaining flux was on the basis of the very efficiently absorbed dietary chenodeoxycholate. The total cholesterol fractional catabolic rate and flux were not appreciably diminished by the administration of either high or low dose chenodeoxycholate to these individuals with cholesterol cholelithiasis.

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INTRODUCTION

Previous studies of the effect of chenodeoxycholate feeding on the bile acid (1) and cholesterol (2) dynamics of humans have demonstrated marked differences in bile acid transport with little influence on total cholesterol flux. A substantial increment in the pool size of chenodeoxycholic acid has been observed, along with reductions in the pool of cholic acid. The flux of chenodeoxycholic acid through the enterohepatic pool was enhanced when this bile acid was fed. From the data obtained, it was impossible to define how much of the chenodeoxycholate flux originated as a result of the fed bile acid and how much from endogenous sterol conversion to the primary bile acid. The studies described here evaluate this issue and the effect of the feeding of two doses of this dihydroxy bile acid (375 mg and 750 mg daily) on the conversion of cholesterol to the primary bile acids and the total loss of cholesterol from the system.

METHODS

Subjects

The subjects were recruited as part of the National Cooperative Gallstone Study and met the criteria for entrance into the Study (3). They all had asymptomatic cholesterol cholelithiasis and some were modestly hypertriglyceridemic and overweight. The details of their clinical characteristics are described in Table 1. Each of the subjects were under treatment with either placebo, low dose (375

TABLE 1

Clinical Characteristics

Patient		Age (yr)	Weight (kg)	% Ideal weight	Cholesterol (mg/dl)	Triglyceride (mg/dl)	Phenocopy
Placebo							
MP	F	56	90	154	328	153	IIB
PS	M	54	77	117	199	186	IV
EG	M	58	101	166	184	169	IV
RA	F	56	49	104	245	114	N
High dose							
DS	M	50	88	120	156	107	N
CG	F	41	78	129	187	89	N
Low dose							
FB	M	67	77	117	188	118	N
DJ	M	48	92	123	164	170	IV

mg, 953 μmol daily), or high dose chenodeoxycholate (750 mg, 1905 μmol daily) for 5 months prior to beginning the turnover studies. The total plasma cholesterol concentration was determined bi-weekly and observed to be within a coefficient of variation of 14%. All subjects were studied as outpatients, and an estimate of their daily cholesterol intake was provided by a dietician. This ranged between 600 and 950 mg daily for the 8 patients. The subjects' weight varied less than 2 kg during the 4-6 months of study.

The plasma was sampled in these patients a mean of 16 times during the 4-6 month study. Bile was obtained 6-10 times during the course of the study. Care was taken to delay the administration of the oral medication and to maintain the fasting state on the mornings of the bile sampling.

Procedure

The studies were performed exactly as previously described (4), with the exception that the time interval of study was extended out to 6 months in most subjects. Autologous plasma was labeled with an acetone dispersion of [4-^{14}C] cholesterol (New England Nuclear Corporation, Boston, MA) for 2 hr at 37C and subsequently kept at room temperature for an additional 6 hr. The plasma was filtered through a 0.22 μ millipore filter and 10-20 μCi intravenously injected after appropriate bacteriologic and pyrogenic evaluation. Duodenal bile was obtained by a standard procedure employing cholecystokinin (Karolinski, Stockholm). Small aliquots of bile were removed after an overnight fast and at least 15 hr after the last ingestion of medication.

At the conclusion of the study (ca. 4-6 months after the ^{14}C cholesterol injection), subjects were injected with 5 μCi of [24-^{14}C] cholic acid and 2 μCi of [2,4-^{3}H] chenodeoxycholic acid (New England Nuclear) dispersed in 5 ml of buffered saline. Samples of duodenal bile were taken out to 5 days after the administration of the radioactive bile acids.

Chemical Methods

The extraction of plasma and bile and the subsequent processing of neutral sterol in bile acid was exactly as previously described (5). A similar thin layer chromatographic system (iso-octane 10, isopropyl ether 4, glacial acetic acid 5, butanol 3, water 1) was employed with a negligible (5%) contamination of the chenodeoxycholate band with deoxycholate when evaluated by gas liquid chromatography of the methylated trifluoroacetylated bile acids as described (4).

Calculations

The initial model employed for the evaluation of the labeled cholesterol and bile acid data utilized a mammilary 3-compartment system previously described for prolonged cholesterol turnover studies (6). It became apparent that for most of the studies, no improvement in the fit of the data was appreciated by this 3-compartment model when compared to a 2-compartment system. A model proposed by Schwartz et al. (7) incorporated an inflow of newly synthesized cholesterol which had not equilibrated with the plasma pools into both acidic and neutral sterol of bile. These studies were in humans with open enterohepatic systems and the model's relevance to the intact human is unknown. It was not necessary to use such a model to obtain satisfactory fits of this data.

The high coefficients of variation (>100%) which were observed for many of the parameters in the 3-compartment model and the similarities of the total irreversible cholesterol flux to that using a 2-compartment model led to the use of a model previously described (5) with some modifications (Fig. 1). The model was modified so that an unlabeled input was provided for the chenodeoxycholate pool to account for the entry of orally

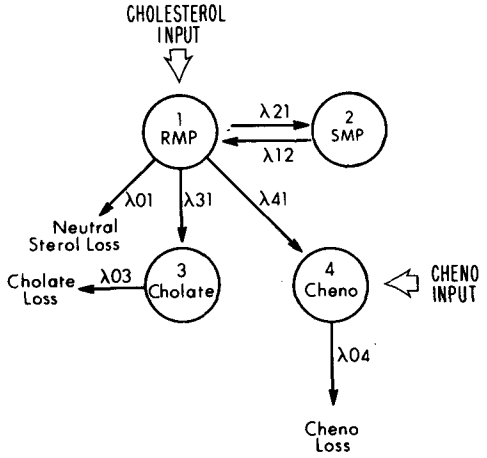

FIG. 1. The model employed to analyze the cholesterol and bile acid activity data of patients taking placebo and chenodeoxycholic acid. Two sources of mass input are indicated, namely cholesterol into compartment 1 of the rapidly miscible compartment and chenodeoxycholic acid into compartment 4. The fractional rates are defined as follows: λ21, fractional rate of transfer of rapidly miscible cholesterol (RMP) to the slowly miscible pool (SMP); λ12, the fractional rate of transfer of SMP cholesterol to the RMP; λ31, fractional rate of cholesterol conversion to cholate; λ41, fractional rate of cholesterol conversion to chenodeoxycholate; λ01, fractional rate of cholesterol loss as neutral sterol; λ03, fractional rate of cholate loss; λ04, fractional rate of chenodeoxycholate loss. The ρ symbol indicates the steady state transport rates in μmol/day for these processes, M represents the size of the pools in μmol.

ingested chenodeoxycholic acid into the system. The solution was run using both the sequential specific activity of biliary cholesterol and primary bile acids obtained from the labeled cholesterol injection and the bile acid activities after the ^{14}C cholate and ^{3}H chenodeoxycholate injections. Dependence relations were written into the input so that the bile acid pool size and fractional turnover rates obtained from the two sets of data would be identical. The SAAM program (8) was employed for the analysis of the data.

RESULTS

All but one of the patients (RA) studied were overweight (Table 1). Three of the 6 patients who had detailed studies of their bile acid kinetics also had modest hypertriglyceridemia (MP; PS; DJ; Table 1). Two of these had a type IV phenotype and the third was IIB by lipoprotein electrophoretic evaluation (9). Changes in plasma cholesterol over the 6 months of the study were less than 10% of the initial value for each of the patients.

The specific activity functions of chenodeoxycholate for the placebo and bile acid treated patients were quite different. Those patients on the placebo had the previously described interception (5) of the chenodeoxycholate specific activity with that of the specific activity curve of the rapidly miscible cholesterol pool (Fig. 2A). The patients taking the chenodeoxycholate, even at a low dose level, had an appreciably lower specific activity of this bile acid when compared to either cholate or the rapidly miscible sterol data (Fig. 2B). No interaction of the bile chenodeoxycholic acid activity with that of cholesterol was observed. This was true for both patients taking the low doses of chenodeoxycholate as well as in the high dose patients.

When the tracer data was analyzed employing the model described in Figure 1 and the SAAM system for analysis, 7 fractional rate constants and their precisions were estimated (Table 2). The coefficients of variations of these parameters were relatively small for those parameters indicating cholesterol catabolism (λ01, λ31, λ41). The fractional removal rates of the bile acids (λ03, λ04) was somewhat less well determined in 3 of the patients (MP, PS and DS). Those patients on a high dose of chenodeoxycholic acid had a somewhat smaller fractional conversion of rapidly miscible cholesterol to both cholic (λ31) and chenodeoxycholic acid (λ41). This was also true for one of the patients taking the low dose of bile acid (FP) whereas the other patient (DJ) had λ31 and λ41 which were well within the placebo patient's range. No trends were apparent for the fractional rate of loss as neutral sterol (λ01) between placebo and chenodeoxycholate-treated patients. This was true for the other fractional rate constants as well.

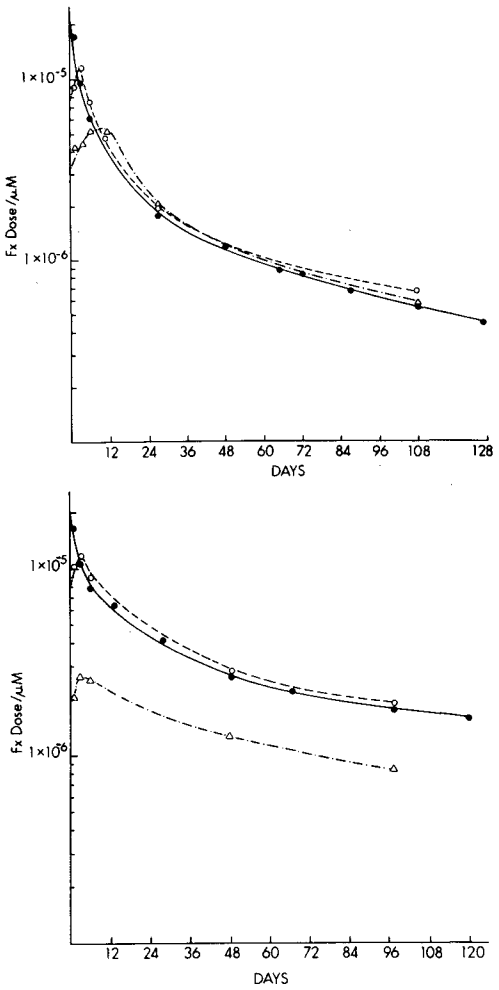

FIG. 2. (A) The sequential plasma cholesterol and biliary bile acid specific activities of patient P.S. taking placebo. ●—● Cholesterol; ○-----○ cholic acid; and △–·–·–△ chenodeoxycholic acid. (B) The sequential plasma cholesterol and biliary bile acid specific activities of patient D.S. taking chenodeoxycholate. ●—● Cholesterol; ○-----○ cholic acid; and △–·–·–△ chenodeoxycholic acid.

The steady-state mass measurements (Table 3) on these 6 patients revealed no trend with respect to the pool size of the rapidly miscible cholesterol pool (M1). This was true as well when this data was normalized with respect to body weight. There was some decrease in the cholate pool (M3) for both the high and low dose chenodeoxycholic acid patients when compared to placebo. An increment in the pool size of chenodeoxycholic acid (M4) was observed for 3 of the patients taking this primary bile acid (Cg, DS, and FB, Table 3) with little

TABLE 2

Cholesterol and Bile Acid Fractional Rate Constants in Placebo and Chenodeoxycholic Acid Treated Patients[a]

Patients	λ21	λ12	λ01	λ31 (day^{-1})	λ41	λ03	λ04
Placebo							
MP	.097 (32)	.035 (27)	.045 (22)	.014 (16)	.0053 (16)	.49 (29)	.24 (39)
PS	.059 (51)	.022 (44)	.009 (12)	.039 (26)	.013 (25)	.63 (35)	.46 (42)
High dose chenodeoxycholate							
CG	.065 (24)	.026 (23)	.021 (19)	.0092 (26)	.0043 (19)	1.05 (19)	.70 (13)
DS	.098 (19)	.035 (21)	.044 (17)	.0064 (23)	.0039 (34)	.42 (44)	.23 (42)
Low dose chenodeoxycholate							
FB	.11 (25)	.056 (24)	.02 (21)	.0059 (16)	.0068 (17)	.41 (19)	.34 (22)
DJ	.073 (20)	.032 (16)	.038 (18)	.018 (23)	.0095 (17)	1.08 (13)	.86 (15)

[a]The rate constant descriptions are provided in Figure 1. The figure within the parentheses represents the coefficient of variation.

TABLE 3

Steady State Cholesterol and Bile Acid Pool Sizes and Fluxes in Placebo and Chenodeoxycholic Acid Treated Patients[a]

Patients	M_1	M_3	M_4	ρ01	ρ31	ρ41	ρ04
	(μ/mol)			(μ mol/day)			
Placebo							
MP	60,241	1,454	1,780	2,710	778	374	
PS	58,823	3,255	2,116	529	2,173	881	
High dose							
CG	74,627	1,000	3,714	1,577	844	321	2,600
DS	67,568	1,041	7,692	2,972	432	264	1,769
Low dose							
FB	56,497	1,062	4,405	1,130	387	384	1,498
DJ	53,191	804	1,710	2,021	913	505	1,471

[a]The nomenclature for pool size and flux is given in Figure 1 and the text.

chenodeoxycholate pool size expansion for the remaining patient (DJ).

The flux of cholesterol as neutral sterol (ρ01) showed little trend for those patients taking the bile acid when compared to the placebo. The flux (μmol/day) of sterol into cholic acid for those patients taking chenodeoxycholic acid was appreciably lower in 2 of the patients (DS and FB) taking a high and low dose of the bile acid, respectively. The flux of the bile acid into the cheno pool was also modestly diminished in both patients taking high dose chenodeoxycholate, but this was less apparent in those taking the low dose of the bile acid. The endogenous synthetic component amounted to a mean of 14% of the total cheno flux (ρ41/ρ04) in the patients taking high dose chenodeoxycholic acid. The endogenous flux comprised a mean of 30% of the total cheno flux in those patients ingesting a lower dose of the bile acid.

Those parameters indicative of the total effective removal of cholesterol from the system, namely the total endogenous catabolic flux (ρ01 + ρ31 + ρ41) as well as the total fractional catabolic rate (λ01 + λ31 + λ41) suggested some decrease in these parameters in 2 of the patients (CG and FB) when compared to the placebo patients (Table 4). These were not particularly striking differences and neither of these patients had the minimal hypertriglyceridemia of the placebo patients which had been associated (10) with an increment in sterol flux. The remaining 2 chenodeoxycholate treated patients (DS and DJ) had catabolic indices fairly similar to those of the placebo-patient treated.

DISCUSSION

The nature of this study did not permit the patients to serve as his or her own control, therefore making the changes induced by chenodeoxycholic acid somewhat difficult to assess. There are some observations which are clear cut and do not require the patient to serve as his own control. It is apparent from the 4 studies of patients taking chenodeoxycholic acid that the absorption and utilization of this bile acid in the enterohepatic circuit at the doses administered was quite good.

TABLE 4

Cholesterol Catabolic Parameters in Placebo and Chenodeoxycholic Acid Treated Patients

Placebo	Total endogenous Flux[a] (mol/kg)	Total fractional catabolic rate (day^{-1})
MP	42.9	.064
PS	46.5	.061
EG	33.9	.053
RA	45.6	.042
High dose		
CG	35.2	.035
DS	41.6	.054
Low dose		
FB	24.7	.033
DJ	37.4	.066

[a]The values represent the sum of neutral sterol output ($\rho 01$) plus that of acidic sterol ($\rho 31 + \rho 41$) divided by the weight in kg. The total fractional catabolic rate represents the sum of $\lambda 01 + \lambda 31 + \lambda 41$.

Three of the 4 patients had a calculated flux of ingested bile acid through the enterohepatic pool which was at least 100% of the oral dose. The remaining patient (DS) calculated to have 80% of his oral dose incorporated into his enterohepatic circuit. This was considerably better than the patients described by Danzinger et al. (1) where absorption ranges from 29% to 84% were noted, with a mean of 60%. These investigators fed considerably more chenodeoxycholate suggesting a saturation of the intestinal uptake mechanism somewhere between the 750 mg to 1500 mg daily load.

The other result that is clear-cut in these studies is that despite the introduction of nearly 2000 μM of chenodeoxycholic acid, the synthesis of chenodeoxycholic acid from cholesterol proceeds. It is possible that this de novo synthesis of chenodeoxycholate is attenuated by the administration of the exogenous bile acid. However, the endogenous synthesis of chenodeoxycholate ($\rho 41$), even in the high dose patients, was not that far from the placebo. Other kinetic studies of cholesterol gallstone patients have demonstrated mean daily chenodeoxycholate synthesis of 186 μM (1) and 264 μM (5) similar to that seen in the patients receiving nearly 2,000 μM of this bile acid. This would indicate that the feedback suppression of bile acid synthesis in man may not be as tightly controlled by bile acid feeding as has been demonstrated (11) for other mammals.

The attenuation in cholic acid synthesis produced by the chenodeoxycholic acid feeding was also relatively modest. The values for all chenodeoxycholate treated patients were within the range of individuals with cholelithiasis previously evaluated by this technique (5). The fractional rates of conversion of cholesterol to cholic acid ($\lambda 31$) were also not substantially different from this previous series. However, the fractional conversion rates for this conversion to cholic acid were somewhat lower in 3 of the chenodeoxycholate treated patients than either of the placebo patients. This variability in cholate-synthetic response was seen as well in the studies of Danzinger et al. (1). Most of the patients in their series showed rather marked decreases in cholate synthesis as well as an enhanced fractional catabolic rate ($\lambda 03$). In this study as well, two of the chenodeoxycholate treated patients showed fractional catabolic rates above 1.0. Although the trends were similar with respect to cholic acid in this study, the decreases in the cholate pool size in the present study using lower doses of chenodeoxycholate were not as striking as seen previously (1) on significantly higher doses. The total flux of bile acid ($\rho 31 + \rho 04$) in this trial was equivalent to the flux of total bile acid reported previously (1) using considerably higher loads of chenodeoxycholate. This might indicate that reasonably similar bile acid deliveries through the enterohepatic system can be achieved with the lesser 750 mg per day load.

There were no real trends observed with respect to the loss of cholesterol as neutral sterol between the chenodeoxycholate treated patients and the placebo patients. The fractional catabolic rate ($\lambda 01$) and the flux ($\rho 01$) were within the rate previously reported for cholelithiasis patients (5). A potential effect of this therapy (12) relates to inhibition of the catabolism of cholesterol when this bile acid was ingested. The data in this study as well as previous data (2) deny this. However, the critical study to define this possibility, namely having the patient as his own control and employing long-term specific activity data, has not been done. However, at this point, there are no strong indications that cholesterol catabolism is significantly impeded or that the readily exchangeable cholesterol pool (M1) is appreciably enhanced. Although some investigators (13) calculate total body sterol content from long-term plasma decay, this calculation is quite tenuous depending upon many assumptions. It is impossible to indicate securely from these data whether the administration of chenodeoxycholic acid does, in fact, expand the total exchangeable sterol pool in man even though catabolic flux was relatively normal.

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Different Fatty Chain Compositions of Alkenylacyl, Alkylacyl and Diacyl Phospholipids in Rabbit Alveolar Macrophages: High Amounts of Arachidonic Acid in Ether Phospholipids

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ABSTRACT

High levels of ether phospholipids were found in rabbit alveolar macrophages. Choline phosphoglycerides (CPG) contained a significant amount of alkylacyl compound (32.5%). On the other hand, ethanolamine phosphoglyceride (EPG) included a very large amount of alkenylacyl compounds (61.2%). Small amounts of alkenylacyl CPG and alkylacyl EPG were also observed. The occurrence of a high amount of alkylacyl CPG may be related to the synthesis or release of platelet-activating factor (PAF) from macrophages. Fatty chains at the 1- and 2-positions in each lipid class of CPG or EPG were considerably different from each other. Particularly, the levels of 20:4 (arachidonic acid) in alkylacyl CPG or alkenylacyl EPG were several times higher than those in corresponding diacyl phospholipids. Large portions of 20:4-containing species have alkenyl or alkyl ether moieties at their 1-position in both CPG (73.6%) and EPG (85.9%). These results suggest the importance of ether-containing phospholipids in rabbit alveolar macrophages.
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INTRODUCTION

Alkenyl or alkyl ether phospholipids are widely distributed in various animal tissues (1), although their biological significance is still obscure. In a preceding study, we have demonstrated that considerable amounts of alkyl ether-containing CPG and alkenyl ether-containing EPG were found in pig lymphocytes (2). Similar results were obtained for rabbit polymorphonuclear leukocytes (3). The high amount of alkylacyl CPG is interesting, since alkylacetyl CPG, known as platelet-activating factor (PAF) (4,5), is released from various types of leukocytes including macrophages (6-10). PAF is now considered to be one of the important mediators of inflammation as well as of acute hypersensitivity. Recently, several investigators demonstrated that 1-alkyl-2-lyso CPG might act as the precursor for PAF synthesis (11-14). It is important, therefore, to examine in detail the properties of ether phospholipids in various kinds of leukocytes.

In the present study, we investigated the contents and the fatty chain compositions of alkenylacyl, alkylacyl and diacyl phospholipids in rabbit alveolar macrophages. We showed that high levels of ether-containing CPG or EPG were observed in these cells and that their fatty acids at the 2-position were considerably different from those of diacyl phospholipids. Particularly, the amount of 20:4 in alkylacyl CPG was ca. 5 times higher than that in corresponding diacyl compounds. Also, alkenylacyl EPG contained ca. 3 times more 20:4 than the diacyl compounds.

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Abbreviations: Fatty chains were designated by number of carbon atoms:number of double bonds, e.g., *18:1 for oleic acid*; CPG, choline phosphoglycerides; EPG, ethanolamine phosphoglycerides; PAF, platelet-activating factor; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade and solvents were distilled before use.

Cells

Alveolar macrophages were prepared by pulmonary lavage with ice-cold 20 mM Hepes-saline (pH 7.2, 4C) from normal healthy rabbits (New Zealand White, 2.5-3.0 kg, female). After filtering through cotton gauze, the cells were collected by centrifugation. Contaminated erythrocytes were removed osmotically, and the cells were washed 3 times with Hepes-saline. The purity of the cells was above 95% by morphological assessment (Wright-Giemsa staining).

Extraction and Fractionation of Lipids

Lipids were extracted immediately after the preparation of the cells by the method of Bligh and Dyer (15). Individual phospholipids were separated by two-dimensional thin-layer chromatography (TLC) (16). Lipid spots were detected under ultraviolet light by spraying with nondestructive fluorescein reagent primuline (17). CPG or EPG were eluted from silica gel with chloroform/methanol/water (1:2:0.8) and extracted by the method of Bligh and Dyer (15). Lipid phosphorus was estimated as described by Rouser et al. (18). The occurrence of lyso-*bis*-phosphatidic acid was con-

firmed according to the earlier study by Mason et al. (19) as follows. (a) The mild alkaline hydrolysis product was identified as glycerophosphoryl glycerol by paper chromatography developed with phenol/water/acetic acid/ethanol (80:20:10:12), using authentic phosphatidylglycerol as a standard. (b) The acetolysis product was identified as acetyl derivative of monoacylglycerol by TLC developed with petroleum ether/ethyl ether/acetic acid (60:40:1). (c) The fatty acid/P ratio was 2.02. These results were very similar to those obtained by Mason et al. (19). Throughout the extraction and fractionation procedures, a small amount of butylated hydroxytoluene was added to the solvents to avoid oxidation.

Separation of Alkenylacyl, Alkylacyl and Diacyl Phospholipids

Alkenylacyl, alkylacyl and diacyl CPG or EPG were separated as 1,2-diradyl-3-acetylglycerol derivatives as described previously (2,20,21). CPG or EPG was hydrolyzed with phospholipase C (*Bacillus cereus*) in 1.0 ml of 0.1 M Tris-HCl buffer (pH 7.2) and 3.0 ml of ethyl ether. Resultant diradylglycerol was extracted and subsequently acetylated with 0.5 ml of acetic anhydride and 0.1 ml of pyridine at 37C for 3 hr. Three types of 1,2-diradyl-3-acetylglycerols as well as 1,3-diradyl-2-acetylglycerols (isomerized products) were separated from each other by TLC developed first with petroleum ether/diethyl ether/acetic acid (90:10:1) and then with toluene and extracted from the plates as described previously (2). The formation of 1,3-diradyl isomers was, however, always very low in this study. The amounts of alkenylacyl, alkylacyl and diacyl compounds were estimated by their fatty acyl quantities, using 17:0 methyl ester as an internal standard (2).

Determination of Fatty Chain Compositions

Fatty acids were analyzed as the methyl ester by gas-liquid chromatography (GLC) (2,21). To investigate the fatty acids at the 1-position of diacyl compounds, snake venom phospholipase A_2 (*Naja naja atra*) was used to remove the fatty acid residues from the 2-position of CPG or EPG. The resulting lysophospholipids were separated by TLC, using the solvent, chloroform/methanol/water (65:25:4) and methylated. The fatty acid compositions at the 2-position of diacyl compounds were calculated from the data for the total fatty acids and those for the fatty acids at the 1-position. Alkylglycerols (glyceryl ethers) were prepared from 1-alkyl-2-acyl-3-acetylglycerols by mild alkaline hydrolysis and analyzed as the trimethylsilyl (TMS) derivatives by GLC (2,20). Fatty aldehydes were liberated from 1-alkenyl-2-acyl-3-acetylglycerols by treating with HCl gas and analyzed by GLC (22).

RESULTS AND DISCUSSION

Phospholipid Composition

Table 1 shows the phospholipid composition of rabbit alveolar macrophages. The lipid phosphorus was estimated as 1.98 $\mu g/10^6$ cells. The predominant components were CPG, EPG and lyso-*bis*-phosphatidic acid. Similar results were demonstrated by Mason et al. (19), although the portion of sphingomyelin was somewhat smaller in our study.

Amounts of Ether Containing and Diacyl Phospholipids

Table 2 shows the class compositions of CPG and EPG of rabbit alveolar macrophages. High levels of alkylacyl compounds were found in CPG (i.e. 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine). On the other hand, EPG included a large amount of alkenylacyl compound (i.e. 1-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine). The amounts of alkenylacyl compounds in CPG and alkylacyl compounds in EPG were small. The total amount of ether-containing CPG and EPG comprised ca. 29% of total phospholipids. In the earlier study, we

TABLE 1

Phospholipid Composition of Rabbit Alveolar Macrophages[a]

Phospholipids	% of total phospholipids[b]
Choline phosphoglycerides	36.1 ± 1.1
Ethanolamine phosphoglycerides	21.7 ± 2.7
Lyso-*bis*-phosphatidic acid	17.7 ± 3.2
Sphingomyelin	8.4 ± 1.2
Serine phosphoglycerides	4.1 ± 0.4
Inositol phosphoglycerides	7.5 ± 1.5
Cardiolipin	4.5 ± 0.8

[a]The mean percentages (weight%) ± SD were taken from different samples (n = 6).
[b]The values were calculated from the lipid phosphorus.

TABLE 2

Class Composition of Choline and Ethanolamine Phosphoglycerides of Rabbit Alveolar Macrophages[a]

Class	CPG[b]	EPG[c]
Alkenylacyl	5.6 ± 1.2[d]	61.2 ± 4.2
Alkylacyl	32.5 ± 1.1	8.1 ± 2.3
Diacyl	61.9 ± 0.7	30.7 ± 3.3

[a]The mean percentages (weight %) ± SD were taken from different samples (n = 6).
[b]Choline phosphoglycerides.
[c]Ethanolamine phosphoglycerides.
[d]The values were calculated from the quantities of acyl moieties in each lipid class.

showed that pig lymphocytes contained high levels of ether phospholipids. In pig lymphocytes, ca. 25% of total phospholipids were accounted for by ether-containing CPG and EPG (2). Recently, Mueller et al. reported similar results for rabbit peritoneal exudate polymorphonuclear leukocytes (3). The biological significance of the high abundance of alkenylacyl EPG has not yet been well elucidated. On the other hand, large amounts of alkylacyl CPG in such leukocytes are particularly noticeable, since a potent lipid mediator, namely PAF, is now regarded as a derivative of alkyl ether containing CPG, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (4,5). It is very probable that PAF is synthesized from alkylacyl CPG by a deacylation-acetylation reaction. Several investigators have already showed the occurrence of acetyl CoA:1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine acetyltransferase (11-14).

Recently, it has been shown that PAF stimulates leukocyte aggregation (23-25), chemotaxis (25-27) and enzyme secretion (23-26). Furthermore, several investigators reported that some of the synthetic alkyl CPG derivatives have antitumor (28,29) or immunopotentiating activity (30-32). Alkyl CPG derivatives also induced the differentiation of leukemic cells (33). It will, therefore, be a very important problem to study the mode of action of PAF as well as the biosynthesis or catabolism of alkyl ether-containing CPG in various types of leukocytes.

Fatty Chain Composition of CPG

The fatty chain composition of CPG of rabbit alveolar macrophages are shown in Table 3. The most predominant fatty chain was 16:0 in each lipid class. On the other hand, 18 carbon fatty chains comprised relatively small proportions. Although the alkyl chain composition of PAF derived from rabbit alveolar macrophages is still unknown, it is interesting to note that naturally occurring PAF from rabbit basophils is mainly composed of the 18 carbon chain (5). Mueller et al. reported that rabbit peritoneal exudate polymorphonuclear leukocytes contained a considerable amount of 20:0 or 22:0 alkyl chains (3). However, we could not find significant amount of alkyl chain other than 16:0, 18:0 and 18:1 in rabbit alveolar macrophages. This difference may be due to the difference in cell types.

The fatty acids at the 2-position of each lipid class were considerably different from each other. The predominant fatty acids at the 2-position of alkylacyl compounds were 20:4 and 18:2. The quantities of 20:4 and 18:2 in alkenylacyl compounds were, however, smaller than those in alkylacyl CPG. Moreover, diacyl compound included only a small amount of 20:4. It is quite noteworthy that the content of 20:4 is very high in alkylacyl CPG compared with alkenylacyl or diacyl compounds. This prominent difference in the quantities of 20:4 between 3 types of CPG was also observed in Ehrlich ascites tumor cells (20). However, the difference was not as remarkable in rabbit peritoneal exudate polymorphonuclear leukocytes (3) or in pig lymphocytes (2) as in rabbit alveolar macrophages. Although 22 carbon polyunsaturated fatty acids comprised only a small portion in each lipid class, their amounts in the ether compounds were higher than those in diacyl CPG.

Fatty Chain Compositions of EPG

Table 4 shows the fatty chain compositions of EPG of rabbit alveolar macrophages. The pre-

TABLE 3

Fatty Chain Composition of Choline Phosphoglycerides[a]

Fatty chain \ Class	Alkenylacyl		Alkylacyl		Diacyl	
Position	1	2	1	2	1	2
	Percentages in each lipid class					
16:0	65.1 ± 3.0	24.6 ± 1.7	83.6 ± 2.0	10.7 ± 0.9	58.2 ± 2.2	22.4 ± 2.8
16:1	–	10.2 ± 1.4	–	1.6 ± 0.6	–	7.6 ± 1.1
18:0	10.4 ± 2.1	6.2 ± 0.7	6.2 ± 0.9	1.0 ± 0.2	22.1 ± 1.2	3.0 ± 1.8
18:1	24.5 ± 3.4	23.0 ± 2.6	10.2 ± 1.6	8.7 ± 1.8	12.7 ± 1.2	20.5 ± 1.9
18:2	–	9.5 ± 2.5	–	25.7 ± 4.6	6.3 ± 0.8	33.8 ± 0.9
18:3 + 20:1	–	1.1 ± 0.2	–	0.7 ± 0.2	0.7 ± 0.3	1.3 ± 0.3
20:3	–	3.0 ± 1.4	–	4.0 ± 0.8	–	1.4 ± 0.6
20:4	–	16.6 ± 2.2	–	38.6 ± 4.9	–	7.8 ± 0.8
22:4	–	0.9 ± 0.5	–	1.9 ± 0.2	–	0.6 ± 0.4
22:5	–	3.4 ± 1.3	–	6.1 ± 1.4	–	1.0 ± 0.8
22:6	–	1.5 ± 0.5	–	1.0 ± 0.2	–	0.6 ± 0.4

[a]The mean percentages (weight%) ± SD were taken from different samples (n = 5). Fatty acids were analyzed as the methyl esters by GLC. Liberated aldehydes were separated by TLC and analyzed by GLC. Glyceryl ethers were analyzed as the trimethylsilyl derivatives by GLC as described in Materials and Methods.

TABLE 4

Fatty Chain Composition of Ethanolamine Phosphoglycerides[a]

Fatty chain	Class	Alkenylacyl		Alkylacyl		Diacyl	
	Position	1	2	1	2	1	2
		Percentages in each lipid class					
16:0		68.1 ± 0.6	3.7 ± 0.5	77.8 ± 2.9	16.7 ± 2.2	10.5 ± 1.9	7.4 ± 1.1
16:1		–	1.8 ± 0.9	–	5.3 ± 1.2	–	4.4 ± 2.2
18:0		30.1 ± 0.5	1.0 ± 0.3	19.1 ± 2.7	4.2 ± 1.2	70.3 ± 3.6	2.2 ± 1.2
18:1		1.8 ± 0.5	11.6 ± 0.9	3.1 ± 0.5	14.7 ± 3.5	14.8 ± 2.0	36.2 ± 1.4
18:2		–	6.3 ± 1.1	–	7.9 ± 1.7	3.7 ± 0.8	20.6 ± 2.3
18:3 + 20:1		–	0.3 ± 0.1	–	0.5 ± 0.2	0.7 ± 0.2	0.9 ± 0.4
20:3		–	2.3 ± 0.5	–	5.3 ± 1.0	–	2.6 ± 1.6
20:4		–	54.1 ± 1.1	–	24.8 ± 0.8	–	18.7 ± 1.0
22:4		–	3.6 ± 1.7	–	4.4 ± 1.5	–	2.2 ± 0.6
22:5		–	13.2 ± 1.1	–	13.5 ± 3.8	–	3.8 ± 0.4
22:6		–	2.1 ± 0.5	–	2.7 ± 1.0	–	1.0 ± 0.4

[a]The mean percentages (weight%) ± SD were taken from different samples (n = 5). Analytical procedures were as in Materials and Methods.

dominant fatty chain at the 1-position was 16:0 in both alkenylacyl and alkylacyl compounds and was 18:0 in the diacyl compound.

The fatty acids at the 2-position of each lipid class showed quite different profiles. The most predominant fatty acid at the 2-position of alkenylacyl compound was 20:4. It comprised more than half of the fatty acyl moieties. On the other hand, the amount of 20:4 in alkylacyl or diacyl compounds was relatively small. Diacyl compounds contained a considerable amount of 18:1 at the 2-position. Furthermore, ether-containing compounds included higher amounts of 22 carbon polyunsaturated fatty acids than diacyl phospholipids.

It seems a unique feature of rabbit alveolar macrophages to contain high amounts of 20:4 in ether phospholipids. High proportions of 20:4-containing species belonged to alkylacyl compounds in CPG (68.5%) or alkenylacyl compounds in EPG (81.0%). Small proportions were also found in alkenylacyl CPG (5.1%) or alkylacyl EPG (4.9%). The content of 20:4-containing species which also possessed acyl moieties at their 1-positions were relatively smaller in both CPG (26.4%) and EPG (14.1%). These observations suggest the possible role of ether-containing phospholipids in the storage of 20:4. A rapid release of 20:4 from stimulated macrophages has already been shown by several investigators (34-39). Enhanced synthesis or release of prostaglandins has been also demonstrated (10,34-39). The metabolism of 20:4 might differ considerably between ether-containing and diacyl phospholipids. Further study will clarify the role and the metabolic features of ether-containing phospholipids in macrophages.

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The Influence of Linoleic Acid Intake on the Kinetics of Adenine Nucleotide Translocase

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ABSTRACT

Dietary enrichment of mitochondrial polyenoic fatty acid content was associated with increased respiratory activity (Abuirmeileh and Elson). The influence of the membrane lipid composition on the adenine nucleotide translocase (AdNT) was studied in rats that were fed diets formulated with beef tallow (BT) to provide low or safflower oil (SO) to provide high contents of linoleic acid. The phosphatidylcholine/phosphatidylethanolamine ratio was 40% higher in the SO mitochondria due primarily to an increase in phosphatidylcholine. SO mitochondria exhibited 25% higher state 3 respiration, 50% higher state 4 respiration and 13% higher net ADP-dependent respiration than did the BT mitochondria. The relative RCR and ADP/O values of the SO mitochondria were slightly but significantly ($p < 0.01$) lower. The kinetics of the AdNT were determined by the back exchange method (Pfaff and Klingenberg). At all assay temperatures, SO mitochondria exhibited a higher K_m for ADP. However, addition of 5 mM carnitine to the assay mixture increased the affinity of the SO-AdNT giving K_m values similar to that of the BT-AdNT. Washing the mitochondria with fat-free BSA had a similar, but lesser, effect. At 25 C and 37 C, the V_{max} of the SO-AdNT were increased by 11-15% ($p < 0.05$) which was independent of either BSA-wash or the presence of carnitine. According to Dixon plot studies, the SO-AdNT had a comparable K_i for atractylate but a slightly lower K_i for palmitoyl-CoA inhibition. The accumulation of acyl-CoA esters in the SO mitochondria was not ruled out. The overall results suggest that changes in mitochondrial membrane lipids accommodated an increased V_{max} of the SO-AdNT, which in turn accounted for that part of the increased state 3 respiration dependent on ADP translocation.
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There is evidence that under physiological conditions the AdNT plays the rate-limiting role for both substrate oxidation and oxidative phosphorylation (1,2). Mak et al. recorded decreased AdNT activity in hepatic mitochondria from thyroidectomized rats (3) and increased AdNT activity in hepatic mitochondria from cold-adapted rats (4), stresses which are recognized to decrease and increase, respectively, rates of oxygen uptake. The lipid patterns of the hypothyroid mitochondria (5) were consistent with a more stable membrane bilayer (6), and those of the cold-adapted mitochondria, with a less stable membrane (4). The kinetic analyses of the AdNT embedded in these lipid matrice revealed changes in the K_m and V_{max} consistent with the changes in AdNT activities (3,4).

The fatty acid (7) and phospholipid (8) patterns of mitochondria respond rapidly in response to alterations in the dietary fat. Abuirmeileh and Elson (9,10) fed diets, isocaloric in fat, but containing 4 or 24% energy as linoleic acid. ADP-succinate-supported oxygen uptake (state 3) was 25% higher and cytochrome c oxidase activity 28% higher in the mitochondria enriched in linoleic acid. Succinate-supported oxygen uptake (state 4), which is independent of the control of the AdNT, was 54% higher in those mitochondria. This observation, coupled with the analyses which showed that the AdNT activity was decreased and phospholipase A activity increased, led Abuirmeileh and Elson (9) to conclude that a remodeling response within the mitochondrial membrane enriched in linoleic acid provided the ADP to support the oxidative activity. However, the AdNT in that study was assayed at 0 C, a temperature well below the membrane transition temperatures which were 13 C and 15.4 C, respectively, according to the Arrhenius plots of cytochrome c oxidase activities in membranes enriched or normal in linoleic acid content (9,10). Calculation of the net ADP-succinate supported oxygen uptake shows that this activity, which could be dependent on ADP translocation, accounted for ca. 70% of the increased state 3 oxygen uptake observed in the linoleic acid-enriched mitochondria. This observation led us to reexamine, at physiological temperature, the influence of the mitochondrial membrane enriched in linoleic acid on AdNT kinetic parameters. In the study below, mitochondria enriched in linoleic acid are shown to have a 13% greater net ADP-dependent oxygen uptake which is consistent with a 11-15% increase in the V_{max} of the AdNT.

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Abbreviations: AdNT, adenine nucleotide translocase; BT, beef tallow; SO, safflower oil; RCR, respiration control ratio; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; and Cn, carnitine.

MATERIALS AND METHODS

Male Sprague-Dawley rats (70 g), purchased from ARS-Sprague-Dawley (Madison, WI), were housed in groups of 3-4 at the Animal Care Unit, U.W. Medical School and maintained on a 12 hr dark-light cycle. The diets, a mixture of 84% fat-free diet mix (Teklad Test Diet, TD 170293, Madison, WI) and 16% fat, provided 35% energy as fat, 49% as carbohydrate and 16% as protein. The mixed diet contained 50.6% sucrose, 0.25% DL-methionine, 16.8% purified casein, 16% fat, 12.6% cellulose, 2.94% mineral mix, AIN-76 (Teklad, 170915) and 0.84% vitamin mix (Teklad, 40060). Edible BT (a gift of Oscar Mayer and Co., Madison, WI) or SO (Hollywood Health Foods, Los Angeles, CA) in the diet provided 1.2 or 26% of total energy as linoleic acid. The diets and water were given ad libitum for 4-5 weeks.

The rats were killed by decapitation at 9 A.M., the livers immediately removed, weighed, washed in chilled medium of 250 mM sucrose, 4 mM Tris-HCl (pH 7.4) and 1 mM EDTA and then homogenized in 8 vol of medium with a Potter Elvehjem-type homogenizer. Liver mitochondria were prepared by differential centrifugation according to the method of Schneider (11). Succinate-supported mitochondrial respiration rate (states 3 and 4) were determined polarographically as described by Estabrook (12).

AdNT activity was measured by the 'back exchange' tehnique of Pfaff and Klingenberg (13). Mitochondria (ca. 60 mg of mitochondrial protein) were loaded with ^{14}C-ATP by incubating at 0-4 C for 45 min with 0.50 μCi of ^{14}C-ATP in 5 ml of medium consisting of 250 mM sucrose, 4 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The ^{14}C-ATP-loaded mitochondria were washed twice and suspended in the above medium at a concentration of 10 mg protein/ml. The back exchange was carried out with 1 mg ^{14}C-ATP-loaded mitochondrial protein ($>$ 6000 dpm) which was preincubated for 5 min at reaction temperature (0-4 C, 10 C, 25 C or 37 C) in 1 ml of medium consisting of 100 mM KCl, 40 mM Tris-HCl (pH 7.4) and 1.0 mM EDTA. The exchange was started by adding specific quantities of ADP and stopped after specified periods of incubation by the addition of 50 μM atractylate. The reaction mixture was then centrifuged, the pellet dissolved in Soluene 100 (Packard Co., Downers Grove, IL) and the radioactivity in the pellet in 10 ml of Bray's scintillation fluid was determined. Each assay was accompained by a control sample in which the addition of atractylate preceded that of ADP at time zero which provided a correction for the atractylate-insensitive efflux of the internal labelled adenine nucleotides. The transport rate is expressed as net % efflux of mitochondrial ^{14}C-labeled adenine nucleotides according to the equation:

$$\% \text{ transport} = 100(\text{dpm}_{\text{control}} - \text{dpm}_{\text{assay}})/\text{dpm}_{\text{control}},$$

where dpm represents the radioactivity in the pellet. The kinetic parameters for the reciprocal plots were calculated within the initial apparent linear rates of transport which were, respectively, 10 min, 1 min, 30 sec and 15 sec for 0-4 C, 10 C, 25 C and 37 C.

The K_i values for atractylate and palmitoyl-CoA were determined according to Dixon (14). The inhibitors were added to the incubation mixtures and preincubated with the ^{14}C-ATP-loaded mitochondria for 5 min at 0-4 C. ADP was added to initiate the transport, the reaction allowed to proceed at 0-4 C, stopped by the addition of 50 μM atractylate, centrifuged, and radioactivity in the pellet determined. Concentrations of the inhibitors and ADP are presented on the appropriate figures.

^{14}C-ADP translocation in liver mitochondria from diabetic rats was increased, according to Lerner et al. (15), by prior treatments which removed endogenous fatty acyl-CoA esters from the membrane. To determine whether or not the endogenous fatty acyl-CoA esters influenced the transport kinetics, aliquots of ^{14}C-ATP-loaded mitochondria pooled from 3-4 rat livers of each group were washed twice with loading medium containing 10% (w/v) fatty acid poor BSA and then twice with loading medium before the back exchange was assayed. Aliquots of these pooled ^{14}C-ATP-loaded mitochondria were also incubated in medium containing 5 mM DL-carnitine for 5 min at the reaction temperature before the exchange assay was initiated by the addition of ADP.

Lipids were extracted from frozen and thawed mitochondrial preparations according to the method of Ames (16) in which methanol, chloroform and the mitochondrial suspension are in the proportions of 2:1:0.8 (v/v/v). Changes in the relative proportions of PC, PE and neutral lipids were studied according to Hanson et al. (17) with some modification. The separation was performed with a Waters Associates liquid chromatographic system comprising an Ultrasil-NH_2 column (Altex) with a 5 μm packing in a stainless-steel tube (250 $\times$ 4.6 mm ID) coupled with a Perkin-Elmer LC-75 Spectrophotometric detector and a Spectra-Physic (SP 4100) computing integrator. The lipids were eluted isocratically with n-hexane/2-propanol/water (6:8:1, v/v/v) at a flow rate of 0.7 ml/min and detected at 206 nm. The separation was complete within 30 min. The PC, PE and neutral lipids were identified using bovine liver PC, egg yolk PE and triolein as standards.

Protein was determined by the Lowry procedure using bovine serum albumin as the standard (18).

Results are expressed as mean values $\pm$ SD. Statistical significances were determined by Student's t-test. Regression lines were calculated by the least squares method (19). The validity of each

regression line was tested by t-test.

^{14}C-ATP (44.8 mCi/mmol) was purchased from New England Nuclear, Boston, MA; ADP and palmitoyl-CoA from P-L Biochemical, Milwaukee, WI; and atractylate, bovine liver PC, egg yolk PE, triolein, BSA-fatty acid-free, Fraction V and DL-carnitine hydrochloride from Sigma, St. Louis, MO. All the HPLC solvents were purchased from Burdick and Jackson Lab., Muskegon, MI.

RESULTS

Effect of the Diets on Rat Growth and on Mitochondrial Respiration

Although the average body weight of rats fed the SO diet was 20% lower than that of BT group, the liver weights of the two groups were similar (Table 1). The liver weight/100 g body weight of the SO group was 10% higher than that of the BT group.

State 3 and state 4 respiration rates for hepatic mitochondria isolated from rats fed the SO diet were 25% and 50%, respectively, higher than those determined for BT group mitochondria (Table 1). The net difference in ADP-dependent respiratory activity (state 3 − state 4) was ca. 10 nanoatoms oxygen (BT: 74.8 ± 7.9 vs SO: 84.3 ± 7.4)/min/mg protein. Confirming our previous observations (9), the ADP/O ratio was 11% higher ($p < 0.01$) and the respiratory control ratio was 28% ($p < 0.01$) higher for the BT group mitochondria. The relative decrease in efficiency of oxidative phosphorylation in rats fed the SO diet appears to correlate in part with the 36% lower gain in weight.

Kinetics of ADP/^{14}C-ATP Transport at Different Temperatures

The uptake of ^{14}C-ATP during equilibrium loading for 45 min at 0-4 C by SO mitochondria was 97 ± 12% (n = 5) that of the BT mitochondria.

Significantly lower K_m values were calculated for the BT-group mitochondrial AdNT at all assay temperatures except 10 and 37 C. At those assay temperatures, the K_m value had high SD but the mean values were lower than that calculated for the SO mitochondrial AdNT (Fig. 1). Whereas the V_{max} was not influenced by dietary treatment at lower assay temperatures, at 25 C and 37 C assay temperatures, the SO-group AdNT V_{max} was ca. 12% higher ($p < 0.05$) (Fig. 1). At all temperatures, the AdNT of the SO-group mitochondria exhibited the higher K_m for ADP.

Examination of the kinetic behavior of the transport activities at higher temperatures revealed that lower levels of ADP (< 10 μM) supported relatively lower rates of transport in mitochondria of the SO group. However, the depressed rate of transport was reversed by high levels of added ADP (> 40 μM). These observations imply that either there was a real difference in affinities for ADP or, alternatively, there was a higher level of competitive inhibitor present in the SO group mitochondrial preparation.

Effects of Carnitine and BSA-Wash on the Kinetics of Transport

The natural inhibitor of the AdNT is the long-chain acyl-CoA ester which accumulates in the mitochondria under certain physiological and pathological conditions (20,21) Studies were undertaken to examine whether or not an accumulation of fatty acyl-CoA esters in the mitochondrial preparations had depressed the rate of transport (Fig. 2).

The kinetic parameters of the BT-group AdNT were not influenced by the BSA-wash. Preincubation with 5 mM Cn slightly increased the transport rate at each assay temperature. The increase occurred in parallel with a 10% decrease in the Km (Fig. 2a). This effect on preincubation with Cn was noted for both control and BSA-wash (data not shown) mitochondrial preparations. On the other hand, the preincubation with Cn markedly stimulated the transport activity of the SO group mitochondrial AdNT (Fig. 2b). This increase in activity, when analyzed by the reciprocal plot technique, was not due to a change in V_{max}, but

TABLE 1

Comparisons of the Influences of BT and SO Diets on Mitochondrial Oxidative Phosphorylation in Rat Liver[a]

Diet groups	Body wt[b]	Liver wt per 100 g	Rate of oxidation +ADP	Rate of oxidation −ADP	Rate of oxidation Net	Respiratory control	ADP:O
BT	276 ± 9	4.05 ± 0.13	97.5 ± 9.1	22.8 ± 1.3	74.8 ± 7.9	4.28 ± 0.19	1.82 ± 0.08
SO	223 ± 15[c]	4.40 ± 0.14[c]	121.0 ± 9.8[d]	36.4 ± 3.2[d]	84.3 ± 7.4	3.33 ± 0.17[d]	1.68 ± 0.06[c]

[a]Respiration rates were determined polarographically at 30 C with succinate as substrate; data are in nanoatoms of oxygen consumed/min/mg protein. Values are means ± SD, n = 4.

[b]Initial weight, 70 g.

[c]p 0.05 between groups.

[d]p 0.01 between groups.

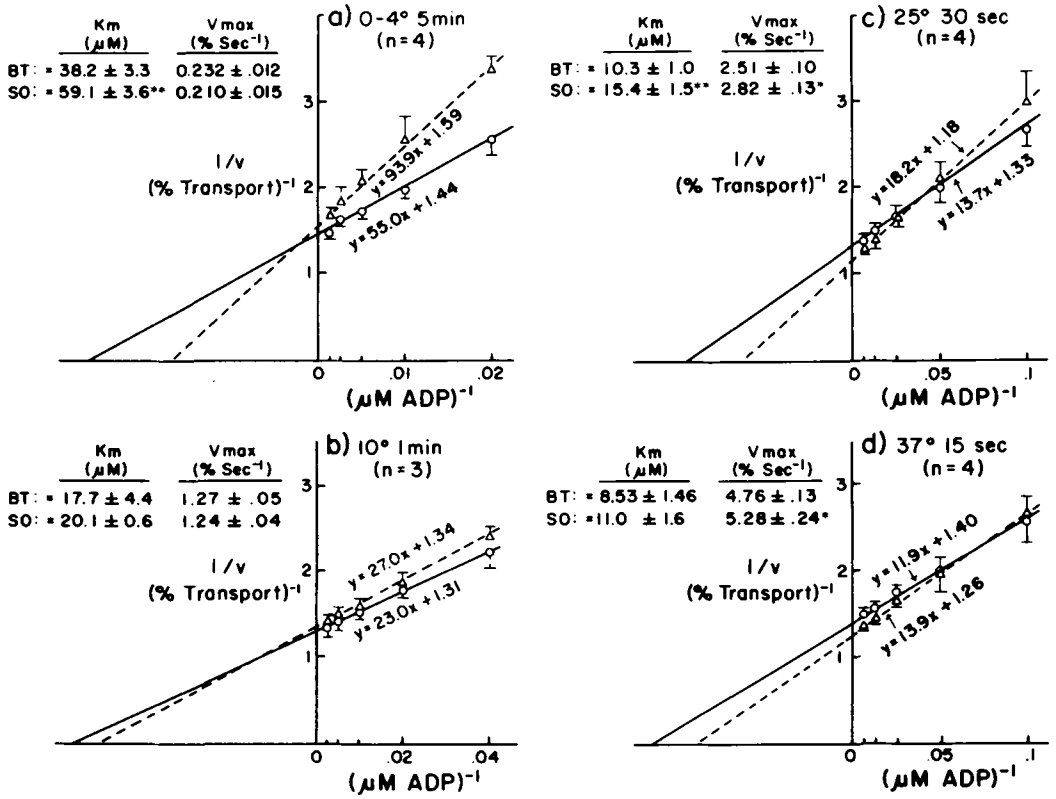

FIG. 1. Determinations of K_m and V_{max} for ADP/^{14}C-ATP transport in liver mitochondria from rats fed BT-diet (solid lines) or SO-diet (broken lines). ^{14}C-ATP-loaded mitochondria (1 mg protein) in 1 ml of incubation medium, pH 7.4, were preincubated for 5 min at the reaction temperature. The transport was started by adding specific quantities of ADP and stopped after specified periods of incubation by the addition of 50 μM atractylate. Data represented mean ± SD; * $p<0.05$, ** $p<0.01$.

instead was due to a lowering of the K_m from 62.8 to 42.2 μM at 0-4 C. Washing the SO mitochondria with BSA lowered the K_m slightly from 62.8 to 57.6 μM. The combination treatment, BSA-wash coupled with preincubation with Cn, yielded kinetic parameters similar to those obtained with Cn preincubation alone (data not shown). At assay temperatures of 25 and 37 C, the SO-group mitochondrial AdNT exhibited a lower binding affinity and a higher reaction rate. Preincubation with Cn increased the apparent binding affinity of this AdNT for ADP to a value similar to that calculated for the BT-group mitochondrial AdNT, the affinity of which was less responsive to the Cn pretreatment. These results imply that the higher K_m recorded for the SO-group mitochondrial AdNT on Figure 1 is due to endogenous fatty acyl-CoA esters.

Inhibitors of the Transport by Atractylate and Palmitoyl-CoA

The mitochondrial preparations used for the experiment reported in Figure 2 were used for the determination of the inhibitory actions of atractylate and palmitoyl-CoA on the AdNT activities. The atractylate K_i values determined according to Dixon (14) for the two groups of mitochondrial AdNT were similar (BT: 0.154 μM vs SO: 0.144 μM) (Fig. 3). However, for palmitoyl-CoA inhibition, the K_i value for the SO-group AdNT was about 2/3 of that for the BT-group (SO: 0.85 μM vs BT: 1.32 μM) (Fig. 4). Presumably, mitochondria of the SO rats were partially saturated with endogenous long-chain fatty acyl-CoA esters; thus, a lower concentration of palmitoyl-CoA was required to bring about an effective inhibition of the transport. K_i values determined for atractylate were little affected by the presence of fatty acyl-CoA esters because the affinity of the AdNT for atractylate is about 10 times that for long-chain fatty acyl-CoA (22).

In all experiments, the transport rate of the BT-group AdNT at low temperature was greater than that calculated for the SO-group transport. However, at a more physiological temperature, the rate of activity of the latter group exceeded that calcu-

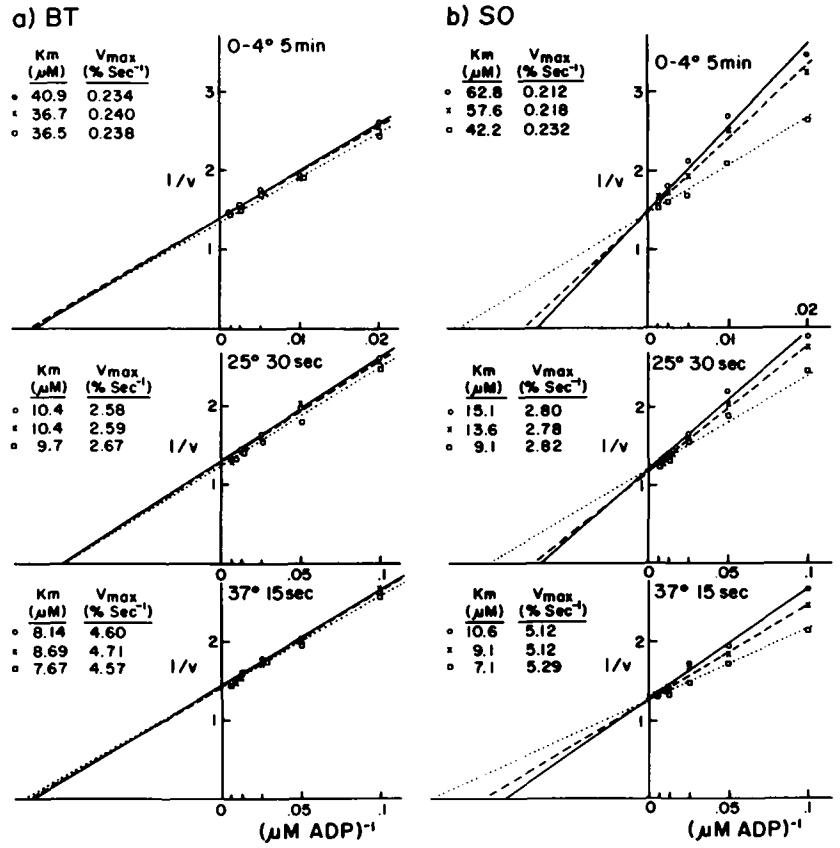

FIG. 2. Effects of BSA-wash and carnitine on the kinetics of the ADP/^{14}C-ATP transport in liver mitochondria from rats fed BT-diet (a) or SO-diet (b). ^{14}C-ATP-loaded mitochondria were either (i) processed as described in Figure 1 (o—o), or (ii) washed twice with 10% fat-free-BSA (x----x), or (iii) preincubated with 5 mM DL-carnitine in the assay mixture (□·····□). Other assay conditions were as in Figure 1. Data represent the average of duplicate determinations from mitochondria pooled from 4 rat livers.

lated for the BT-group AdNT. This result, coupled with the results gained by preincubation with Cn, is consistent with an enhanced oxidation of fatty acids by the SO-group mitochondria. Our calculations based on assays at 25 C and 37 C show that AdNT activity and V_{max} are elevated by 11-15% in the SO group. This difference in activity is consistent with the net ADP stimulation of oxygen uptake (Table 1), and the increase in cytochrome c oxidase activity and the electron transport components, cytochromes cc_1 and aa_3 previously reported (9,10).

Effect of Diet Treatment on Mitochondrial PC, PE and Neutral Lipids Distribution

Qualitative changes in the lipid patterns were noted; the distribution of neutral lipids, PE and PC in the BT mitochondrial lipids was 34.7%, 30.3% and 35.0%, respectively. The distribution in the SO mitochondrial lipids was 31.0% neutral lipid, 27.5% PE and 41.5% PC. Some perspective on these qualitative changes is forthcoming from the analyses of the standards; when monitored at 206 nm, the integrator recorded 400 counts/μg triolein, 280/μg egg yolk PE and 300/μg beef liver PC. Applying these standard responses to the mitochondrial lipid profiles, the values recorded on Table 2 were calculated.

DISCUSSION

The net ADP-succinate-supported respiratory activity of the SO mitochondria was 13% greater than that of the BT mitochondria [(state 3-state 4)$_{SO}$ − (state 3-state 4)$_{BT}$]/[(state 3-state 4)$_{BT}$]. This difference in ADP-linked respiratory activity was recorded at physiological temperature. At lower temperature, the V_{max} of the AdNT of the SO AdNT was not significantly different from that of

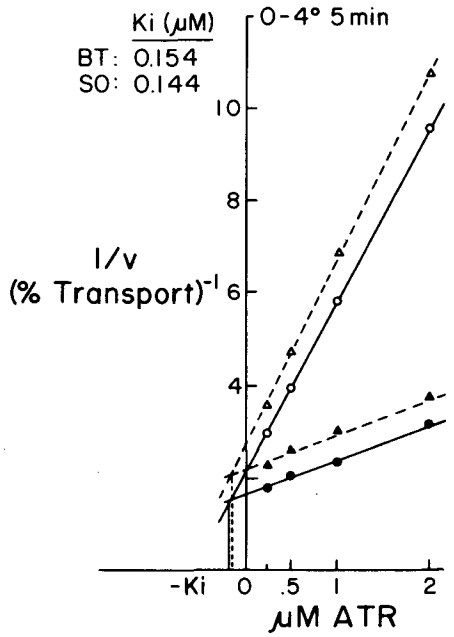

Fig. 3. Determination of K_i values for atractylate inhibition of the ADP/^{14}C-ATP transport in liver mitochondria from rats fed BT-diet (solid lines) or SO-diet (broken lines). Final ADP concentrations were either 50 μM (o, Δ) or 200 μM (●, ▲). Assay conditions are described under Materials and Methods. Values are determined from mitochondria pooled from 4 rat livers.

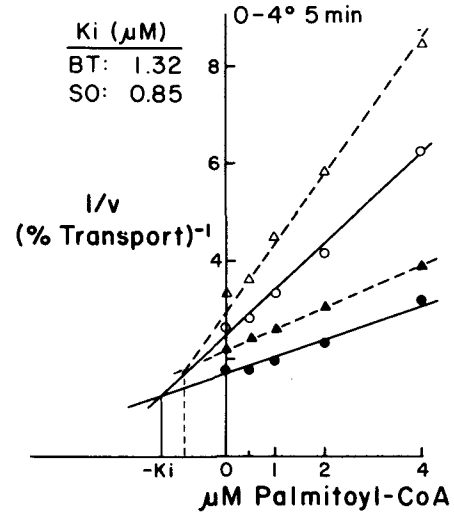

Fig. 4. Determination of K_i values for palmitoyl-CoA inhibition of the ADP/^{14}C-ATP transport in liver mitochondria from rats fed BT-diet (solid lines) or SO-diet (broken lines). Final ADP concentrations were either 50 μM (o, Δ) or 200 μM (●, ▲). The mitochondrial preparations were as for Figure 3. Assay conditions are described under Materials and Methods.

TABLE 2

Effect of Diet Treatments on Mitochondrial Neutral Lipids, PC and PE Distributions (as wt%)

Rat group	Neutral lipids	PE	PC	PC/PE
BT diet	27.9	34.7	37.4	1.07
SO diet	24.7	31.3	44.0	1.41

Data represent the average of duplicate determinations from mitochondira pooled from 3 rat livers.

the BT AdNT (Fig. 1a, 1b). Abuirmeileh and Elson (10) reported phase transition temperatures of 13 C and 15.4 C for cytochrome c oxidase activities in mitochondrial membranes enriched or normal in linoleic acid content. Plots (not shown) of the temperature-V_{max} relationship (Figs. 1 and 2) indicate the rank of the activities changed at a temperature falling within the range 13-15 C.

Fatty acid patterns of mitochondrial lipids reflect the patterns of the dietary fats (7,9,10). More recently, the membrane phospholipids were observed to change in response to changes in dietary fat (8). The SO mitochondrial lipids contained higher proportions of linoleic and arachidonic acids so that the degree of unsaturation was elevated 85% above that present in BT mitochondria (9). Concomitantly, the proportions of PE and PC shifted so that the ratio of PC/PE was 40% higher in the SO mitochondrial lipids (Table 2). In hypothyroidism (3,5), a decrease in PC and increase in PE plus acidic phospholipid contents in the mitochondria were associated with the decrease in V_{max} of the AdNT. In reconstitution studies (23), an increase in unsaturated bonds in the phospholipids increased the V_{max} of the reconstituted ATPase activity. Our results are consistent with the above observations.

Although the binding of ADP to the SO mitochondrial AdNT was characterized with a higher K_m, after preincubation with Cn, the binding affinities were similar. Consistent with this result was the agreement in the K_i for atractylate binding. The lower K_i for palmitoyl-CoA binding to the SO AdNT might reflect a higher concentration of endogenous acyl-CoA esters. At all assay temperatures, procedures taken to remove the endogenous acyl-CoA esters lowered the K_m of the SO AdNT but not that of the BT AdNT (Fig. 2). Our interpretation of these results is that the differences in net ADP-succinate supported respiration is due only to an increase in the V_{max} which is a consequence of the changes in the mitochondrial membrane characteristics.

The difference in the state 3 respiration rate was 23.5 nanoatoms oxygen/mg protein/min in the direction of the SO mitochondria. The difference in the net ADP-succinate supported respiration which

we attributed to the higher AdNT activity of the SO mitochondria was 9.9 nanoatoms oxygen/mg protein/min. The remainder of the difference in the respiration rate is attributed to the 13.6 nanoatoms oxygen/mg protein/min differential in the state 4 respiration, rates which are independent of the AdNT activity. This difference, we suggest, traces to the energy expenditure within the mitochondrion, a consequence of the phospholipase A-mediated membrane remodeling activity which Abuirmeileh and Elson (9) observed in the SO mitochondria. A similar activity directed towards the extramitochondrial membrane lipids and/or other cytosolic ATP-consuming activities may be concurrently elevated to provide increased ADP to support the higher rate of ADP-ATP exchange. Documentation of such activities would integrate the overall increase in energy expenditure. This expenditure of energy, we propose, underlies the lower rates of weight gain (9; Table 1) and the lower feed efficiency (10) supported by the diet enriched in linoleic acid.

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Metabolism of Erucic Acid in Adipocytes Isolated from Rat Epididymal Fat

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ABSTRACT

The metabolism of [14-^{14}C]erucic acid and [U-^{14}C]palmitic acid has been investigated in adipocytes isolated from rat epididymal fat. The rate of acylation of [^{14}C]erucic acid in cellular lipids and oxidation to CO_2 and acid-soluble activity was ca. $^1/_3$ of the rate with [^{14}C]palmitic acid as substrate. A maximal incorporation of fatty acids in triacylglycerol was found at a fatty acid concentration of 0.8 mM in the medium, both with [^{14}C]erucic acid and [^{14}C]palmitic acid as substrate. Glucose added to the medium increased the esterification and decreased the oxidation of both fatty acids. No significant chain-shortening of [^{14}C]erucic acid to shorter monoenes was identified in the fat cells. Increasing concentrations of unlabeled palmitic acid in the incubation medium markedly inhibited the esterification of [^{14}C]erucic acid, whereas unlabeled erucic acid had little effect on the rate of esterification of [^{14}C]palmitic acid.
Lipids 18: 137-141, 1983.

INTRODUCTION

In 1970, Abdellatif and Vles (1) reported that rats fed a diet rich in erucic acid (22:1ω9) develop a transient cardiac lipidosis. Later, the metabolism of several 22:1 fatty acids and particularly of erucic acid in liver and heart was extensively studied both in perfused organs, in subcellular fractions and in isolated liver cells.

In isolated liver cells (2-5), both the rate of esterification and mitochondrial oxidation of 22:1 fatty acids is distinctly slower, ca. $^1/_3$ of the rate with palmitic acid. In the perfused heart (6-8), erucic acid is esterified as rapidly as palmitic acid whereas the rate of mitochondrial oxidation of erucic acid is as in liver, ca. $^1/_3$ of the rate with palmitic acid. In liver and heart, 22:1 fatty acids are shortened to 18:1 (oleic acid) and smaller amounts of 20:1 and 16:1 fatty acids, probably by a peroxisomal chain-shortening system (2-10).

In addition to liver and muscle, fat tissue is the main system metabolizing fatty acids. The purpose of the present work was to study the metabolism of 22:1 fatty acids in fat cells isolated from rat epididymal fat.

MATERIALS AND METHODS

Chemicals

[U-^{14}C]Palmitic acid (99% purity from the manufacturer) was from the Radiochemical Center, Amersham, England. [14-^{14}C]Erucic acid (99% purity from the manufacturer) was from Centre d'Etudes Nucleaires de Saclay, France. The [14-^{14}C]erucic acid was purified by thin layer chromatography (TLC) using hexane/diethylether/acetic acid (80:20:1, v/v/v). The band corresponding to free fatty acid was scraped off and extracted with $CHCl_3/CH_3OH$ (2:1, v/v). Essentially fatty acid free bovine serum albumin, erucic acid and palmitic acid were purchased from Sigma Chemical Co., St. Louis, MO. Collagenase prepared from *Clostridium histolyticum* was obtained from Worthington Biochemical Corporation (Freehold, NJ).

Preparation of Fat Cells

Male Wistar rats from Møllegaard Laboratory, Denmark (weight 120-150 g), were used. The rats had free access to food and water until they were sacrificed. Adipocytes from epididymal fat were isolated with collagenase as described by Rodbell (11). The fat cells were incubated in plastic (Nalgene) vials in Krebs-Ringer bicarbonate buffer, containing 2% (w/v) defatted bovine serum albumin. The fatty acids were added to the fat cells as a 3% albumin solution. The specific activities were 760 cpm/nmol and 360 cpm/nmol of the [14-^{14}C]erucic acid and the [U-^{14}C]palmitic acid, respectively.

Analytical Procedure

Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter, Model 3385, and Insta-Gel II was used as scintillation solution. The measurements of radioactive acid soluble products and radioactive CO_2 were performed as in (12). Lipids were extracted from the fat cells according to Folch et al. (13), and separated by TLC on silica gel (Stahl H) using hexane/diethylether/acetic acid (80:20:1, v/v/v) as a solvent system. Fractions of free fatty acids and triacylglycerols were extracted from the gel with $CHCl_3/CH_3OH$ (2:1, v/v) and phospholipids with $CHCl_3/CH_3OH$/acetic acid/H_2O (65:25:2:2, v/v/v/v). Aliquots of the

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total lipid extract and of the free fatty acid, triacylglycerol and phospholipid fractions were *trans*-methylated (14) and analyzed by radio-gas chromatography as described (7). The DNA-content was measured by the method of Burton (15).

RESULTS

The time course of the oxidation and esterification of [^{14}C]erucic acid and [^{14}C]palmitic acid in isolated adipocytes from rat epididymal fat pads is given in Figure 1. Erucic acid was esterified to triacylglycerol at a distinctly slower rate than palmitic acid. The esterification of erucic acid in diacylglycerol and phospholipids also occurred at ca. $^1/_3$ of the rate with palmitic acid as substrate. The incorporation in diacylglycerol was ca. 10% and the incorporation in the phospholipids ca. 1% of the rate of esterification in triacylglycerol, both with erucic acid and with palmitic acid as substrate.

Figure 1D shows that the rate of oxidation of the [^{14}C]erucic acid to CO_2 and acid-soluble intermediates was slow compared to the rates with [^{14}C]palmitic acid. With both substrates, the oxidation products accounted for only 1-3% of the total amounts of fatty acid substrate metabolized. In the absence of glucose (Table 1), the oxidation of the fatty acid substrates to CO_2 and acid-soluble intermediates was higher than when glucose was added, whereas the esterification to triacylglycerol, diacylglycerol and phospholipids was markedly reduced. The total amount of fatty acid metabolized was reduced by ca. 60-70% without glucose in the incubation medium, both with palmitic acid and erucic acid as substrate.

The possible existence of a chain-shortening of erucic acid in isolated adipocytes was studied by using radio-gas chromatography (7). No significant chain-shortening of erucic acid to shorter monoenes could be detected, neither in the free fatty acid, triacylglycerol, diacylglycerol and phospholipid fraction, nor in the total lipid extract (results not shown).

The dose-dependency of the incorporation of fatty acids into triacylglycerol is shown in Figure 2. A maximal rate of triacylglycerol synthesis was obtained at a fatty acid concentration of ca. 0.8 mM, both with erucic acid and with palmitic acid as substrate.

Figure 3 shows that increasing concentrations of unlabeled palmitic acid in the incubation medium

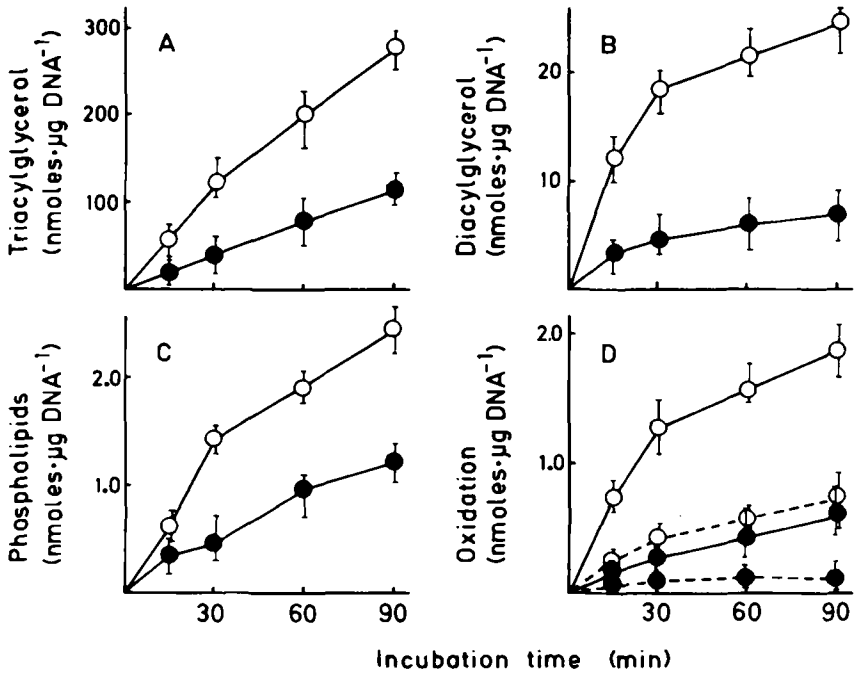

FIG. 1. Time course of [14-^{14}C]erucic acid (●) and [U-^{14}C]palmitic acid (○) incorporation in cellular lipids (A-C) and oxidation products (D). 1 ml fat cell (4.8 µg DNA) was incubated with 1 ml fatty acid (final concentration 1 mM), the medium contained 5 mM glucose. The oxidation products measured (D) were $^{14}CO_2$ (----) and radioactive acid-soluble products (——). The results presented are means of 3 experiments (the bars indicate the observed range).

TABLE I.

The Effect of Glucose (5 mM) on the Incorporation of [14-^{14}C] Erucic Acid and [U-^{14}C] Palmitic Acid into Cellular Lipids and Oxidation Products

	^{14}C-Erucic acid				^{14}C-Palmitic acid			
	+glucose		−glucose		+glucose		−glucose	
Experiment	1	2	1	2	1	2	1	2
Triacylglycerol	92.4	96.4	32.5	26.1	195.0	215.0	73.7	81.9
Diacylglycerol	6.5	5.7	3.0	3.8	20.1	24.1	7.8	7.6
Phospholipids	0.92	1.08	0.41	0.59	1.95	1.85	0.96	1.24
$^{14}CO_2$	0.23	0.17	0.28	0.32	0.51	0.49	1.28	1.12
Acid-soluble radioactivity	0.54	0.66	1.45	1.35	1.49	1.71	2.98	3.22
Total fatty acid metabolized	100.6	104.0	37.6	32.2	219.1	243.2	86.7	95.1

1 ml fat cells (ca. 6.5 μm DNA) was incubated with fatty acids (final concentration 1 mM), incubation time 60 min, with or without glucose (5 mM) in the incubation medium. The results are given as nmol fatty acid metabolized per μg DNA in 2 experiments. For experimental details, see Materials and Methods.

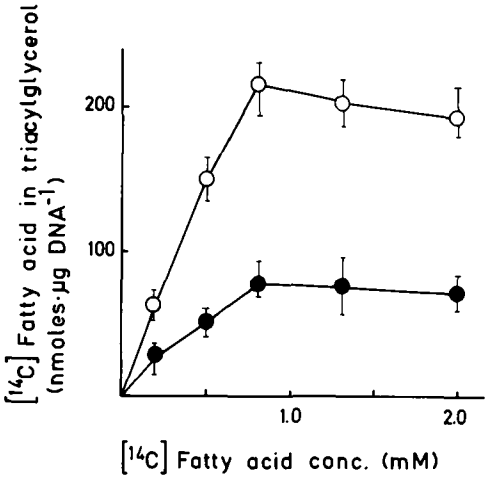

FIG. 2. The incorporation of [14-^{14}C]erucic acid (●) and [U-^{14}C]palmitic acid (○) in triacylglycerol in isolated adipocytes with increasing fatty acid concentration. 1 ml fat cells corresponding to ca. 5.0 μg DNA per sample was used. The incubation time was 60 min. The results presented are means of 3 experiments (the bars indicate the observed range).

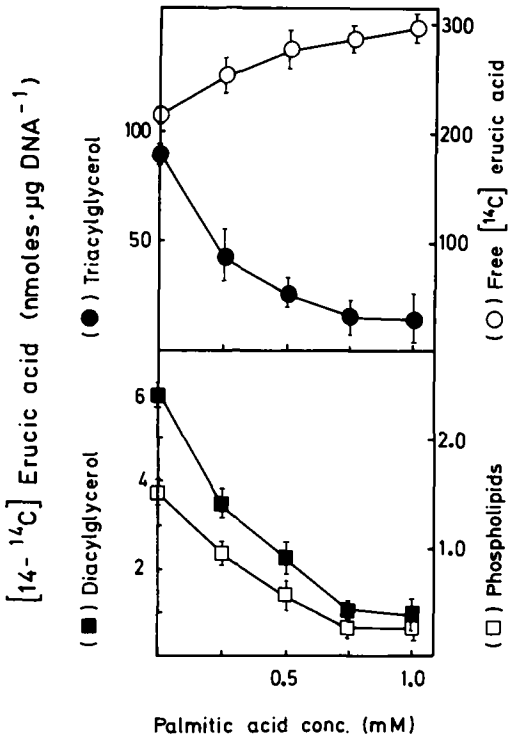

FIG. 3. The esterification of [14-^{14}C]erucic acid (final concentration 1 mM) to cellular lipids in isolated rat adipocytes with increasing concentration of unlabeled palmitic acid in the incubation medium. The incubation time was 60 min. 1 ml fat cells corresponding to ca. 5.3 μg DNA was used per sample. The medium contained 5 mM glucose. The results presented are means of 2 experiments (the bars indicate the observed range). ○—○ free fatty acid, ●—● triacylglycerol, ■—■ diacylglycerol, □—□ phospholipids.

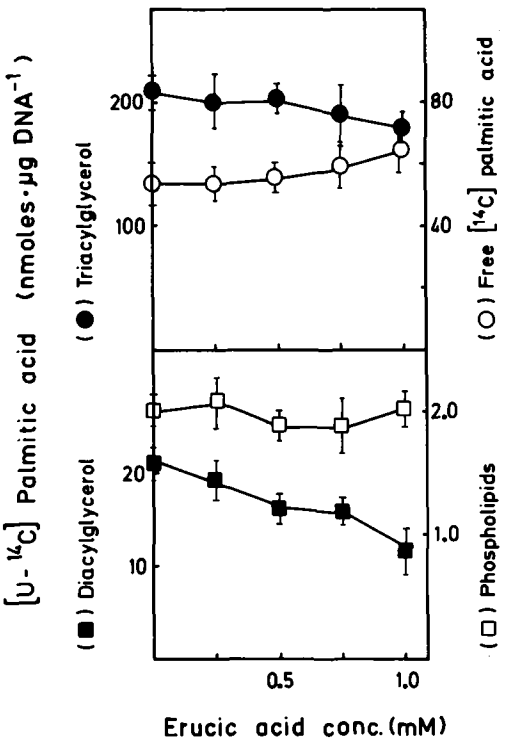

FIG. 4. The esterification of [U-^{14}C]palmitic acid (final concentration 1 mM) to cellular lipids in cellular rat adipocytes with increasing concentration of unlabeled erucic acid in the incubation medium. The incubation time was 60 min. 1 ml fat cells corresponding to ca. 4.5 µg DNA was used per sample. The medium contained 5 mM glucose. The results presented are means of 2 experiments (the bars indicate the observed range). ○- ○ free fatty acid, ●-●-● triacylglycerol, ■-■ diacylglycerol, □-□-□ phospholipids.

markedly inhibits the esterification of [^{14}C] erucic acid. With equimolar concentrations of the two fatty acids present (1 mM), the rate of esterification of erucic acid to triacylglycerol, diacylglycerol and phospholipids was ca. 15-20% of the rate observed in the absence of palmitic acid.

In contrast to this, increasing concentrations of erucic acid had little effect on the rate of esterification of [^{14}C]palmitic acid (Fig. 4). Thus, the esterification of palmitic acid (1 mM) to triacylglycerol and phospholipids was not significantly reduced by the presence of erucic acid at the same concentration. Only the formation of diacylglycerol from [^{14}C]palmitic acid was reduced to some extent by addition of unlabeled erucic acid. This is in agreement with earlier observations showing that erucic acid is more easily incorporated in diacylglycerol than in triacylglycerol or phospholipids (16).

DISCUSSION

The present study with adipocytes supports earlier findings with liver and heart showing that erucic acid is generally a poor substrate for mitochondrial β-oxidation. In fat cells, however, only a very small amount (1-2%) of the fatty acids is oxidized to CO_2 and acid-soluble activity compared to the amounts esterified. The finding of a slow rate of esterification of erucic acid compared to the rate with palmitic acid in adipocytes is similar to the observation in liver (2-5) and differs from the findings in heart (6-8) where the two fatty acids are esterified at the same rate.

The addition of glucose decreased the rate of oxidation and increased the esterification of both palmitic acid and erucic acid in isolated fat cells. This is in agreement with Abumrad et al. (17), who found that addition of glucose increased the esterification of [^{14}C]oleate in isolated rat adipocytes by 75%. In the absence of glucose, the α-glycerophosphate necessary for esterification is probably provided from endogenous glycerol by the action of glycerolkinase which has been found, also in rat adipose tissue, in substantial amounts (18-20). Smaller amounts of α-glycerophosphate may also be produced by glycolysis of glucose derived from glycogen in the rat adipocytes.

Craig et al. (21) investigated the fatty acid composition of cutaneous, abdominal and carcass fats in rats fed rapeseed oil for 21 weeks and found that an unexpectedly high proportion of oleic acid was present. These findings indicate that erucic acid is chain-shortened to shorter monoenes in adipose tissue or that chain-shortening products from liver are taken up by the fat cells. The present work supports the view that an extramitochondrial chain-shortening of 22:1 fatty acids probably does not occur to any significant extent in fat tissue itself. Although microperoxisomes have been described in a number of tissues (22), and are thought to be almost ubiquitous in mammalian cells, as far as we know they have never been identified in white adipose tissue. Fatty acids from VLDL-triacylglycerol are taken up by adipose tissue (in a fed state) and the free fatty acids released from adipose tissue in fasting may thus, in part, originate from VLDL-triacylglycerol fatty acids which have passed through the liver at an earlier stage. Thus, the metabolism of the liver probably has a strong influence on the fatty acid pattern, both of the triacylglycerol and the free fatty acid circulating in the blood, and thus on the composition of fatty acids reaching both the adipose tissue and the heart. It is interesting that Hülsmann et al. (23) have found that feeding rapeseed oil, rich in erucic acid, resulted in increased lipoprotein lipase activity and decreased hormone stimulated tissue lipase activity in fat cells.

ACKNOWLEDGMENT

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COMMUNICATIONS

Phospholipid Composition of Liver in Rats Fed High Levels of 13-*cis* Retinoic Acid

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ABSTRACT

The composition of liver phospholipids was studied in rats fed for 4 weeks diets containing 0, 100 or 300 mg 13-*cis* retinoic acid per kg diet. There was a significant decrease in phosphatidylcholine content, whereas the levels of phosphatidylethanolamine were slightly increased in liver phospholipids of rats fed 13-*cis* retinoic acid. The fatty acid composition of total phospholipids, PC, PE, and PI + PS fractions revealed a general increase in the levels of 18:2 and 20:3ω6, whereas the levels of 20:4ω6 and C_{22} fatty acids were reduced in most of the hepatic phospholipids isolated from rats fed 13-*cis* retinoic acid containing diets. A decrease in the double-bond index of fatty acids was also observed in phospholipids of rats fed 13-*cis* retinoic acid. The data suggest that high levels of 13-*cis* retinoic acid may possibly be influencing the activities of microsomal desaturating and chain-elongating enzymes in the liver.
Lipids 18: 142-145, 1983.

A number of retinoids, including retinoic acid, have been used as cancer chemopreventive agents in experimental animals (1-4). Due to its lower toxicity (5), the 13-*cis* isomer has been found to be more promising than all *trans* retinoic acid. 13-*cis* Retinoic acid is also being used for the treatment of cystic and conglobate acne in man (6).

When used in large amounts, 13-*cis* retinoic acid has a number of undesirable side effects, including hypertriglyceridemia (7,8). We have recently observed (9) changes in fatty acid composition of total lipids in plasma and liver of rats fed diets containing 13-*cis* retinoic acid. The levels of 16:1, 18:1 and 20:3ω6 were increased, whereas those of 20:4 were decreased. In the present communication, we report the changes in phospholipid composition of liver as a result of feeding an excess of 13-*cis* retinoic acid.

MATERIALS AND METHODS

Male, weanling, Sprague-Dawley rats (5 per group) were fed ad libitum purified diets containing 0, 100 or 300 mg of 13-*cis* retinoic acid per kg diet. Composition of the basal diet has been described previously (9). It consisted of (in percent): sucrose, 63.8; casein (vitamin-free), 20.0; corn oil, 7.0; cellulose, 4.0; mineral mixture, 4.0; vitamin mixture, 1.0; choline chloride, 0.2; and butylated hydroxytoluene (BHT) 0.002. 13-*cis* Retinoic acid was a generous gift from Hoffman LaRoche Inc. The control group (0 retinoic acid) was fed the same diet as the other two groups, except that it had no 13-*cis* retinoic acid added to it. After 4 weeks of feeding, the rats were decapitated, livers were excised, rinsed with physiological saline, weighed and homogenized with deionized water. Homogenates were extracted for total lipids using Bligh and Dyer's procedure (10). Total lipid extracts from livers were fractionated into neutral lipids, glycolipids and phospholipids by using silicic acid column chromatography on Bio-Sil A columns (11). The phospholipid fraction was further subjected to thin layer chromatography (TLC) on 0.25 mm thick Silica Gel H plates for the separation of various phospholipids. Plates were developed under nitrogen in a solvent system of chloroform/methanol/acetic acid/water (25:15:4:1.75, v/v) containing 0.02% butylated hydroxytoluene (BHT) as an antioxidant. After a brief (15 sec) exposure to iodine vapors, the spots for phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol plus phosphatidylserine (PI + PS) and sphingomyelin (SM) were scraped off and transferred to glass tubes with teflon lined stoppers. Phospholipids were extracted twice with 3 ml of methanol/chloroform/water (2:1:0.8, v/v), the 2 extracts were combined and 1.6 ml each of chloroform and water was added in order to separate the chloroform layer from the aqueous layer. The chloroform phase containing

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the phospholipids was used for the determination of phosphorus content using Bartlett's procedure (12). The recovery of phospholipid standards from the TLC plates using this extraction procedure was 90-95%. Aliquots of total phospholipids and various phospholipid fractions isolated by TLC were transesterified with boron trifluoride/methanol (13) and the fatty acid composition of methyl esters was determined by gas chromatography as previously described (9). Due to small amount of SM fraction available, its fatty acid composition was not determined. The double-bond index was calculated from the sum of (percentage of each unsaturated fatty acid) × (number of double bonds).

Results are shown as mean ± SEM. Significance of difference among the groups was calculated using analysis of variance, Newman-Keul's test (14).

RESULTS

Rats fed diets containing 13-*cis* retinoic acid for 4 weeks gained slightly less body weight as compared with their controls. There was no significant difference in the total lipid or phospholipid content of liver. These findings have been reported previously (9).

The phospholipid composition of liver as affected by an excess of 13-*cis* retinoic acid feeding is shown in Table 1. PC, PE, PI + PS, and SM fractions constituted 70-80% of the total phospholipids. Other phospholipids normally present in liver such as cardiolipin, lyso PC, lyso PE and phosphatidic acid probably made up the difference. These were, however, not determined.

There was a significant reduction in the proportions of PC in the liver of rats fed the two levels of retinoic acid. The levels of PI + PS fraction were also slightly decreased, whereas those of PE were relatively higher in the liver of rats fed the higher level of retinoic acid. The SM fraction, which constitutes a minor phospholipid fraction in liver, was also slightly lower in one group fed retinoic acid.

The fatty acid composition of total phospholipids, PC, PE and PI + PS fractions is shown in Table 2. In the total phospholipids, the levels of 18:2 and 20:3ω6 were higher in the 2 groups of rats fed retinoic acid containing diets as compared with the control. On the other hand, the levels of 22:4ω6 and 22:6ω3 were lower in the groups fed retinoic acid. In the PC fraction, in addition to the above changes, the proportions of 18:1 were higher and those of 20:4ω6 were lower in the groups fed retinoic acid as compared with the controls. Similar changes in fatty acid composition were observed in PE with respect to the levels of 18:2, 20:3ω6 and 22:4ω6. The fatty acid patterns of PI + PS fraction also revealed higher levels of 18:1 and 20:3ω6 in the hepatic phospholipids of rats fed retinoic acid while the levels of 20:4ω6 tended to be lower. An additional observation was that, in all the phospholipids except PI + PS fraction, there was a significant reduction in the degree of unsaturation as indicated by a lower double-bond index in the groups fed retinoic acid.

TABLE 1

Effect of 13-*cis* Retinoic Acid Feeding on Phospholipid Composition of Rat Liver

Dietary 13-*cis* retinoic acid (mg/kg)	PC	PE	PI + PS	SM
	% of total phospholipids			
0	51.1[a,b] ±0.94	21.1[b] ±0.68	5.3[b] ±0.23	2.6[a] ±0.22
100	45.7[a] ±1.24	20.8[a] ±1.01	4.0[a] ±0.44	2.0[a] ±0.07
300	42.8[b] ±1.28	24.0[a,b] ±0.77	2.7[a,b] ±0.09	2.3 ±0.12

Values are mean ± SEM of 5 rats/group. Values sharing a common superscript within the same column are significantly different from each other, using analysis of variance, Newman-Keul's test ($p < 0.05$).

DISCUSSION

Our previous work (9) has shown that the intake of diets containing an excess of 13-*cis* retinoic acid resulted in a modification of fatty acid composition of total lipids in plasma and liver of rats. The changes in fatty acid patterns of other tissues (lung, kidney and heart) were not so profound. Gerber et al. (15) have observed similar changes in fatty acid composition of triglycerides, free fatty acids, cholesteryl esters and phospholipids of serum in rats fed an excess (25 mg/kg diet) of all *trans* retinoic acid for 8 days.

The present studies show that 13-*cis* retinoic acid induces fatty acid composition changes in total phospholipids, PC, PE and PI + PS fractions of liver. In most phospholipids, these changes consisted of an increase in the levels of 18:2 and 20:3ω6 with a corresponding decrease in the levels of 20:4ω6, 22:4ω6 and 22:6ω3. There was a general decrease in the degree of unsaturation of various phospholipids in the groups fed retinoic acid, as shown by a lower double-bond index.

Increase in the proportion of 18:2 and 20:3ω6 with a concomitant decrease in 20:4ω6 and 22:4ω6 suggests that 13-*cis* retinoic acid may be inhibiting the conversion of linoleic acid to arachidonic acid. This would, therefore, result in lower levels of 22:4ω6. Since the metabolic transformation of linoleic acid to arachidonic acid is catalyzed by microsomal desaturases and chain-elongating en-

TABLE 2

Effect of 13-*cis* Retinoic Acid Feeding on the Fatty Acid Composition of Various Phospholipids in Rat Liver

Dietary 13-*cis* retinoic acid (mg/kg)	16:0	18:0	18:1	18:2	20:3 ω9	20:3 ω6	20:4 ω6	22:4 ω6	22:6 ω3	Double-bond index
					Total phospholipids					
0	20.4 ±0.32	19.6 ±0.40	11.7 ±0.35	11.2^{a,b} ±0.58	0.5 ±0.04	0.7^{a,b} ±0.04	28.2 ±0.67	5.0^{a,b} ±0.45	2.1^{a,b} ±0.12	183.1^{a} ±2.59
100	22.5 ±0.75	19.7 ±0.17	11.3 ±0.75	14.3^{a} ±0.34	0.7 ±0.04	1.6^{a} ±0.14	26.4 ±0.68	1.2^{a} ±0.31	1.5^{a} ±0.16	166.2^{a} ±4.68
300	20.0 ±1.36	19.7 ±0.66	11.2 ±0.52	15.5^{b} ±0.57	0.6 ±0.05	1.9^{b} ±0.06	27.1 ±1.25	2.1^{b} ±0.16	1.4^{b} ±0.11	174.9 ±5.33
					Phosphatidylcholine					
0	28.5 ±0.52	19.2 ±0.59	10.0^{a,b} ±0.46	9.7^{a,b} ±0.98	0.4 ±0.07	0.7^{a,b} ±0.06	26.2^{a} ±0.60	3.4^{a,b} ±0.48	1.2^{a,b} ±0.17	158.3^{a,b} ±2.55
100	26.6 ±0.99	20.2 ±0.47	12.3^{a} ±0.38	14.6^{a} ±0.23	0.7 ±0.06	1.4^{a,c} ±0.12	22.9 ±0.61	0.7^{a} ±0.10	0.7^{a} ±0.09	146.4^{a} ±2.66
300	27.1 ±0.77	19.0 ±0.77	11.8^{b} ±0.5	15.5^{b} ±0.72	0.5 ±0.03	1.7^{b,c} ±0.10	21.6^{a} ±1.80	0.5^{b} ±0.07	0.6^{b} ±0.02	141.4^{b} ±5.46
					Phosphatidylethanolamine					
0	15.2 ±1.44	29.2 ±1.18	10.1 ±0.62	5.4^{a,b} ±0.43	0.1 ±0.08	-^{a,b}	29.2 ±1.15	7.2^{a,b} ±0.79	3.0 ±0.38	184.8^{a,b} ±5.20
100	18.9 ±1.26	29.3 ±0.42	10.6 ±0.23	7.1^{a} ±0.35	0.3 ±0.03	0.5^{a} ±0.07	29.4 ±0.62	1.5^{a} ±0.29	2.3 ±0.31	164.6^{a} ±4.61
300	19.4 ±1.60	27.6 ±0.93	9.7 ±0.51	7.2^{b} ±0.29	0.3 ±0.08	0.7^{b} ±0.04	30.8 ±1.14	1.1^{b} ±0.15	1.8 ±0.16	165.5^{b} ±5.32
					Phosphatidylinositol + phosphatidylserine					
0	8.7 ±1.58	51.2 ±2.56	8.6^{a} ±1.51	3.2 ±0.38	-	-^{a,b}	30.0^{a} ±1.8	-	-	135.0 ±2.94
100	11.0 ±1.56	48.4 ±1.13	10.1^{b} ±1.71	2.9 ±0.43	-	1.0^{a} ±0.38	24.2 ±2.87	-	-	115.7 ±13.0
300	11.3 ±1.44	48.6 ±2.77	14.3^{a,b} ±0.48	3.2 ±0.24	-	1.3^{b} ±0.13	21.4^{a} ±2.68	-	-	110.2 ±11.0

Values are percent by weight (mean ± SEM of 5 rats per group). Values sharing a common superscript within a column are significantly different from each other using analysis of variance, Newman-Keul's test. -: Not detected.

zymes, it is likely that an excess of 13-*cis* retinoic acid may be inhibiting the activities of these enzymes in the liver. There is some evidence that a number of factors, including the degree of unsaturation of the dietary fat (16), proteins (17) and carbohydrate metabolism (18) may influence the activities of Δ6 desaturase in rat liver microsomes.

The levels of PC in liver of rats fed 13-*cis* retinoic acid were significantly decreased in our study. This was accompanied with a slight increase in the levels of PE. It is known that PC may be synthesized via two pathways, a stepwise methylation of PE using S-adenosylmethionine as a methyl donor and secondly from diacylglycerol and CDP-choline. It was recently reported by Fell and Steele (19) that the levels of S-adenosyl methionine were decreased in liver of rats fed an excess of retinol (1000 IU/g diet for 10 days). It was suggested by these investigators that the availability of methyl pool for phospholipid synthesis via phospholipid methyltransferase may be affected by high levels of retinol. If 13-*cis* retinoic acid has a similar effect on S-adenosylmethionine pool in the liver, this could account for a reduced synthesis of PC from PE and consequently for the lower PC levels in liver which were observed in our study. A reduced incorporation of choline into phospholipids by retinoic acid has also been observed in bovine lymphocytes treated with tumor promoting phorbol esters (20).

Significant reduction in the double-bond index of total phospholipids and the two major phospholipid fractions of liver, PC and PE, would suggest that membrane fluidity may be altered by 13-*cis* retinoic acid since phospholipids are part of cell membranes. If so, it is likely to have important implications in terms of a number of basic cell functions such as ionic transport, cell-cell interaction and differentiation. These processes play an important role in normal and abnormal cellular growth and development. A modification of fluidity

of cell membranes may be one of the important modes of action of 13-*cis* retinoic acid and other retinoids in terms of their cancer chemopreventive effects. Indeed, a number of retinoids, including 13-*cis* retinoic acid, have recently been found to influence the microviscosity of embryonic carcinoma cells (21). At physiological concentrations, the retinoids increased the microviscosity whereas, at higher concentrations, the same retinoids decreased the microviscosity. The exact mechanism as to how retinoids may affect the membrane lipid composition and fluidity needs to be further investigated.

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Effect of Dietary Vitamin E on Sulfolipid Synthesis in Rat Submandibular Salivary Gland

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ABSTRACT

The effect of feeding diets containing various levels of vitamin E for 6 months on the in vitro synthesis of sulfolipids in rat submandibular salivary glands (SMG) was elucidated. The incorporation of [^{35}S]sulfate into sulfolipid of SMG from rats on deficient or "normal" vitamin E diets was quite similar, however, the uptake was significantly increased in glands from rats on diets high in vitamin E. Whereas, in many instances, antioxidants can mimic the effect of vitamin E, in the present study, the antioxidant N,N'-diphenyl-*p*-phenylene diamine (DPPD) was actually found significantly to depress sulfolipid-labeling below that noted in SMG from all other diets. The results suggest that in the synthesis of SMG sulfolipid the action of vitamin E may be more than that of a simple antioxidant.
Lipids 18: 146-148, 1983.

There is little doubt that one function of vitamin E is as a natural tissue antioxidant protecting the integrity of susceptible cellular membrane lipids against peroxidation (1). However, its exact mode of action in situ is not yet fully understood. While there have been many studies on the effects of vitamin E deficiency, few reports are available dealing with excessive intake of vitamin E and only one (2) relates to submandibular salivary gland (SMG). In this latter report, it was found that feeding 250 U vitamin E/kg diet, a level ca. 80 times higher than the suggested requirement (3), for 7 weeks caused little change in total lipid but there was a decrease in phospholipid content and some changes in unsaturated fatty acids when compared to diets with less vitamin E. Although these changes were significant, they were not excessively drastic nor did the health of the rats appear to be affected.

The SMG of the rat exhibit an active lipid metabolism (4,5) and sulfolipids are readily synthesized under both in vitro (6,7) and in vivo (8) conditions. While the precise function of SMG sulfolipid is presently unknown, it has been suggested that many of them are secreted into the oral cavity (9). If so, their immediate presecretory site would most likely be within membrane-bound vesicles (10,11).

The present communication shows that relatively long-term feeding of very high levels of vitamin E to nonalbino rats markedly increased the ability of SMG to incorporate [^{35}S]sulfate into sulfolipid. This effect could not be duplicated by feeding the antioxidant N,N'-diphenyl-*p*-phenylenediamine (DPPD). The results suggest the possibility that vitamin E may also act in ways other than that of an antioxidant.

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MATERIALS AND METHODS

Diet Preparation

The basal diet is given in Table 1. All ingredients were obtained from ICN Canada (Montreal, Canada) except for the stripped corn oil which was from Fisher Scientific Co. (Winnipeg, Canada). DL-α-tocopherol acetate (Sigma Chemical Co., St. Louis, MO) was added to the basal diet as shown in Table 2. Diets were freshly mixed every week to prevent possible oxidation of tocopherol. The mixed diets were kept at 4C in air- and light-tight containers.

TABLE 1.

Composition of Basal Diet

Ingredient	Percent by weight
Vitamin-free casein	20.0
Dl.-methionine	0.3
Corn starch	13.5
Dextrose	50.0
Alphacel (fiber)	5.0
Salt mixture (4164)	4.0
Stripped corn oil	5.0
Vitamin mixture (no tocopherol)	2.2
	100.0
Selenium	0.1 mg/kg diet

DL-α-tocopherol acetate and DPPD was added to basal diet as shown in Table 2. Salt mixture (g/kg diet) consists of: calcium carbonate, 6.54; cupric sulfate, 0.0072; calcium diphosphate, 14.22; potassium diphosphate, 3.11; ferric citrate, 0.64; magnesium carbonate, 1.64; manganese sulfate, 0.055; potassium citrate, 9.46; potassium iodide, 0.0016; sodium chloride, 4.32; zinc carbonate, 0.0176. Vitamin mixture contains (mg/kg diet): L-ascorbic acid, 1000; choline chloride, 1665; calcium pantothenate, 66.6; i-inositol, 110.9; menadione, 49.9; niacin, 99.89; PABA, 110.99; pyridoxine HCl, 22.2; riboflavin, 22.2; thiamin HCl, 22.2; biotin, 0.44; folic acid, 2.0; vitamin B_{12}, 0.03; vitamin A acetate, 20,000 IU and D_2 calciferol, 2,220 IU/kg diet.

Animals

Male rats of the Long-Evans strain (nonalbino), bred and housed in the Faculty of Dentistry's animal facility, were employed in this study. They were 20-25 days of age at the start of feeding trials. Food and water were fed ad libitum. The diet mixtures were supplied fresh daily to each cage of 3-4 rats in newly washed ceramic food containers. Rats were transferred to fresh cages twice weekly.

Preparation of Tissue

Rats were lightly anesthetized and ensanguinated by aortic puncture and the glands quickly removed, cleaned and separated from the adhering sublingual glands. Tissue slices were prepared, weighed and placed in warm Krebs-Ringer saline buffered with 50 mM Tes, pH 7.4, and containing 5 mM glucose (7). Each 10 ml vessel contained 100 ± 5 mg slices in 1 ml buffered saline. After the addition of 0.5 μCi of inorganic [^{35}S]sulfate/vessel, the incubation at 37C was continued for 90 min. All incubations were done in triplicate from each gland.

Isolation of Sulfolipid

The contents of the incubation vessels were homogenized in chloroform/methanol (2:1, v/v) with the aid of Potter Elvehjem homogenizers with Teflon pestles. The homogenates were decanted into individual 25 ml glass-stoppered cylinders and the homogenizing tubes rinsed several times with the extracting solvent. The final volume was made to 20 ml with chloroform/methanol and allowed to stand overnight at 4C. Each lipid extract was filtered and the lipids purified according to the Folch procedure (12). The lower phases from all tests were reduced to dryness in a rotary evaporator, redissolved in chloroform/methanol (2:1, v/v, containing 1% water) and made to 1 ml volume with the same solvent. Suitable portions of these extracts were streaked on Whatman LK5 silica gel thin layer chromatographic (TLC) plates using a Camag Linomat III sample applicator (Terochem Laboratories Ltd., Winnipeg, Canada). Development was with chloroform/methanol/water (65:25:4, v/v/v). Radioactive lipids were located by radioautography, scraped from the plates and placed in scintillation vials. After the addition of 7 ml Aquasol-1 (NEN Canada, Montreal), the radioactivity was measured by scintillation spectrophotometry.

Other Analyses

Estimations of protein (13), pyruvic kinase (14) and α-tocopherol (15) levels were done by published procedures.

RESULTS

In agreement with the work of others (2), all animals, regardless of dietary regimen, appeared healthy and showed no significant differences in body weight after 6 months on the diets, although there were distinct biochemical differences (Table 2). In the absence of vitamin, plasma pyruvate kinase, an indicator of myopathy (15,16), was increased 10-fold ($P>0.005$) over rats fed either vitamin E or the antioxidant DPPD (Table 2). Plasma α-tocopherol concentrations were linear with the logarithm of dietary vitamin E concentrations. The levels of plasma α-tocopherol were 9 times greater in the DPPD-fed rats than in those on vitamin E-free diets ($P<0.01$), indicating that this antioxidant partly conserved the stores of vitamin E.

Figure 1 shows that there is one major SMG sulfolipid in the lower phase of a Folch extract and it contains over 90% of the radioactivity. Figure 2 demonstrates that SMG from rats fed high to excess levels of vitamin E were able to synthesize significantly more sulfolipid from precursor[^{35}S] sulfate than those from low (50 ppm) or no vitamin E diets ($P<0.01$). The antioxidant DPPD in this instance did not mimic the effect of vitamin E but tended to inhibit sulfolipid formation.

DISCUSSION

Although the level of α-tocopherol in SMG was not estimated in the present investigation, another group (2), who fed rats 0, 250 U and 2500 U vitamin E/kg diet, reported that the vitamin E content of SMG was 1.3, 33.1 and 77.4 μg/g, respectively. This means that SMG are remarkable scavengers of vitamin E from the plasma as other investigators

TABLE 2.

Effect of Dietary Vitamin E on Plasma α-Tocopherol and Pyruvate Kinase Levels and Body Weight

Dietary vitamin E (mg/kg)	n	α-Tocopherol (μg/mg total plasma lipid)	Pyruvate kinase (U/ml plasma)	Body wt (g)
0	4	0.14 ± 0.02[1]	0.62 ± 0.06[1]	542 ± 14
50	3	6.33 ± 1.67[2]	-	572 ± 12
100	3	7.68 ± 0.64[2]	0.06 ± 0.01	558 ± 14
1000	4	10.55 ± 0.56[1]	0.05 ± 0.01	585 ± 30
5000	5	14.82 ± 0.68[1]	0.06 ± 0.01	558 ± 17
0 + DPPD	3	1.28 ± 0.27[1]	0.05 ± 0.01	561 ± 26

Rats were placed on purified diets at 20-25 days of age (60-70 g) and fed the diets for 25 weeks. DPPD (N,N'-diphenyl-p-phenylene diamine) was added to the diet at a level of 100 mg/kg. Results are mean ± SD (n as shown).

[1]Indicates value significantly different from all other values in column ($P<0.01$).

[2]Indicates values are not different from each other but significantly different from all other values in column ($P<0.01$).

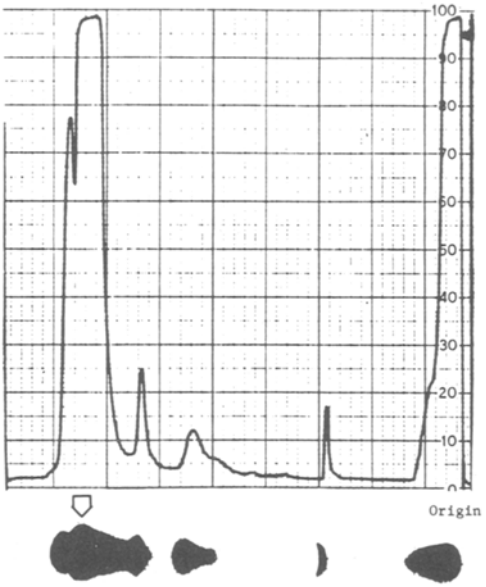

FIG. 1. Densitometric scan of radioautograph of TLC plate. [^{35}S]labeled SMG sulfolipids developed with chloroform/methanol/water (65:25:4, v/v/v). R_f of major sulfolipid (arrow), which contains 90-93% of the radioactivity, was 0.67-0.70.

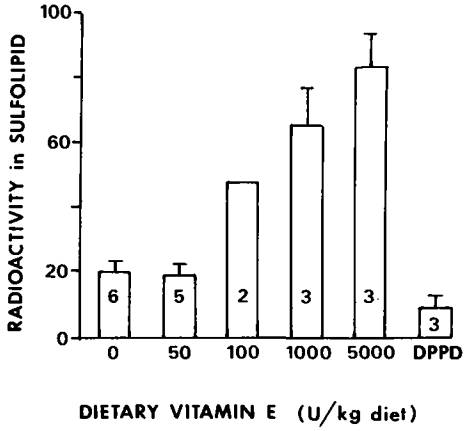

FIG. 2. Labeling of sulfolipid from inorganic [^{35}S]-sulfate in SMG slices obtained from rats fed various levels of vitamin E and the antioxidant DPPD. Conditions as given in Table 2. U is equivalent to 1 mg DL-α-tocopherol acetate. Radioactivity is expressed as dpm $\times 10^8$/100 mg tissue after a 90 min incubation in the presence of 0.5 μCi [^{35}S]sulfate. Values are mean $\pm$ SEM (n as shown within bars).

(17) have reported that rat heart, liver and adrenals have a limited capacity for vitamin E storage. Therefore, the SMG from rats on high vitamin E diets would be expected to contain very high levels of tocopherol. How this effects the sulfolipid-synthesizing system is unknown. It may be that the "extra" vitamin E, which is a very lipophilic molecule, induces changes in the Golgi membranes where most sulfation reportedly occurs (18), allowing an increased sulfation of acceptor lipid. However, the fact that DPPD, an antioxidant that has consistently been shown to mimic the effects of vitamin E (19-21), did not stimulate sulfolipid synthesis, argues against a purely membrane antioxidant phenomenon for the action of vitamin E in this tissue.

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Absence of Wax Esters in Pelagic Lake Baikal Fauna

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ABSTRACT

The absence of wax esters in meso- and bathypelagic organisms from Lake Baikal may be a clue to the historical development of wax ester metabolism in the marine ecosystem.
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Wax esters are common lipid components of many marine organisms[1-5], particularly those inhabiting areas where the food supply is erratic, i.e., meso- and bathypelagic communities. The manner of their distribution in the marine ecosystem has given rise to suggestions that they are a more efficient energy store than the conventional triglycerides[6,7]; that due to their low specific gravity[8], their accumulation in organisms may be a means of attaining neutral buoyancy and hence, an aid to energy conservation[9].

The problems of life in deep-water ecosystems are not confined to the oceans. They also apply to deep freshwater ecosystems, although the problem of buoyancy related to energy conservation is more serious due to the lower specific gravity of freshwater compared to sea water. While such ecosystems are rare, a particularly fine example exists in Lake Baikal (Siberia) which is a very large inland sea, well isolated from the oceans and more than 1600 m deep. Palaeontological data[10] show that by the Pliocene, and perhaps even in the Miocene, the fauna of Lake Baikal had become ecologically distinct from the fauna of the surrounding shallow Siberian lakes. At that time, Baikal must have already existed as a relatively large and deep lake or series of lakes[10] and separation from the marine system is likely to have occurred considerably earlier. Thus, the ecosystem of Lake Baikal has evolved in isolation from the marine ecosystem for many millions of years; indeed, over 75% of the present plant and animal species found are peculiar to Lake Baikal.

Of immediate relevance to the likely function of wax esters in marine animals is the question of how the deep-water pelagic fauna in Lake Baikal have evolved biochemically to cope with low biomass, energy conservation and buoyancy. Lipid analyses of the important meso- and bathypelagic crustaceans and fish (Table 1) indicate the complete absence of wax esters. However, high levels of lipid were found (26-73% dry wt), with triglycerides being the major component (>70% total lipid). The fatty acid compositions of the phospholipids and triglycerides closely resembled those of most oceanic animals.

The midwater fauna of Lake Baikal appear to need greatly enhanced levels of triglyceride. These high triglyceride levels, together with their light skeletons, must result in fairly neutral buoyancy. Indeed, the golomyanka fish (*Comephorus* sp.) has been observed hanging motionless in midwater. Adult males of this species were found generally to have smaller fat deposits than the females, although the more developed the 'eggs' (this species gives birth to live young), the less stored fat. It would appear that the triglyceride stores are functioning, in part, as an energy reserve for egg formation and general metabolism.

Thus, the central question is, for two pelagic ecosystems possessing similar survival problems and similar capacities for fatty acid synthesis, why

TABLE 1

Triglyceride Content of Some Pelagic Organisms from Lake Baikal

Sample	Comments	Depth caught (m)	Lipid content (% dry wt)	Triglyceride content (% total lipid)
Epischura baicalensis (calanoid copepod)	Dominant mesopelagic crustacean	0-200	35	82
Macrohectopus branickii (gammarid)	Dominant meso- bathypelagic gammarid	0-1000	26	70
Comephorus baicalensis (fish)	Dominant meso bathypelagic fish	0-1000	73	>95

do marine animals have a wax ester metabolism in addition to the more general one of triglyceride, while Lake Baikal animals merely build up their triglyceride content? A possible explanation is that the fauna of Lake Baikal have not developed the appropriate biochemical mechanisms for the reduction of normal fatty acids to corresponding alcohols, thus not allowing subsequent coupling of acids and alcohols to give wax esters. If correct, this would strongly imply that such mechanisms were only relatively recently developed in the marine ecosystem after the isolation of Lake Baikal (ca. 40-60 million years). Certainly, the earliest reported wax esters in marine sediments (based on available DSDP data, Dr. S. Brassell personal communication) occur during the lower Pliocene/upper Miocene (ca. 10 million years).

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The Effects of Partially Hydrogenated Marine Oils on the Mitochondrial Function and Membrane Phospholipid Fatty Acids in Rat Heart

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ABSTRACT

The influence of dietary partially hydrogenated marine oils containing docosenoic acid on rat heart mitochondrial membrane phospholipid fatty acid composition was studied with particular reference to cardiolipin and oxidative phosphorylation. Five groups of male weanling rats were fed diets containing 20% (w/w) peanut oil (PO), partially hydrogenated peanut oil (HPO), partially hydrogenated Norwegian capelin oil (HCO), partially hydrogenated herring oil (HHO), and rapeseed oil (RSO) for 10 weeks. All the cardiac phospholipids investigated were influenced by the experimental diets. An increased amount of arachidonic acid observed in phosphatidylethanolamine (PE) after feeding partially hydrogenated oils suggests a changed regulation of the arachidonic acid metabolism in comparison with PO treatment. 22:1 originating from the dietary oils was incorporated only to a small extent into phosphatidylcholine (PC) and PE. A selective incorporation of 18:1 isomers into the 1- and 2-positions of PC and PE with respect to geometry and position of the double bond was observed. Large amounts of 18:1 *trans* were incorporated into the 1-position of PC and PE, irrespective of the amount of 18:2 supplemented to the diets, replacing a considerable proportion of stearic acid in this position. After feeding HHO and RSO, the content of 22:1 in mitochondrial cardiolipin of rat heart was found to be 3% (mainly cetoleic acid) and 10% (mainly erucic acid), respectively, indicating a high affinity for *cis* isomers of 22:1, but also a considerable resistance against incorporation of *trans* isomers was observed. The ability of rat cardiac mitochondria to oxidize palmitoylcarnitine and to synthesize ATP was depressed after feeding HHO and RSO. Dietary *cis* isomers of 22:1 seem to have a specific ability to interfere with cardiac ATP synthesis and also to alter the fatty acid composition of cardiolipin of rat heart.
Lipids 18:151-170, 1983.

INTRODUCTION

Fatty acids with *trans* double bonds do occur in some natural oils from leaves and seeds of plants (1) and ruminant fats (2,3), but the main source in our diets is from partial hydrogenation of vegetable and marine oils. During this process, a wide variety of geometrical and positional isomers of fatty acids arises, depending on the original composition of the oils (4-10).

Our knowledge on the biological and metabolic effect of geometrical and positional fatty acid isomers on the heart and liver mitochondrial membranes is not extensive. It is apparent that several membrane functions are dependent on the lipid composition of the membrane (11).

In earlier studies (12), we have shown that dietary erucic acid influences the phospholipid class distribution of cardiac mitochondrial membranes in rats. In these experiments, there was a decrease in phosphatidylethanolamine (PE) and an increase in the phosphatidylcholine (PC) content. Erucic acid was incorporated into the different phospholipids but had a specific affinity to the cardiolipin molecule of the inner mitochondrial membrane. In a series of papers, Innis and Clandinin (13,14) have demonstrated that phospholipid fatty acyl composition of cardiac mitochondrial inner membrane is dynamically influenced by dietary fat and indicating similar changes in the PE/PC ratio.

Rapeseed oil, high in erucic acid, produces decreased rates of ATP synthesis, as first indicated by Houtsmuller et al. (15) and decreased ADP/O ratio (16,17) and rate of energy transport by the membrane adenine nucleotide translocase/creatine phosphokinase energy transport system (18) in isolated cardiac mitochondria. The described biochemical changes occur together with cardiac lipidosis and pathological changes including necrosis and fibrosis of heart muscle and changes in the mitochondrial ultrastructure (for review, see ref. 19). The connection between the biochemical and the morphological changes has been the matter of a lot of dispute (19) and the mechanism involved is not quite clear.

The altered fatty acid composition associated with the incorporation of different dietary long-chain fatty acids and their metabolites in PC, PE and cardiolipin may have important effects on the

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integrity and function of the heart cell membranes. It must, therefore, be considered very important to elucidate in detail the metabolism and biochemical effect of partially hydrogenated marine oils, especially as several of these oils contain isomers of erucic acid.

Conacher et al. (20) have investigated in detail the distribution of geometrical and positional isomers of monounsaturated fatty acids of varying chain length in total lipid of rat heart after feeding partially hydrogenated herring oil. The incorporation of geometrical and positional isomers of octadecenoic acid derived from partially hydrogenated vegetable oils have been studied in different lipid classes from whole organs (21-26) and in the membrane total lipid of rat liver mitochondria (27). So far, however, the dietary influence of partially hydrogenated marine oils on the fatty acid composition of mitochondrial membrane phospholipids of rat heart has not been investigated in detail with an adequate supply of linoleic acid in the diet.

In the present paper, we have studied the incorporation of dietary isomeric fatty acids derived from different partially hydrogenated marine oils into individual phospholipids of mitochondrial membranes of rat heart. Furthermore, the distribution of the individual phospholipids and the cholesterol/phospholipid ratio have been followed in the mitochondrial membranes. Whether or not mitochondrial function is impaired in rats given partially hydrogenated marine oils has remained a matter of controversy (28-31). In the present report, we have also studied the influence of dietary partially hydrogenated marine oils on the ability of isolated rat heart mitochondria to oxidize different substrates and to synthesize ATP.

EXPERIEMENTAL PROCEDURES

Materials

Partially hydrogenated peanut oil was a gift from Dr. B. Andersen (Aarhus Oliefabrik A/S, Aarhus, Denmark); peanut oil, safflower oil, rapeseed oil and partially hydrogenated Norwegian capelin oil, were gifts from Dr. R. Ohlson (AB Karlshamns Oljefabriker, Karlshamn, Sweden); and partially hydrogenated herring oil, a gift from Dr. J. Beare-Rogers (Department of Health and Welfare, Ottawa, Canada). D-Mannitol was obtained from Difco Laboratories (Detroit, MI) and sucrose from Fisher Scientific Co. (Fair Lane, NJ). Bacterial protease (Subtilisin BPN), bovine serum albumin (fraction V, essentially fatty acid-free), ADP, L-malate, L-glutamate, pyruvate, succinate, L-palmitoylcarnitine, rotenone, phospholipase A_2 (Ophiophagus Hannah), and DL-α-tocopherol-acetate were obtained from Sigma Chemical Co. (St. Louis, MO). Methanolic sodium methoxide (0.5 M) was obtained from Applied Science Europe B.V. (Oud-Beijerland, The Netherlands). Methanolic hydrogen chloride (1 M) was prepared from hydrogen chloride, purity 99% (AGA, Special Gas, Lidingö, Sweden). BHT (2,6-di-*tert*-butyl-*p*-cresol) was obtained from Fluka AG (CH-9470 Buchs, Switzerland). All other reagents and chemicals used were purchased from Merck (Darmstadt, GFR).

Animals and Diets

White male Sprague-Dawley rats weighing 50 g at the start of the experiment were used (Anticimex, Stockholm, Sweden). The animals were subjected to reversed lighting periods (light was automatically switched on at 6 P.M. and off at 6 A.M.). The animals, divided into 5 different groups (10 in each group), were fed a semisynthetic diet containing 20% (w/w) of different fats for 10 weeks. The complete composition of the basal diet was as previously described (32), except that DL-α-tocopherolacetate was added as an antioxidant to the diets (0.02% w/w). The different fats used were: peanut oil (PO), partially hydrogenated peanut oil (HPO), partially hydrogenated Norwegian capelin oil (HCO), partially hydrogenated herring oil (HHO), and rapeseed oil (RSO). The diets containing partially hydrogenated oils were supplemented with safflower oil (17% (w/w) partially hydrogenated oil + 3% (w/w) safflower oil) in order to prevent essential fatty acid deficiency. The safflower oil contained 77% of linoleic acid, 18:2, (32) corresponding to 4.6 cal % 18:2 in the supplmented diets. In an additional experiment, a diet containing partially HCO supplemented with a small amount of safflower oil (HCO + 0.8 cal % 18:2) was also fed to a group of 10 rats. The fatty acid composition of the different diets is shown in Table 1. The food for each rat was restricted to 15 g/day except during the last 3 weeks of the feeding period when the rats had free access to food. Water was provided ad libitum. The animals were weighed and examined weekly during the experimental period. The rats were fasted overnight prior to being killed. The animals were sacrificed by decapitation and exsanguination at about 9A.M. on the day of the experiment. Blood was collected for serum cholesterol and triglyceride analyses.

Preparation of Mitochondria

The hearts from 2 rats in each group were immediately removed, placed in ice cold 0.21 M mannitol/70 mM sucrose/0.1 mM EDTA, weighed, and cut into pieces to pooling for mitochondrial preparation. Before further treatment, part of the myocardium (ca. 0.5 g) was removed, frozen and stored in saline at −20C until utilized for lipid analyses. The cardiac mitochondria was isolated mainly according to Pande and Blanchaer (33).

TABLE 1

Fatty Acid Composition (% w/w) of Diets Containing 20% (w/w) Fat: PO, HPO + 4.6 cal% 18:2, HCO + 4.6 cal% 18:2, HHO + 4.6 cal% 18:2 and RSO

	Diets				
Fatty[b] acid	PO	HPO + 4.6 cal% 18:2[a]	HCO + 4.6 cal% 18:2[a]	HHO + 4.6 cal% 18:2[a]	RSO
12:0	-	-	0.3	-	-
14:0	-	0.1	5.0	5.6	-
16:0	10.3	12.0	15.6	12.1	2.3
16:1 *trans*	-	-	4.9	3.5	-
16:1 *cis*	-	-	1.3	3.3	0.1
18:0	2.5	11.6	7.6	2.7	0.9
18:1 *trans*	-	43.5	11.3	5.3	-
18:1 *cis*	41.5	13.1	7.4	8.1	17.0
18:2ω6	37.8	11.3	11.0	11.5	15.1
18:2[c]	-	1.7	1.5	2.2	-
18:3 ω3	0.7	-	-	-	8.2
20:0	1.2	1.4	5.5	2.0	0.7
20:1 *trans*	-	0.7	11.3	6.5	-
20:1 *cis*	1.1	0.3	3.5	10.3	9.7
20:2	-	-	0.2	0.4	0.6
22:0	3.2	2.9	3.5	2.6	0.7
22:1 *trans*	-	0.3	7.1	8.8	-
22:1 *cis*	0.2		2.7	14.5	43.1
24:0	1.5	1.1	0.2	0.1	0.2
24:1	-	-	0.1	0.5	1.4
Σ *trans*	-	44.2	34.6	24.1	-
Σ 22:1	0.2	0.3	9.8	23.3	43.1

[a]The partially hydrogenated diets were supplemented with safflower oil.

[b]The shorthand notation used for the fatty acids indicates chain length: number of double bonds. ω = First double bond position beginning from the hydrocarbon end; methylene interruption is assumed if not otherwise specified.

[c]18:2 isomers other than linoleic acid.

Before treatment with Subtilisin, the hearts were minced by garlic press instead of chopping, and the mitochondrial pellet was washed only once. Care was taken to avoid contamination of the preparations with lipids. The resulting mitochondrial pellet was suspended in 0.5 ml of the solution described above which had been made 10 mM with Tris-Cl (pH 7.4). Liver mitochondria from peanut and rapeseed oil treated animals were isolated in the same way. The yield was ca. 13 mg of mitochondrial protein/g rat heart and 19 mg of mitochondrial protein/g rat liver. Protein was determined according to Lowry et al. (34).

Measurement of Mitochondrial Oxygen Uptake and ATP Synthesis

The oxidation rates of different substrates by the isolated mitochondria were determined by measuring the rate of O_2 consumption by means of a Clark electrode (Yellow Springs Instruments Co., Inc, Yellow Springs, OH) mainly as described by Swarttouw (35). The mitochondria (ca. 0.25 mg of heart mitochondrial protein and 0.5 mg of liver mitochondrial protein) was incubated at 37C in a medium containing 7.7 mM sucrose, 7.7 mM K_3PO_4 (pH 7.4), 45 mM KCl, 63 mM Tris-Cl (pH 7.4), 3.3 mM $MgCl_2$, and 0.17% w/w bovine serum albumin (essentially fatty acid-free). The addition of cytochrome c was found to be unnecessary and, therefore, was omitted. 6 mM succinate with 1.5 μM rotenone, 6 mM glutamate with 0.20 mM malate, 6 mM pyruvate with 0.20 mM malate or 36 μM palmitoylcarnitine with 0.20 mM malate were used as substrates. The total incubation volume was 1.66 ml and the oxygen content of the air-saturated medium was calculated to be 385 ng atoms of oxygen/ml at 37C according to Estabrook (36). The reaction was started with the addition of 400 nmol ADP. The state 3 respiratory rate in the presence of ADP, the state 4 respiratory rate after exhaustion of ADP, the respiratory control ratio, the ADP/O ratio, and the ATP synthesis rate were calculated as described by Estabrook (36).

Lipid Analysis

Mitochondria and homogenate were extracted for total lipid according to Folch et al. (37). Using silicic acid chromatography (12), neutral lipids were eluted with chloroform and phospholipids with methanol. BHT was added to the eluates to a

final concentration of 20 μg/ml. Individual phospholipids were separated from total phospholipids (12) on Silica Gel H plates with chloroform/methanol/25% aqueous ammonia (14:6:1,v/v/v) followed by chloroform/methanol/acetic acid/water (80:13:8:0.3, v/v/v/v) as solvent systems. Cochromatographed authentic cardiolipin, PE and PC and the outer edges of the different zones corresponding to separated phospholipids from the sample were visualized with iodine vapor. Care was taken not to contaminate the separated phospholipids with iodine vapor. The chromatographic zones corresponding to cardiolipin, PE and PC were collected and extracted according to Burns et al. (38). Phosphorus analysis was performed on the appropriate silica gel zones without prior elution of the phospholipids according to Vikrot (39).

Analysis of the positional distribution of fatty acids in the isolated PC and PE (1-2 mg) was performed by enzymatic hydrolysis with phospholipase A_2 (Ophiophagus Hannah snake venom) mainly as described by Christie (40). The resultant fatty acids from the 2-position and the lysophospholipids were extracted using chloroform/methanol (2:1, v/v) after dilution with water (37). To improve the TLC separation of incubation products from PE diisopropyl ether/acetic acid (96:4, v/v) was used as the first solvent system. The chromatographic zones corresponding to the liberated fatty acids and the lysophospholipids were visualized with iodine vapor as described above, and scraped directly into screw-capped test tubes. Fatty acid methyl esters were prepared as previously described (10).

Analysis of Fatty Acid Distribution and the Content of Geometrical and Positional Isomers

Gas liquid chromatography (GLC) of fatty acid methyl esters was performed utilizing glass capillary columns coated with Silar-5 CP as stationary phase (41). The fatty acid distribution was expressed as area percentages. Unsaturation index (UI) and mean chain length (MCL) were calculated according to Innis and Clandinin (13). The content of geometrical and positional isomers of monounsaturated fatty acids in dietary oils, heart neutral lipid, and mitochondrial phospholipids was determined by high performance liquid chromatography (HPLC) and glass capillary gas chromatography (10). Unfortunately, cholesterol cochromatographed with *trans* docosenoic acid methyl esters in the HPLC under the conditions used. Thus, prior to analysis with HPLC of fatty acid methyl esters from rat heart neutral lipid, the methyl esters were purified by thin layer chromatography (TLC) on Silica Gel G plates using hexane/diethyl ether (90:10, v/v) as solvent system.

Analysis of Cholesterol and Triglycerides

Cholesterol content in whole heart and heat mitochondria was determined by isotope dilution-mass spectrometry (42), serum triglycerides according to Wahlefeld (43), and serum cholesterol according to Svensson et al. (44).

RESULTS

Fatty Acid Composition of Dietary Fat

The different experimental diets were extracted and the lipids obtained were analyzed with respect to fatty acid composition by glass capillary gas chromatography and HPLC (Table 1). There was no detectable amount of *trans* fatty acids in the unhydrogenated oils (PO and RSO), but there were small amounts of ω7-positional isomers of *cis* monounsaturated fatty acids. PO contained 0.9% 18:1ω7 *cis*, and the content of 18:1ω7 *cis*, 20:1ω7 *cis*, and 22:1ω7 *cis* in RSO was 1.2%, 1.1%, and 1.0%, respectively. The partially hydrogenated oils used in the experimental diets were further characterized with respect to the content of positional and geometrical isomers of monounsaturated fatty acids of chain length 18-22 by means of HPLC and glass capillary gas chromatography (Table 2). However, all positional isomers could not be resolved in the glass capillary gas chromatography. 18:1ω4 *trans* cochromatographed with 18:1ω5 *trans*, and positional isomers with the double bond in a relatively high ω-position, irrespective of geometry and chain length, had a tendency to coincide. Generally cetoleic acid (22:1ω11 *cis*) separated from 22:1 *cis* isomers with the double bond located in a lower ω-position, but overlapped to some extent with 22:1ω12 *cis* and 22:1ω13 *cis*, as could be judged from the peak shapes obtained in the chromatogram. The 18:1 *cis* fractions contained the ω9 isomer as a major constituent, but part of the 18:1ω9 *cis* found in the partially hydrogenated oils originated from the added safflower oil, which contained ca. 12% oleic acid as the predominant 18:1 isomer. Generally the *trans* fractions had a wider range of positional isomers as compared to the *cis* fractions. HHO contained less *trans* fatty acids as compared to HPO and HCO. The double bonds in the *cis* fraction of 20:1 and 22:1 from HHO were mainly in ω9- and ω11-position, respectively, the original double bond positions in 20:1 and 22:1 from unhydrogenated marine oils. The HCO which was hydrogenated to a higher degree contained less isomers with the original double bond positions as compared to HHO.

Food Consumption, Body Weight, Heart Size, Serum Cholesterol, and Serum Triglycerides

The effects of the different diets on food con-

TABLE 2

Content of Monounsaturated Fatty Acid Isomers in Diets Containing Partially Hydrogenated Fat: HPO +4.6 cal% 18:2, HCO+4.6 cal% 18:2, and HHO+4.6 cal% 18:2

Double bond position (ω)[c]	Diets													
	HPO+4.6 cal% 18:2[a]		HCO+4.6 cal% 18:2[a]						HHO+4.6 cal% 18:2[a]					
	18:1[b] (56.6%)[d]		18:1 (18.7%)		20:1 (14.8%)		22:1 (9.8%)		18:1 (13.4%)		20:1 (16.8%)		22:1 (23.3%)	
	trans (76.9%)[e]	*cis* (23.1%)[e]	*trans* (60.4%)	*cis* (39.6%)	*trans* (76.4%)	*cis* (23.6%)	*trans* (72.4%)	*cis* (27.6%)	*trans* (39.6%)	*cis* (60.4%)	*trans* (38.6%)	*cis* (61.4%)	*trans* (37.8%)	*cis* (62.2%)
3	1.1	-	10.6	-	-	-	-	-	-	-	-	-	-	-
4	T	-	T	-	-	-	-	-	T	-	-	-	-	-
5	7.2	1.5	9.0	1.5	4.7	-	-	-	11.1	2.3	2.0	-	-	-
6	8.7	7.7	13.0	7.0	4.1	3.5	-	-	9.5	3.4	2.4	-	-	-
7	13.5	10.1	20.3	8.1	7.9	9.3	-	3.1	14.5	13.4	6.1	3.6	-	0.4
8	25.4	14.7	27.4	8.8	17.1	19.1	4.0	4.7	18.4	3.7	14.4	4.1	-	-
9	T	56.0	T	67.5	31.7	35.3	11.7	16.9	T	70.7	T	79.5	10.2	4.9
10	44.1	T	19.7	T	T	T	22.2	21.0	46.5	T	75.1	T	15.4	2.2
11		10		7.1	34.5	32.8	T	T		6.5		12.8	T	T
12							62.1	54.3					74.4	92.5
13	-	-	-	-	-	-			-	-	-	-		

[a]The partially hydrogenated diets were supplemented with safflower oil.
[b]The shorthand notation used for the fatty acids indicates chain length: number of double bonds.
[c]The double bond position (ω) is stated from the hydrocarbon end of the fatty acid molecule.
[d]Figures in parentheses after each monounsaturated fatty acid refer to percent in total fatty acids.
[e]Figures in parentheses after *cis* and *trans* refer to percent within each monounsaturated fatty acid.

sumption, body weight, heart size, serum cholesterol, and serum triglycerides are summarized in Table 3. The average food consumption was 15.5 g per day and rat, and there was only small differences between the various dietary treatments. The body weight of animals kept on HPO and HCO diets were slightly higher than those of animals fed PO. Hearts from rats fed RSO were significantly enlarged, in agreement with the findings of Beare-Rogers and Nera (45). Also, the hearts from rats subjected to docosenoic acid rich HHO were significantly enlarged, but the hearts from rats fed HPO were significantly smaller as compared to the heart size when kept on PO diet. The serum cholesterol levels were significantly lower in the groups of rats fed partially hydrogenated oils as compared to animals kept on PO and RSO diets. On the contrary, the serum triglyceride pattern of the different groups of rats was reversed. Dietary polyunsaturated fat has been reported to increase the serum cholesterol level in rat as compared to dietary saturated fat (46,47). Thus, the serum cholesterol lowering effect observed after feeding partially hydrogenated oils to rats as compared to animals kept on unhydrogenated oils might be better related to the content of polyunsaturated fatty acids than to the content of *trans* fatty acids in the experimental diets.

Content of Total Lipid, Neutral Lipid, Phospholipid, and Cholesterol in Heart

The effects of the different diets on the content of total lipid, neutral lipid, phospholipid and cholesterol in rat heart are presented in Table 4. There was a tendency to higher content of total lipid in hearts from rats fed diets containing docosenoic acid (HCO, HHO, and RSO) as compared to the corresponding lipid from rats kept on PO and

TABLE 3

Food Consumption, Body Weight, Heart Size, Serum Cholesterol, and Serum Triglycerides of Rats Fed Diets Containing 20% (w/w) PO, HPO + 4.6 cal% 18:2, HCO + 4.6 cal% 18:2, HHO + 4.6 cal% 18:2, and RSO for 70 Days

	PO	HPO + 4.6 cal% 18:2[a]	HCO + 4.6 cal% 18:2[a]	HHO + 4.6 cal% 18:2[a]	RSO
Food consumption[b] (g/day)	15.3	15.3	15.9	15.5	15.4
Body weight[c] (g)	368 ± 15	381 ± 11[e]	391 ± 22[e]	370 ± 11	361 ± 13
Heart size[c] (% of body weight)	0.33 ± 0.01	0.30 ± 0.01[g]	0.32 ± 0.01	0.35 ± 0.01[e]	0.40 ± 0.01[i]
Serum cholesterol[d] (mmol/l)	2.42 ± 0.14	1.87 ± 0.10[i]	2.02 ± 0.08[h]	2.00 ± 0.18[h]	2.65 ± 0.09[i]
Serum triglycerides[d] (mmol/l)	0.37 ± 0.04	0.49 ± 0.09[f]	0.53 ± 0.06[h]	0.46 ± 0.12	0.36 ± 0.02

[a]The partially hydrogenated diets were supplemented with safflower oil.
[b]Results are mean from 10 rats.
[c]Results are mean ± standard deviation from 10 rats.
[d]Results are mean ± standard deviation from 6 rats.
[e-i]Probability level as calculated according to Student's t-test; e, f, g, h, and i are significantly different from PO values: $p < 0.05$, $p < 0.01$, $p < 0.005$, $p < 0.001$, and $p < 0.0001$, respectively.

TABLE 4

Total Lipid, Neutral Lipid, Phospholipid, and Cholesterol Content of Heart from Rats Fed Diets Containing 20% (w/w) PO, HPO + 4.6 cal% 18:2, HCO + 4.6 cal% 18:2, HHO + 4.6 cal% 18:2, and RSO for 70 Days

	PO	HPO + 4.6 cal% 18:2[a]	HCO + 4.6 cal% 18:2[a]	HHO + 4.6 cal% 18:2[a]	RSO
Total lipid[b] (μg/mg protein)	189	187	197	215	231
Total neutral lipid[b] (μg/mg protein)	41	52	56	67	96
Total phospholipid[b] (μg/mg protein)	148	135	141	148	136
Total cholesterol[b] (μg/mg protein)	6.7	6.8	7.1	6.7	7.6

[a]The partially hydrogenated diets were supplemented with safflower oil.
[b]The results obtained are from analysis of pooled homogenate from 10 rat hearts.

HPO diets. The increased total lipid was mainly due to an increased content of neutral lipid, principally triglycerides, in hearts from rats fed docosenoic acid-rich diets, demonstrating the occurrence of cardiac lipid accumulation even after long-term dietary treatment in agreement with observations made by others (45,48). There was no tendency to altered content of phospholipid and cholesterol in rat hearts caused by the different dietary treatments.

Content of Monounsaturated Fatty Acid Isomers in Heart Neutral Lipid

The content of positional and geometrical monounsaturated fatty acids of chain length 18-22 in heart neutral lipid from rats fed partially hydrogenated oils (HPO, HCO, and HHO) supplemented with 4.6 cal % 18:2 is summarized in Table 5. The total contents of docosenoic acid as percent of total fatty acids in heart neutral lipid after feeding HCO, HHO, and RSO were 2.4%, 8.7%, and 29.9%, respectively. The docosenoic acid found in RSO fed animals was mainly erucic acid. The total *trans* fatty acid content as percent of total fatty acids in the corresponding lipids from rats fed HPO, HCO, and HHO diets was 16.2%, 10.0% and 10.5%, respectively. As compared to the distribution of geometrical isomers in the dietary partially hydrogenated oils, less *trans* than *cis* monounsaturated fatty acids were incorporated. With the exception of 18:1ω8 *cis*, which was excluded to some extent, there was a fair resemblance between the isomer composition of octadecenoic acid in neutral fat from rats fed HPO and the dietary HPO. Positional isomers of docosenoic acid were incorporated into the neutral lipid approximately to the same extent as their relative proportions in the dietary marine oils. Positional isomers of octadecenoic and eicosenoic acid with the double bond in a relatively high position (ω10-ω12) were deposited in the neutral lipid in a higher degree as compared to their relative distribution in the dietary HCO and HHO. These isomers were probably derived from chain-shortening of the major 22:1 isomers (ω10-ω13) found in the corresponding dietary oils. These results are consistent with the work of Craig and Beare (49) and Carreau et al. (50), who presented evidence for the chain-shortening in rats of 22:1ω11 *cis* and 22:1ω9 *cis*, and the wok of Conacher et al. (20), who found increased levels of *cis* as well as *trans* isomers of 18:1ω11 and 20:1ω11 in rat heart total lipid after feeding partially hydrogenated herring oil rich in 22:1ω11.

Content of Total Lipid, Neutral Lipid, Phospholipid, and Cholesterol of Cardiac Mitochondria

The effects of the different dietary treatments on the content of total lipid, neutral lipid, phospholipid, and cholesterol of cardiac mitochondria are presented in Table 6. No significant amount of esterified cholesterol could be detected by TLC. There was a tendency to a decreased content of total lipid and phospholipid in cardiac mitochondria isolated from rats fed HPO, HCO, HHO, and RSO as compared to the corresponding content in animals kept on dietary PO. The individual phospholipids followed this pattern to some extent. However, there was a tendency to a decreased ratio between PE and PC after feeding RSO in accordance with earlier observations (12,14). The content of neutral lipids seemed not to be affected by the different diets. There was a tendency to elevated cholesterol content and cholesterol/phospholipid ratio in cardiac mitochondria in rats fed RSO.

Positional Distribution of Fatty Acids in PC and PE of Cardiac Mitochondria

The relative distribution of fatty acids as percent of total fatty acids in the 1- and 2-positions of PC from cardiac mitochondria isolated from rats fed the different experimental diets are summarized in Table 7. The results from a group of rats fed HCO supplemented with a low amount of linoleic acid (HCO + 0.8 cal % 18:2) were also included for comparison. The fatty acids from PO and RSO fed animals were found to be distributed according to the general pattern having the saturated fatty acids located predominately in the 1-position and the polyunsaturated acids almost entirely in the 2-position. In contrast to the neutral lipid of rat heart, only a small amount of docosenoic acid was incorporated into mitochondrial PC. *Trans* fatty acids, mainly octadecenoic acids, were preferentially incorporated in the 1-position, replacing a considerable proportion of stearic acid in this position. The amount of linoleic acid supplemented to the diets did not have any major influence on the degree of incorporation of *trans* monounsaturated fatty acids. However, the rats fed HCO + 0.8 cal % 18:2 suffered to some extent from essential fatty acid deficiency, as could be judged from the relatively high content of 20:3ω9 (7.0%), low content of 20:4ω6, and the markedly reduced mean chain length and unsaturation index in the 2-position of PC. A high amount of 20:4ω6 was observed and only a trace amount of 20:3ω9 could be detected in rats fed partially hydrogenated diets supplemented with 4.6 cal % 18:2, indicating that the diets had been supplemented with an adequate amount of linoleic acid. Only small amounts of *trans* fatty acids could be found in animals kept on unhydrogenated diets (PO and RSO). The values obtained were comparable to those reported for PC and PE from rat cardiac tissue by Wood et al. (51) after feeding a chow diet with no detectable quantities of *trans* fatty acids. The total content of ω6 fatty acids in the 2-position of PC in the groups

TABLE 5

Content of Monounsaturated Fatty Acid Isomers in Neutral Lipid from Rat Heart After Feeding Diets Containing 20% (w w) HPO + 4.6 cal% 18:2, HCO + 4.6 cal% 18:2, and HHO + 4.6 cal% 18:2 for 70 Days

Double bond position (ω)[c]	Monounsaturated fatty acid isomers in heart neutral lipid after feeding different oils													
	HPO + 4.6 cal% 18:2[a]		HCO + 4.6 cal% 18:2[a]						HHO + 4.6 cal% 18:2[a]					
	18:1[b] (28.9%)[d]		18:1 (16.2%)		20:1 (4.8%)		22:1 (2.4%)		18:1 (18.8%)		20:1 (9.3%)		22:1 (8.7%)	
	trans (56.0%)[e]	*cis* (44.0%)[e]	*trans* (39.7%)	*cis* (60.3%)	*trans* (46.0%)	*cis* (54.0%)	*trans* (58.5%)	*cis* (41.5%)	*trans* (31.0%)	*cis* (69.0%)	*trans* (22.0%)	*cis* (78.0%)	*trans* (29.7%)	*cis* (70.3%)
3	1.1[f]	-	2.4	-	-	-	-	-	2.4	-	-	-	-	-
4	7.4	-	12.1	-	-	-	-	-	8.4	-	-	-	-	-
5		1.3		-	5.1	-	-	-		-	-	-	-	-
6	9.8	5.9	9.3	3.4	7.0	2.8	-	-	5.5	1.4	3.0	-	-	-
7	14.8	18.8	13.4	15.8	12.4	16.2	-	3.9	10.4	13.9	6.8	6.8	-	-
8		8.3		69.7	10.5	16.3	8.2	2.7	54.5	67.0	8.7	5.3	-	-
9		51.5			21.8	19.6	17.9	13.2			43.5	50.1	11.9	10.3
10	66.9		62.8			22.9	13.4	21.7					18.5	4.9
11		14.2		11.1	43.2	22.2			18.8	17.7	38.0	37.8		
12							60.5	58.5					69.6	84.8
13	-	-	-	-	-	-			-	-	-	-		

[a]The partially hydrogenated diets were supplemented with safflower oil.
[b]The shorthand notation used for the fatty acids indicates chain length: number of double bonds.
[c]The double bond position (ω) is stated from the hydrocarbon end of the fatty acid molecule.
[d]Figures in parentheses after each monounsaturated fatty acid refer to percent in total fatty acids.
[e]Figures in parentheses after *cis* and *trans* refer to percent within each monounsaturated fatty acid.
[f]The values obtained are from analysis of pooled homogenate from 10 rat hearts.

TABLE 6

Total Lipid, Neutral Lipid, Phospholipid, and Cholesterol Content of Cardiac Mitochondria Isolated from Rats Fed Diets Containing 20% (w/w) PO, HPO+4.6 cal% 18:2, HCO+4.6 cal% 18:2, HHO+4.6 cal% 18:2 and RSO for 70 Days

	PO	HPO+ 4.6 cal% 18:2[a]	HCO+ 4.6 cal% 18:2[a]	HHO+ 4.6 cal% 18:2[a]	RSO
Total lipid[b] (μg/mg protein)	377	293	338	297	296
Total neutral lipid[b] (μg/mg protein)	34	25	25	31	32
Cardiolipin[b,c] (μg/mg protein)	55	46	55	46	47
Phosphatidylethanolamine[b,c] (μg/mg protein)	123	102	117	94	82
Phosphatidylcholine[b,c] (μg/mg protein)	151	107	130	116	126
Unidentified phospholipid[b,c,d] (μg/mg protein)	14	13	11	10	9
Total phospholipid[b,c] (μg/mg protein)	343	268	313	266	264
Total cholesterol[b] (μg/mg protein)	1.8	1.6	1.4	1.6	2.2
Total cholesterol × 10²/total phospholipid	5.2	5.9	4.4	6.0	8.3

[a]The partially hydrogenated diets were supplemented with safflower oil.
[b]The results obtained are from analysis of pooled mitochondria from 10 rat hearts.
[c]The amount of phosphorus analyzed was converted to μg phospholipid by the use of the following molecular weights: 1585, 744, 787, and 750 for cardiolipin, PE, PC, and unidentified phospholipid, respectively.
[d]The unidentified phospholipid had a slightly lower R_f value than PC in the TLC.

of fats fed partially hydrogenated oils supplemented with 4.6 cal % 18:2 did not seem to be affected by the dietary treatment. However, after feeding partially hydrogenated marine oils (HCO and HHO), there was a tendency to a decreased content of 22:4ω6 and 22:5ω6. Similar changes were observed by Astorg and Cluzan (52), after feeding partially hydrogenated herring oil to rats. The presence of *trans* fatty acids in the diet, if supplemented with an adequate amount of 18:2, generally increased the UI of PC in comparison with PO treated animals. As can be deduced from Table 7, the increased UI in the 1-position of PC from animals fed HPO and HCO was mainly a consequence of the large incorporation of 18:1 *trans*, but for animals kept on HHO, *trans* as well as *cis* isomers contributed to the elevated UI in this position. Feeding RSO increased the UI in both positions of PC in comparison with animals fed PO. A decrease of the relative amounts of 22:4ω6, and 2:5ω6 followed by a corresponding increase of 20:5ω3, 22:5ω3, and 22:6ω3 could be observed in rats fed RSO. The relatively high proportions of ω3 fatty acids of RSO treated animals might be related to elongation desaturation of 18:3ω3, a considerable constituent of the RSO diet.

Generally, PE from cardiac mitochondria contained lower amount of 20:4ω6 and higher amount of 22:5 and 22:6 as compared to PC (Table 8). The influence of the dietary treatment on the fatty acid distribution in the 1- and 2-positions of PE was similar to that found in PC. However, PE from rats fed partially hydrogenated oils had a higher proportion of *trans* fatty acids as compared to PC. UI of the 2-position of PE was not affected by the different partially hydrogenated oils fed to the rats. However, an alteration in the distribution of the ω6-polyunsaturated fatty acids was observed. The content of 20:4ω6 increased and the content of ω6 docosapolyenoic acids decreased after feeding partially hydrogenated diets as compared to rats fed PO. This observation might indicate a partial blockage in the elongation desaturation process of ω6 fatty acids to docosapolyenoic acid or an increased retroconversion of docosapolyenoic acids. Feeding RSO resulted in a marked increase in ω3 polyunsaturated fatty acids and UI of PE.

Relative Distribution of Positional and Geometrical Isomers of Octadecenoic Acid in 1- and 2-position of PC and PE of Cardiac Mitochondria

The distribution of positional isomers of 18:1 *trans* in the 1-position of PC and PE is recorded in Table 9. The small amount available of *trans* fatty acids from the 2-position did not permit accurate analysis of positional isomers. As mentioned above, 18:1ω4 *trans* and 18:1ω5 *trans* could not be resolved and 18:1ω8-12 coincided in the glass capillary gas chromatography. Irrespective of dietary treatment, the isomers with the double bond located around the center of the molecule (18:1ω8-12) made up a

TABLE 7

Relative Distribution of Fatty Acids as Percent of Total Fatty Acids in the 1- and 2-Positions of PC from Cardiac Mitochondria Isolated from Rats Fed Diets Containing 20% (w/w) PO, HPO, HCO, HHO and RSO for 70 Days

Fatty[b] acid	PO		HPO+ 4.6 cal% 18:2[a]		HCO+ 0.8 cal% 18:2[a]		HCO+ 4.6 cal% 18:2[a]		HHO+ 4.6 cal% 18:2[a]		RSO	
	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos
12:0	-	-	0.02	0.20	-	0.80	-	-	-	-	-	-
14:0	0.12[c]	0.29	0.11	0.23	0.22	0.80	0.30	0.36	0.24	0.29	-	-
14:1	-[d]	-	-	-	0.03	0.68	-	-	-	-	-	-
15:0ai	-	-	-	-	-	0.11	-	0.09	-	-	-	-
15:0	0.12	0.22	0.07	0.09	0.14	0.29	0.14	0.18	0.19	0.15	-	-
16:0	19.55	5.78	16.81	3.87	18.15	6.04	17.05	4.95	13.58	4.39	7.03	2.89
16:1	0.26	0.13	1.12	0.30	2.90	3.46	2.12	1.00	1.92	0.78	0.37	-
17:0ai	-	-	-	-	0.20	0.17	0.14	0.08	0.10	0.06	-	-
17:0	0.41	0.10	0.15	0.07	0.41	0.22	0.39	0.11	0.67	0.09	0.39	0.10
18:0	62.32	3.31	39.66	1.69	37.39	4.01	46.77	2.06	44.56	1.67	56.33	2.06
18:1	7.24	9.55	33.81	11.61	23.82	33.48	20.73	7.31	20.49	6.48	10.65	9.62
18:2[e]	-	-	0.70	0.10	1.27	2.68	0.99	0.14	1.18	-	-	-
18:2 ω 6	7.58	10.44	6.27	8.98	11.24	23.07	8.50	13.52	10.83	10.62	11.62	8.43
18:3 ω 6	-	-	-	-	-	0.19	-	-	-	-	-	-
18:3 ω 3	-	-	-	-	0.02	0.50	-	-	-	-	0.87	0.14
20:0 iso	-	-	-	-	-	-	-	-	-	-	-	-
20:0	0.59	0.10	0.07	-	0.49	0.24	0.67	0.05	0.71	0.07	0.90	0.11
20:1	0.74	-	0.19	-	2.05	1.42	1.60	0.22	3.73	1.16	7.33	1.12
20:2	-	-	-	-	0.12	0.52	-	-	-	-	-	-
20:2 ω 6	0.08	0.35	-	0.13	0.21	0.21	0.06	0.15	0.31	0.25	0.78	1.20
20:3 ω 9	-	-	-	0.07	0.17	6.95	-	0.07	0.06	0.15	-	0.14
20:3 ω 6	0.11	0.28	0.03	0.38	0.14	2.11	0.04	0.84	0.11	1.10	0.19	0.80
20:4 ω 6	0.61	62.05	0.86	64.13	0.17	9.30	0.48	63.42	0.69	67.87	0.89	59.17
20:5 ω 3	-	-	-	-	-	0.22	-	-	-	0.07	0.08	1.56
22:0	-	-	-	-	0.06	0.12	-	-	-	-	-	-
22:1	0.07	-	-	-	0.28	0.11	-	-	0.52	0.65	2.06	2.07
22:2 ω 6	-	-	-	-	-	-	-	-	-	-	0.04	0.21
22:4 ω 6	0.10	1.94	0.05	1.63	0.07	0.21	0.06	0.87	0.04	0.58	0.16	0.18
22:5 ω 6	0.03	2.93	0.06	4.00	0.12	0.36	-	2.49	-	1.23	-	0.09
22:5 ω 3	-	0.58	-	0.43	0.21	0.33	-	0.35	-	0.38	0.04	3.58
22:6 ω 3	0.03	1.97	0.04	2.10	0.14	1.19	-	1.73	0.06	1.84	0.21	6.54
23:0	-	-	-	-	-	-	-	-	-	-	-	-
24:0	-	-	-	-	-	0.22	-	-	-	-	-	-
24:1	-	-	-	-	-	-	-	-	-	-	0.07	-
Saturated	83.11	9.80	56.89	6.15	57.06	13.02	65.43	7.88	60.05	6.72	64.65	5.16
Unsaturated												
ω 9 *cis*	6.55	5.71	2.74	4.77	7.26	31.32	2.60	3.51	4.59	4.13	14.83	9.60
ω 6 *cis*	8.51	77.99	8.25	79.53	12.78	38.85	9.52	81.65	12.48	81.93	13.68	70.08
ω 3 *cis*	0.03	2.55	0.04	2.60	0.37	2.24	-	2.08	0.06	2.29	1.20	11.82
18:1 *trans*	0.50	-	26.26	2.16	15.56	2.60	16.91	1.38	16.32	0.90	2.00	-
20:1 *trans*	0.33	-	0.19	-	1.37	0.15	1.03	0.11	0.90	0.23	-	-
Other mono-unsaturated	0.93	3.97	4.96	4.70	4.20	8.63	3.52	3.24	4.41	3.80	3.63	3.32
Others	-	-	0.70	0.10	1.39	3.20	0.99	0.14	1.18	-	-	-
MCL[f]	17.6	19.4	17.7	19.5	17.7	18.2	17.7	19.4	17.8	19.5	18.2	19.7
UI[f]	27	317	53	330	59	171	46	321	55	328	54	339

[a]The partially hydrogenated diets were supplemented with safflower oil.

[b]The shorthand notation used for the fatty acids indicates chain length:number of double bonds. ω = First double bond position beginning from the hydrocarbon end; methylene interruption and *cis* geometry are assumed if not otherwise specified.

[c]The values obtained are from analysis of pooled mitochondria from 10 rat hearts.

[d]Indicates values less than 0.005%.

[e]18:2 isomers other than linoleic acid.

[f]MCL and UI were calculated according to Innis and Clandinin (13).

major part of the *trans* isomers in PC and PE. Reichwald-Hacker et al. (25) have reported that the incorporation of 18:1ω8 *trans* was discriminated over the other positional isomers in PC of rat heart after feeding partially hydrogenated soybean oil which contained a considerable amount of this isomer. However, in the present study, the presence of 18:1ω8 *trans* could not be determined with

TABLE 8

Relative Distribution of Fatty Acids as Percent of Total Fatty Acids in the 1- and 2-Positions of PE from Cardiac Mitochondria Isolated from Rats Fed Diets Containing 20% (w/w) PO, HPO, HCO, HHO, and RSO for 70 Days

Fatty[b] acid	PO		HPO+ 4.6 cal% 18:2[a]		HCO+ 0.8 cal% 18:2[a]		HCO+ 4.6 cal% 18:2[a]		HHO+ 4.6 cal% 18:2[a]		RSO	
	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos
12:0	-	-	-	0.10	-	0.39	-	-	-	0.18	-	-
14:0	0.04[c]	0.15	0.02	0.28	0.08	0.93	0.06	0.33	0.07	0.38	-	0.17
14:1	-[d]	-	-	-	-	0.06	-	-	-	-	-	-
15:0ai	-	-	-	-	-	0.09	-	-	-	-	-	-
15:0	0.04	0.15	0.02	0.12	0.06	0.43	0.04	0.13	0.05	0.15	-	0.14
16:0	9.60	2.97	5.16	1.97	7.16	4.67	7.10	2.24	6.45	2.25	4.61	2.40
16:1	-	0.40	0.09	0.28	0.65	1.89	0.66	0.36	0.60	0.35	0.08	0.17
17:0ai	-	-	-	-	0.03	0.10	0.06	0.05	0.09	0.05	-	-
17:0	0.42	0.27	0.15	0.09	0.49	0.32	0.43	0.11	0.70	0.14	0.45	0.20
18:0	67.14	3.31	41.96	2.67	42.73	3.68	48.48	2.33	44.27	2.15	48.39	3.18
18:1	11.06	8.68	40.79	6.54	35.09	12.97	26.52	4.65	26.88	4.14	21.35	5.87
18:2[e]	-	-	0.62	-	1.57	1.02	1.04	0.14	1.29	0.07	-	-
18:2 ω 6	5.73	8.59	2.85	8.01	4.88	13.08	5.09	10.52	5.99	8.98	6.64	8.91
18:3 ω 6	-	-	-	-	0.06	0.05	-	-	-	-	-	-
18:3 ω 3	0.05	0.11	-	0.05	-	0.09	-	-	-	-	0.93	0.31
20:0 iso	-	-	-	-	-	-	-	-	-	-	-	-
20:0	0.85	0.23	0.18	0.06	0.62	0.26	0.78	0.12	0.88	0.09	0.88	0.22
20:1	0.78	0.37	0.11	-	2.99	1.34	2.42	0.45	5.15	2.01	6.26	1.89
20:2	-	-	-	-	0.16	0.23	-	-	-	-	-	-
20:2 ω 6	0.03	0.72	-	0.05	0.23	0.07	0.05	0.12	0.37	0.30	0.65	1.13
20:3 ω 9	-	0.03	-	-	0.41	9.65	-	-	0.08	0.10	0.03	0.04
20:3 ω 6	0.12	0.11	-	0.15	0.36	1.29	0.04	0.26	0.18	0.35	0.21	0.27
20:4 ω 6	2.89	34.53	6.65	48.40	0.95	38.57	5.78	46.99	4.83	51.56	5.40	30.80
20:5 ω 3	-	-	-	-	0.09	0.41	-	-	-	-	0.17	0.87
22:0	0.06	-	-	-	0.23	0.04	-	-	0.15	-	-	-
22:1	0.19	-	-	-	0.71	0.10	0.31	-	1.17	1.01	2.31	1.41
22:2 ω 6	-	0.09	-	-	-	0.05	-	-	-	-	-	-
22:4 ω 6	0.38	3.94	0.30	2.66	0.11	0.31	0.23	2.08	0.13	1.59	0.23	0.48
22:5 ω 6	0.22	19.74	0.55	18.86	0.04	1.35	0.44	18.08	0.20	10.40	0.05	0.29
22:5 ω 3	0.07	1.34	0.11	0.71	0.03	0.37	0.10	0.80	0.12	1.11	0.56	8.17
22:6 ω 3	0.33	14.22	0.43	9.05	0.12	5.88	0.37	10.24	0.35	12.63	0.80	33.09
23:0	-	-	-	-	0.08	0.07	-	-	-	-	-	-
24:0	-	0.06	-	-	0.02	0.19	-	-	-	-	-	-
24:1	-	-	-	-	0.03	0.03	-	-	-	-	-	-
Saturated	78.15	7.14	47.49	5.29	51.50	11.17	56.95	5.31	52.66	5.39	54.33	6.31
Unsaturated												
ω 9 *cis*	10.39	5.86	0.41	2.23	8.63	18.15	3.91	2.21	7.21	3.38	24.39	7.02
ω 6 *cis*	9.37	67.72	10.49	78.45	7.08	55.94	12.17	78.25	12.19	73.37	13.18	41.88
ω 3 *cis*	0.45	15.67	0.54	9.81	0.24	6.75	0.47	11.04	0.47	13.74	2.46	42.44
18:1 *trans*	0.80	-	39.71	1.10	23.90	1.25	21.24	0.68	19.92	0.30	0.90	-
20:1 *trans*	0.38	-	0.11	-	2.12	0.60	1.52	0.23	1.66	0.48	-	-
Other mono-unsaturated	0.50	3.64	0.62	3.03	4.78	4.87	2.71	2.14	4.60	3.26	4.79	2.39
Others	-	-	0.62	-	1.73	1.25	1.04	0.14	1.29	0.07	-	-
MCL[f]	17.9	20.2	18.1	20.2	18.0	19.2	18.1	20.1	18.2	20.1	18.3	20.4
UI[f]	41	374	82	380	61	280	71	380	73	374	79	402

[a]The partially hydrogenated diets were supplemented with safflower oil.

[b]The shorthand notation used for the fatty acids indicates chain length:number of double bonds. ω = First double bond position beginning from the hydrocarbon end; methylene interruption and *cis* geometry are assumed if not otherwise specified.

[c]The values obtained are from analysis of pooled mitochondria from 10 rat hearts.

[d]Indicates values less than 0.005%.

[e]18:2 isomers other than linoleic acid.

[f]MCL and UI were calculated according to Innis and Clandinin (13).

certainty, because in the capillary-GC this isomer could only be resolved when present in relatively large amounts as in the partially hydrogenated oils fed to the animals. There was a tendency to an increased proportion of 18:1ω4-5 *trans* in PC as well as in PE as compared to the isomeric distribution in the partially hydrogenated oils, in agreement with the results reported by Wood (23)

TABLE 9

Relative Distribution of Positional *trans*-Isomers of Octadecenoic Acid as Percent of Total Fatty Acids in the 1-Position of PC and PE from Cardiac Mitochondria Isolated from Rats fed Diets Containing 20% (w w) of HPO, HCO, and HHO for 70 Days

1-Position of PC				
18:1 *trans*[b]	HPO + 4.6 cal% 18:2[a]	HCO + 0.8 cal% 18:2[a]	HCO + 4.6 cal% 18:2[a]	HHO + 4.6 cal% 18:2[a]
ω 3	-	0.5	0.5	0.5
ω 4-5	5.0	2.9	4.4	2.8
ω 6	3.4	1.9	1.9	1.1
ω 7	6.3	2.2	2.2	1.8
ω 8-12	11.6	8.1	7.9	10.1
Σ 18:1 *trans*	26.3	15.6	16.9	16.3

1-Position of PE				
18:1 *trans*[b]	HPO + 4.6 cal% 18:2[a]	HCO + 0.8 cal% 18:2[a]	HCO + 4.6 cal% 18:2[a]	HHO + 4.6 cal% 18:2[a]
ω 3	-	0.5	0.4	0.4
ω 4-5	6.0	3.8	4.0	2.8
ω 6	4.8	2.4	2.3	1.2
ω 7	6.3	3.3	3.0	2.8
ω 8-12	22.6	13.9	11.5	12.7
Σ 18:1 *trans*	39.7	23.9	21.2	19.9

[a]The partially hydrogenated diets were supplemented with safflower oil.
[b]The shorthand notation used for the fatty acids indicates chain length: number of double bonds. ω - = Double bond position stated from the hydrocarbon end.
[c]The results obtained are from analysis of pooled mitochondria from 10 rat hearts.

and by Reichwald-Hacker et al. (25). The proportion of linoleic acid in the HCO diet (0.8 cal % and 4.6 cal %) did not influence the isomeric pattern of *trans* octadecenoic acids in the two phospholipids.

The relative distribution of positional isomers of 18:1 *cis* in the 1- and 2-positions of PC is summarized in Table 10. The major proportion of the total 18:1 *cis* isomers was found in the 2-position of PC, irrespective of dietary treatment. Generally, except for the content of 18:1ω9 *cis* after feeding PO, there was a higher content of 18:1ω9 *cis* and 18:1ω7 *cis*, fatty acids which can be formed endogenously, in the 2-position as compared to the content in the 1-position of PC. The relatively high level of 18:1ω7 *cis* observed in the 2-position of PC might be attributed to extensive endogenous synthesis of this isomer in the cardiac tissue (53). The exogenous fatty acid isomers, 18:1ω6 *cis* and 18:1ω10-12 *cis*, which are derived from the partially hydrogenated dietary oils, were preferentially incorporated into the 1-position. 18:1ω8 *cis* has been reported to be almost excluded from rat heart phospholipids after feeding partially hydrogenated safflower fatty acids of which 18:1ω8 *cis* was a significant dietary component (23). In the present study, 18:1ω8 *cis* was incorporated to some extent in the 1-position of PC after feeding partially hydrogenated oils. It should be pointed out, however, that only partial separation was achieved between 18:1ω8 *cis* and 18:1ω9 *cis* in the capillary-GC. Therefore, with the technique used in this study for separation of positional isomers, the possibility exists to overestimate the smaller 18:1ω8 *cis* peak, which eluted after the larger 18:1ω9 *cis* peak and nearly coincided. A large amount of 18:1ω9 *cis* was deposited in the 2-position of PC from rats fed the partially hydrogenated oil diet supplemented with an inadequate amount of linoleic acid (HCO + 0.8 cal % 18:2).

In contrast to the isomeric distribution of PC the major part of the total 18:1 *cis* isomers were deposited in the 1-position of PE after feeding the experimental diets with the exception of the HPO diet (Table 11). The 1-position of PE from animals kept on the HPO diet contained a very small

TABLE 10

Relative Distribution of Positional *cis*-Isomers of Octadecenoic Acid as Percent of Total Fatty Acids in the 1- and 2-Positions of PC from Cardiac Mitochondria Isolated from Rats Fed Diets Containing 20% (w/w) PO, HPO, HCO, HHO, and RSO for 70 Days

18:1 *cis*[b]	PO		HPO+ 4.6 cal% 18:2[a]		HCO+ 0.8 cal% 18:2[a]		HCO+ 4.6 cal% 18:2[a]		HHO+ 4.6 cal% 18:2[a]		RSO	
	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos
ω 6	-	-	1.0	0.3	0.8	3.4	0.4	0.3	0.5	0.3	-	-
ω 7	0.7[c]	4.1	1.0	3.8	1.0	5.3	0.5	2.6	0.6	2.4	1.7	2.3
ω 8	-	-	1.8	-	-	-	0.5	-	0.9	-	-	-
ω 9	6.0	5.4	2.6	4.6	6.1	22.2	1.9	2.8	1.5	2.7	6.9	7.3
ω10-12	-	-	1.2	0.8	0.3	-	0.5	0.2	0.7	0.2	-	-
Σ18:1 *cis*	6.7	9.5	7.6	9.5	8.2	30.9	3.8	5.9	4.2	5.6	8.6	9.6

[a]The partially hydrogenated diets were supplemented with safflower oil.
[b]The shorthand notation used for the fatty acids indicates chain length: number of double bonds. ω = Double bond position stated from the hydrocarbon end.
[c]The results obtained are from analysis of pooled mitochondria from 10 rat hearts.

TABLE 11

Relative Distribution of Positional *cis*-Isomers of Octadecenoic Acid as Percent of Total Fatty Acids in the 1- and 2-Positions of PE from Cardiac Mitochondria Isolated from Rats Fed Diets Containing 20% (w/w) PO, HPO, HCO, HHO, and RSO for 70 Days

18:1 *cis*[b]	PO		HPO+ 4.6 cal% 18:2[a]		HCO+ 0.8 cal% 18:2[a]		HCO+ 4.6 cal% 18:2[a]		HHO+ 4.6 cal% 18:2[a]		RSO	
	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos	1-Pos	2-Pos
ω 6	-	-	0.1	0.3	0.4	1.2	0.5	0.2	0.5	0.2	-	-
ω 7	0.9[c]	3.4	0.1	2.7	1.4	2.9	0.6	1.7	1.1	1.9	3.1	1.9
ω 8	-	-	0.2	-	-	-	-	-	-	-	-	-
ω 9	9.4	5.3	0.4	2.1	7.6	7.3	3.2	2.0	4.1	1.6	17.4	4.0
ω10-12	-	-	0.3	0.3	1.8	0.3	1.0	0.1	1.3	0.1	-	-
Σ18:1 *cis*	10.3	8.7	1.1	5.4	11.2	11.7	5.3	4.0	7.0	3.8	20.5	5.9

[a]The partially hydrogenated diets were supplemented with safflower oil.
[b]The shorthand notation used for the fatty acids indicates chain length: number of double bonds. ω = Double bond position stated from the hydrocarbon end.
[c]The results obtained are from analysis of pooled mitochondria from 10 rat hearts.

proportion of 18:1 *cis* isomers (1.1%) but a large amount of 18:1 *trans* isomers (39.7%). Except for the content of 18:1ω9 *cis* after feeding HPO and 18:1ω7 *cis* after feeding RSO, there was a tendency to deposition of 18:1ω9 *cis* in the 1-position and 18:1ω7 *cis* in the 2-position of PE. 18:1ω8 *cis* could only be detected as a small proportion in the 1-position of PE after feeding HPO. As for PC, the other exogenous 18:1 *cis* isomers derived from the diets had a tendency to be incorporated into the 1-position of PE. After feeding HCO + 0.8 cal % 18:2, there was a remarkable increase of 18:1ω9 *cis* in the 1- and 2-positions as compared to the corresponding content in PE in rats fed HCO + 4.6 cal % 18:2. Animals kept on RSO diet had a high content of 18:1ω9 *cis* in the 1-position of PE, probably derived from the high content of ω9 monounsaturated fatty acids in the RSO diet.

Relative distribution of Fatty Acids in Cardiolipin of Cardiac Mitochondria

The relative distribution of fatty acids as percent of total fatty acids of cardiolipin from cardiac mitochondria isolated from rats fed the different experimental diets is presented in Table 12. Compared to the other two major phospholipids after feeding partially hydrogenated oils, only small amounts of *trans* fatty acids were incorporated into cardiolipin, less than 1% of total fatty acids, in agreement with the results reported by Høy and Hølmer (54) after feeding partially hydrogenated vegetable oils to rats but in contrast to the results reported by Hsu and Kummerow (55), who observed considerably higher incorporation of *trans* fatty acids upon feeding partially hydrogenated fat. Feeding a diet with an insufficient amount of linoleic acid (HCO + 0.8 cal % 18:2) did not have

TABLE 12

Relative Distribution of Fatty Acids as Percent of Total Fatty Acids of Cardiolipin from Cardiac Mitochondria Isolated from Rats Fed Diets Containing 20% (w/w) PO, HPO, HCO, HHO and RSO for 70 Days

Fatty[b] acid	PO	HPO + 4.6 cal% 18:2[a]	HCO + 0.8 cal% 18:2[a]	HCO + 4.6 cal% 18:2[a]	HHO + 4.6 cal% 18:2[a]	RSO
12:0	-	0.08	-	0.08	-	-
14:0	0.44[c]	0.24	0.50	0.41	0.34	0.17
14:1	-	0.08	-	0.10	0.12	-
15:0	0.21	0.08	0.23	0.11	0.13	0.08
16:0	3.21	1.10	3.32	1.42	1.58	1.10
16:1	0.96	0.46	4.25	0.92	1.31	0.70
17:0	-	0.12	0.20	0.24	0.19	0.21
18:0	3.88	1.21	2.75	1.29	2.04	1.48
18:1 *trans*	-	0.77	0.67	0.28	0.49	-
18:1 *cis*	8.08	5.58	9.47	2.99	3.91	7.54
18:2 ω 6	75.66	83.68	68.51	84.56	76.49	65.82
18:3 ω 6	0.10	0.04	0.23	0.09	0.09	0.04
18:3 ω 3	0.08	0.07	0.21	0.06	0.09	1.97
20:0	0.60	0.08	0.30	0.19	0.18	0.11
20:1 *trans*	-	-	-	0.21	0.23	-
20:1 *cis*	1.07	0.25	1.45	0.84	2.71	3.09
20:2	-	-	0.57	-	-	-
20:2 ω 6	1.00	0.25	0.21	0.25	0.20	1.30
20:3 ω 9	0.12	0.07	0.39	0.07	0.09	0.03
20:3 ω 6	0.58	1.09	3.63	1.55	2.65	1.85
20:4 ω 6	1.91	2.65	0.97	1.73	2.49	2.15
20:5 ω 3	-	-	-	-	-	0.06
22:0	0.15	0.13	0.38	0.17	0.23	0.18
22:1 ω 9	0.17	0.12	-	-	-	10.25
22:1 ω 11	-	-	1.13	1.24	3.14	-
22:2 ω 6	-	-	-	-	-	0.22
22:4 ω 6	0.25	0.24	0.12	0.17	0.14	0.08
22:5 ω 6	0.87	1.03	-	0.59	0.49	0.02
22:5 ω 3	0.09	0.10	-	0.07	0.11	0.36
22:6 ω 3	0.40	0.43	0.31	0.31	0.57	1.16
24:0	0.17	0.05	0.20	0.06	0.06	0.03

[a]The partially hydrogenated diets were supplemented with safflower oil.
[b]The shorthand notation used for the fatty acids indicates chain length: number of double bonds. ω = First double bond position beginning from the hydrocarbon end; methylene interruption and *cis* geometry are assumed if not otherwise stated.
[c]The values obtained are from analysis of pooled mitochondria from 10 rat hearts.

any major influence on the content of *trans* fatty acids and docosenoic acid in cardiolipin, as compared to rats fed a diet with an adequate supplementation of linoleic acid (HCO + 4.6 cal % 18:2). Cardiolipin isolated after feeding unhydrogenated oils (PO and RSO) contained no detectable amount of *trans* fatty acids. Considerable amount of erucic acid (10.3%) was incorporated into cardiolipin of rat heart mitochondria after feeding RSO, confirming earlier results (12). Docosenoic acid derived from the diets containing partially hydrogenated marine oils was also incorporated into cardiolipin. The incorporation of docosenoic acid, mainly 22:1ω11 *cis*, in myocardial cardiolipin was found to be 1.2% and 3.1% after feeding HCO + 4.6 cal % 18:2 and HHO + 4.6 cal % 18:2, respectively. In contrast to rat heart, liver mitochondrial cardiolipin contained only 0.5% erucic acid after feeding RSO (results not shown). Suprisingly, the linoleic acid content of cardiolipin was increased after feeding partially hydrogenated oils (containing ca. 11% 18:2) as compared to the group of rats fed unhydrogenated peanut oil (containing 38% 18:2). This form of altered fatty acid composition of cardiolipin was also observed by Dewailly et al. (56), who found a higher content of linoleic acid in cardiolipin from rat heart mitochondria after feeding low erucic acid rapeseed oil as compared to animals kept on peanut oil. Within the groups of rats fed partially hydrogenated oils supplemented with an adequate amount of linoleic acid, there was a tendency to a decreased content of linoleic acid in cardiolipin from rats fed HHO containing a substantial amount of docosenoic acid.

Oxygen Uptake and ATP Synthesis in Isolated Cardiac Mitochondria

Table 13 records the values obtained for state 3 oxygen uptake rate, respiratory control ratio, ADP/O ratio, and ATP synthesis rate after incubation with succinate plus rotenone, glutamate plus malate, pyruvate plus malate, and palmitoylcarnitine plus malate as substrates. The rate of

TABLE 13

Oxidative Activity of Cardiac Mitochondria Isolated from Rats Fed Diets Containing 20% (w/w) PO, HPO+4.6 cal% 18:2, HCO+4.6 cal% 18:2, HHO+4.6 cal% 18:2 and RSO for 70 Days

Substrate	Diet[a]	State 3 oxygen uptake[b] (ng atoms/mg protein/min)	Respiratory control ratio[b]	ADP/O[b]	ATP synthesis[b] (nmol/mg protein/min)
Succinate plus rotenone	PO	641 ± 52	2.3 ± 0.2	1.71 ± 0.04	1095 ± 101
	HPO+4.6 cal% 18:2	601 ± 43	2.2 ± 0.1	1.71 ± 0.12	1025 ± 45
	HCO+4.6 cal% 18:2	613 ± 39	2.2 ± 0.2	1.67 ± 0.03	1024 ± 66
	HHO+4.6 cal% 18:2	567 ± 49	2.0 ± 0.2	1.61 ± 0.05[d]	915 ± 106[c]
	RSO	619 ± 64	2.1 ± 0.3	1.62 ± 0.10	1003 ± 117
Glutamate plus malate	PO	395 ± 81	3.2 ± 0.9	2.54 ± 0.11	1014 ± 231
	HPO+4.6 cal% 18:2	390 ± 68	3.0 ± 0.4	2.57 ± 0.12	999 ± 193
	HCO+4.6 cal% 18:2	403 ± 64	3.3 ± 0.8	2.52 ± 0.12	1020 ± 202
	HHO+4.6 cal% 18:2	355 ± 35	2.9 ± 0.6	2.47 ± 0.13	877 ± 125
	RSO	365 ± 72	2.8 ± 0.7	2.47 ± 0.22	912 ± 246
Pyruvate plus malate	PO	494 ± 76	3.5 ± 0.4	2.83 ± 0.09	1361 ± 198
	HPO+4.6 cal% 18:2	432 ± 24	3.2 ± 0.3	2.85 ± 0.07	1230 ± 96
	HCO+4.6 cal% 18:2	441 ± 32	3.4 ± 0.8	2.80 ± 0.12	1241 ± 74
	HHO+4.6 cal% 18:2	387 ± 39[c]	3.0 ± 0.6	2.78 ± 0.50	1080 ± 146[c]
	RSO	424 ± 38[c]	3.1 ± 0.7	2.69 ± 0.10	1141 ± 100
Palmitoylcarnitine plus malate	PO	487 ± 57	3.9 ± 0.6	2.74 ± 0.18	1327 ± 92
	HPO+4.6 cal% 18:2	463 ± 60	3.6 ± 0.3	2.58 ± 0.06	1197 ± 177
	HCO+4.6 cal% 18:2	470 ± 45	3.5 ± 0.7	2.61 ± 0.12	1225 ± 159
	HHO+4.6 cal% 18:2	414 ± 37[c]	3.0 ± 0.6[c]	2.56 ± 0.10[c]	1058 ± 111[d]
	RSO	421 ± 49	2.8 ± 0.6[c]	2.43 ± 0.17[c]	1026 ± 164[d]

[a]The partially hydrogenated diets were supplemented with safflower oil.

[b]Results are mean ± standard deviation of 5 preparations, each containing 2 pooled hearts.

[c,d]Probability level as calculated according to Student's t-test; c and d are significantly different from PO values: $p < 0.05$ and $p < 0.01$, respectively.

oxygen uptake obtained in the present study were higher than the rates reported by Houtsmuller et al. (15), but were compatible with the rates reported by other workers (16,55). The relatively low respiratory control index obtained might be explained by increased Mg^{2+} induced oligomycin sensitive ATP-ase activity at physiological temperature (57).

After incubation with succinate plus rotenone as well as pyruvate plus malate as substrates, there was a small but significant decrease in ATP synthesis rate of cardiac mitochondria isolated from rats fed HHO as compared to PO dietary treatment as the control. For succinate plus rotenone as substrate, there was also a decreased ADP/O ratio after feeding HHO. The state 3 oxygen uptake rate was depressed in the presence of pyruvate + malate as substrate after HHO and RSO dietary treatments. A slightly more pronounced effect on the cardiac mitochondrial oxidative activity was observed in animals kept on HHO and RSO diets using the fatty acid substrate palmitoylcarnitine in the presence of malate. Except of state 3 oxygen uptake rate after feeding RSO, all the oxidative paramethers investigated with the use of palmitoylcarnitine as substrate were significantly decreased after treatment with dietary HHO and RSO as compared to treatment with PO.

The oxidative activities of rat liver mitochondria after feeding RSO were not, in any case, irrespective of substrate used, significantly different from those obtained after PO dietary treatment (results not shown).

DISCUSSION

The biological and biochemical effects of dietary long-chain isomeric fatty acids have been extensively studied in different experimental animals and biochemical systems. In many experiments, the metabolic fate of isomeric fatty acids has been studied against a background of essential fatty acid deficiency (58-61). The present investigation was designed in such a way that the linoleic acid content in the diet should be adequate. The diet in our experiments contained 4.6 cal % linoleic acid which is of the same magnitude as in the experiments by Høy and Hølmer (27).

The rats in our experiments did not show any clinical signs of essential fatty acid deficiency and it was not possible to detect any significant amount of 20:3ω9 (62) in the lipid classes investigated. In order to evaluate the presence of linoleic acid in the diet, one group of rats was fed a diet with HCO supplemented with only 0.8 cal % linoleic acid. In

this group, the presence of 20:3ω9 and reduced level of 20:4ω6 in the heart lipids indicated an essential fatty acid deficiency.

In comparing the composition of the fed partially hydrogenated oils and the fatty acids of the different lipid classes of the rat heart, it is important to realize that there might be differences already in the absorption and digestibility of the different isomeric long-chain fatty acids.

With regard to octadecenoic acids, Ono and Fredrickson (63) did not find any differences in the intestinal absorption of *cis* and *trans* isomers of octadecenoic acid and palmitic acid. Furthermore, the absorption of individual *trans* isomers of octadecenoic acid was reported to be independent of the double bond position (21). On the other hand, docosenoic acids have a slower absorption as compared to octadecenoic acids. Docosenoic acid in partially hydrogenated herring oil was reported to be 79% digested when supplemented with corn oil (64), but little is known about the absorption of individual *cis* and *trans* isomers of docosenoic acid formed during partial hydrogenation.

In the present investigation, the rats fed partially hydrogenated marine oils showed a normal weight gain in comparison with the PO group and showed no signs of malabsorption. It should be pointed out, however, that brassidic acid, the *trans* isomer of erucic acid, when fed as tribrassidin has a lower digestibility as compared to erucic acid and did not incorporate as well as erucic acid in either cardiac or adipose tissue (65,66).

The distribution of isomeric fatty acids in different lipid classes of rat heart might also be reflected by differences in the rate of oxidation of individual dietary long chain fatty acid isomers in liver and heart after feeding partially hydrogenated oils.

The most efficient fatty acid oxidation occurs in mitochondria. In some early work of Ono and Fredrickson (63) and Coots (67), ^{14}C-labeled *cis* and *trans* isomers of octadecenoic acid were found to be similarly degraded to CO_2 in adult male rats. However, most *trans* isomers of octadecenoic acid are oxidized slower than the corresponding *cis* isomers by isolated rat heart mitochondria (68,69). A selectivity in the rate of β-oxidation with regard to individual *cis* and *trans* positional isomers of octadecenoic acid was reported by Lawson and Holman (70). Generally the differences in β-oxidation rates obtained in vitro for different isomers could not be correlated with the content of the corresponding isomers in tissue lipids (70).

In contrast to octadecenoic acids, erucic and cetoleic acid which are slowly oxidized by isolated rat heart mitochondria and inhibit the oxidation of other fatty acids are rapidly deposited together with other fatty acids in heart triglycerides after feeding rapeseed oil or partially hydrogenated marine oils rich in docosenoic acids (19,71). However, on prolonged feeding on such diets, the lipidosis is reversed to near normal triglyceride levels (19).

Recently, Lazarow and de Duve (72) found that peroxisomes purified from rat liver are capable of oxidizing long-chain acyl-CoA esters. In contrast to mitochondrial β-oxidation, peroxisomal β-oxidation does not oxidize fatty acids to completion (73). With regard to substrate specificity, *trans* isomers of monounsaturated fatty acids are oxidized at rates that are faster than, or similar to, those obtained with corresponding *cis* isomers (74).

Prolonged feeding of partially hydrogenated marine oil and, to a less extent, rapeseed oil have been reported to induce increased peroxisomal chain-shortening of erucic acid in perfused rat liver and isolated hepatocytes as compared to rats fed peanut oil (75,76). Chain-shortening of erucic acid has also been observed under similar dietary conditions in perfused rat heart (77). It was suggested that the increased peroxisomal chain-shortening in liver and heart from rats fed partially hydrogenated marine oil or rapeseed oil might play an important role in the reversal of the initial heart lipidosis caused by these oils (75,77). Furthermore, it has been proposed that liver peroxisomal β-oxidation may be of significance with regard to metabolism of *trans* fatty acids or other dietary fatty acids that are poorly oxidized by mitochondrial β-oxidation (74).

In the present study, all the groups of rats fed docosenoic acid rich diets for 10 weeks showed a slight accumulation of cardiac neutral lipid, although most pronounced in the group of rats kept on RSO which contained the highest amount of docosenoic acid. In comparison with the rapeseed oil group, rats fed partially hydrogenated marine oils accumulated less docosenoic acid in the myocardium than could be expected from the corresponding content in the diets. Furthermore, in the cardiac neutral lipids of rats fed partially hydrogenated marine oils, we observed increased amounts of those octadecenoic and eicosenoic acid isomers which could be derived by chain-shortening of dietary docosenoic acid as compared to the occurrence of the corresponding isomers in the dietary oils. These findings suggest that the peroxisomal chain-shortening system might be of importance in the metabolism of dietary long-chain isomeric fatty acids that are not well β-oxidized by mitochondria (75-77). However, in spite of these auxiliary mechanisms for oxidation, our data clearly indicate that a variety of *cis* and *trans* isomers are incorporated into the mitochondrial membrane phospholipids of rat heart after feeding partially hydrogenated marine oils. Apparently the peroxisomal β-oxidation system has a limited capacity to oxidize certain fatty acids when the influx of these acids are too high.

Positional Distribution of Fatty Acids in PC and PE

That dietary ^{14}C-labeled oleic and palmitic acid were asymmetrically distributed into PC of liver, intestinal tract and lymph chylomicrons was first shown unequivocally by Hanahan and Blomstrand (78), then extended using ^{14}C-labeled stearic and linoleic acid in human beings by Blomstrand et al. (79). Already in these original experiments, it was observed that phospholipase A released nearly all the ^{14}C-labeled linoleic acid but also a significant amount of the incorporated labeled oleic acid from the PC molecule. It is now generally accepted that long-chain dietary saturated fatty acids are preferentially incorporated into the 1-position of PC and PE and that polyunsaturated fatty acids are preferentially incorporated into the 2-position. Oleic acid, however, is distributed between the two positions. The mechanisms behind the asymmetric distribution of the fatty acids into these two major phospholipids has been the matter of intensive investigation. When it was found that arachidonic acid was the main precursor for several prostaglandins, it was realized that a very important problem to elucidate was to find out which fatty acids originating from the diet were candidates to compete with linoleic and arachidonic acid about the strategic 2-position of these phospholipids.

It is quite clear from the results of the present investigation that, in the experimental rat groups fed partially hydrogenated marine oils with an adequate amount of linoleic acid, the content of linoleic and arachidonic acid in the PC and PE molecules was not decreased. On the contrary, there was a significant increase in the content of arachidonic acid in the PE in the rats fed partially hydrogenated marine oils.

The acyl groups placed in the 2-position of such important membrane constitutents as PC and PE will depend not only on the molecular structure of the fatty acid but also on the relative concentrations of different fatty acids, the selectivity of the enzymes and the rate of transfer of fatty acids (for review, see ref. 80).

The present study shows that isomers of docosenoic acid were incorporated into the 1- and 2-positions of PC and PE, the major phospholipids in rat heart mitochondria, to a relatively low degree as compared to isomeric octadecenoic acids. A considerably low rate of activation of docosenoic acid in rat liver microsomes have been reported by Normann et al. (81). Marchand and Beare-Rogers (82) observed that CoA derivatives of *cis* and *trans* docosenoic acid did not react with rat liver microsomal acyl-CoA-1-phosphatidylglycerol-3-phosphate acyltransferase. It is thus possible that these observations from in vitro experiments with rat liver microsomes also could be valid for the present study of rat heart mitochondria.

Furthermore, the present study shows that *trans* isomers of octadecenoic acid were preferentially incorporated into the 1-position of PC and PE from rat heart mitochondria mainly replacing stearic acid in this position, but were not excluded from the 2-position in accordance with the results reported by Reichwald-Hacker et al. (26) for the positional distribution of *cis* and *trans* octadecenoic acids in PC from rat heart after feeding partially hydrogenated soybean oil. The incorporation of individual *trans* isomers of octadecenoic acid appeared to be selective in a similar way as have been reported by other investigators (23-25). Unusual 18:1 *cis* isomers were mainly incorporated into the 1-position of the phospholipids. At the 2-position, only small amounts of unusual *cis* isomers of octadecenoic acid (mainly ω6- and ω10-ω12-isomers) were observed. The ω8-isomer was discriminated against, as compared to the other unusual *cis* isomers. This is in accordance with recent reports on the composition of phospholipids in rat heart (23) and rat liver (27) after feeding different partially hydrogenated vegetable oils. However, Reitz et al. (83) found that 18:1ω8 *cis* was transferred to the 1-position of 2-acylglycero-3-phosphorylcholine at a considerably high rate in vitro. These observations demonstrate that acyltransferase activities measured in vitro under simplified experimental conditions not always have the predictive value with respect to the ultimate distribution of fatty acids in phospholipids in vivo as suggested by Lands et al. (84). In contrast to 18:1ω8 *trans*, which was suggested to be preferentially chain-shortened to 16:1ω8 *trans*, the metabolic fate of 18:1ω8 *cis* is unknown (22).

It is generally considered that in order to function properly a biomembrane should have an appropriate fluidity. The fluidity is to a high extent influenced by the physicochemical properties of the phospholipid acyl chains within the bilayer. The calculated unsaturation index used in this work is only a gross estimator of changes in the proportion of unsaturated bonds, irrespective of geometry and position in the fatty acyl chain in membrane phospholipids after different dietary treatments. The introduction of one *cis* double bond into a saturated fatty acid will have a profound influence on the transition temperature and surface area of synthetic phospholipids. The introduction of additional *cis* double bonds beyond the first double bond have little effect on the surface area of phospholipids (85). Thus, the somewhat higher unsaturation index obtained at the 2-position of PC in rat heart mitochondria after feeding partially hydrogenated oils as compared to the group of rats kept on peanut oil does not necessary imply an increased membrane fluidity. Furthermore, the relatively small amounts of *trans* isomers and unusual *cis* isomers of octadecenoic acid observed in the 2-position of the phospholipids after feeding

partially hydrogenated oils are not very likely to influence the fluidity of the mitochondrial membranes.

The fluidity of the membranes is, however, not solely influenced by the physicochemical properties of the phospholipid acyl chains, but also by the specific classes of phospholipids and the content of cholesterol. Experiments with monolayers have shown that PE have a higher transition temperature and lower surface area than PC with the same fatty acid composition (85). Furthermore, the addition of cholesterol to the monolayer will have a stabilizing effect on the film (85). According to these physical experiments, the marked reduction of the relative concentration of PE and the lower content of saturated fatty acids observed in the mitochondrial phospholipids of rat heart after feeding RSO are likely to increase the membrane fluidity. However, the increased mitochondrial cholesterol content found in the RSO group might be a sign of a compensatory mechanism in maintaining an adequate fluidity of the corresponding membranes.

Cardiolipin

In confirmation of earlier studies (12), it was shown that erucic acid had a high affinity for cardiolipin of rat heart mitochondria after feeding rapeseed oil. Docosenoic acid, mainly 22:1ω11 *cis*, from partially hydrogenated capelin and herring oil was also found in the cardiolipin, but to a less extent than erucic acid from rapeseed oil. In contrast to PE and PC, cardiolipin was rather resistant to incorporation of *trans* isomers. The *cis* configuration and position of the double bond in the erucic and cetoleic acid thus seem to be the most important factors together with the chain length determining the incorporation into the cardiolipin molecule.

Recently, Vik et al. (86) showed that beef heart cytochrome c oxidase requires a small number of cardiolipin molecules for optimal activity. A functional role for these two or three molecules of cardiolipin in cytochrome c oxidase activity has recently been suggested and it would be of great interest to study the influence of cardiolipin with different acyl groups on this enzymatic activity.

In this connection, it is interesting to note that the effect on the mitochondrial oxidative activity seemed to be larger in rats kept on partially hydrogenated herring oil and rapeseed oil which contain high amount of docosenoic acids. In these two groups of animals, a significant decrease of ATP-synthesis was observed using fatty acid substrate palmitoylcarnitine in the presence of malate. It has been shown by Christophersen and Christiansen (87) that the inhibitory effect of erucoylcarnitine on the mitochondrial oxidation of CoA dependent substrates such as pyruvate, acetylcarnitines and α-ketoglutarate may be due to sequestration of free CoA in the form of slowly metabolized erucoyl-CoA. Alternatively, in the case of acylcarnitines, there may be an inhibition at the level of acyl-CoA dehydrogenase. In the present study, it was found that the neutral fat fraction, mainly free fatty acids, of mitochondria isolated from the groups of animals kept on HHO and RSO, contained docosenoic acid corresponding to 0.7 μM cetoleic acid and 2.1 μM erucic acid in the incubation mixture, respectively. Regardless of whether or not the major part of these free fatty acids were produced by hydrolysis during preparation, the possibility cannot be excluded that docosenoic acids may interfere with the mitochondrial oxidation of palmitoylcarnitine and other substrates, during the in vitro conditions used.

On the contrary, isolated liver mitochondria from RSO treated rats did not show any tendency to decreased oxidative activities in comparison with PO fed animals and only trace amount of erucic acid could be detected in the neutral fat fraction of liver mitochondria (unpublished data). Furthermore, considerably less erucic acid was incorporated into liver mitochondrial cardiolipin as compared to cardiolipin isolated from rat heart mitochondria after feeding rapeseed oil. These discrepancies observed between liver and heart mitochondria support the suggestion that docosenoic acid might act either directly as an inhibitor of mitochondrial respiration in rat heart or indirectly by altering the fatty acid distribution of cardiolipin which is generally assumed to be required for electron transport and for coupling of this process to ATP synthesis (86,88).

It should be pointed out, however, that the effect of the docosenoic acid rich diets on the mitochondrial energy production observed in the present study was of a low magnitude. If the state 3 oxygen uptake is calculated on a basis of mitochondrial phospholipid content instead of protein content, the differences obtained between the various dietary treatments in the present study are almost abolished. In addition, it was observed that the mitochondrial yield, expressed as mg mitochondrial protein/g rat heart was 10-15% higher after feeding rapeseed oil and partially hydrogenated herring oil. Heart mitochondria isolated from rats fed rapeseed oil have been reported to be enriched in mitochondria of great size (89). In such mitochondria, the relation between phospholipids and protein may be different from that in normal mitochondria, which may in part explain the results of the present study.

In conclusion, results from the present feeding experiments demonstrate that a wide range of positional *cis* and *trans* isomers of long-chain fatty acids derived from partially hydrogenated vegetable and marine oils supplemented with an adequate

amount of linoleic acid is not only incorporated into phospholipids which are important mitochondrial membrane constituents, but also may be involved in the regulation of the synthesis of polyunsaturated fatty acids. With regard to the cardiac energy production, as measured in vitro, it seems as only partially hydrogenated herring oil containing a high amount of cetoleic acid and rapeseed oil, high in erucic acid interfere with the ATP synthesis. No effect on oxidative activities was observed after feeding HCO. In view of the importance of polyunsaturated fatty acids as regulators of membrane fluidity and precursors for prostaglandin synthesis, we have in an accompanying paper more extensively studied the influence of this type of dietary oils on fatty acid desaturase activities in rat liver microsomes (90).

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The Effect of Dietary Partially Hydrogenated Marine Oils on Desaturation of Fatty Acids in Rat Liver Microsomes

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ABSTRACT

The influence of dietary partially hydrogenated marine oils on distribution of phospholipid fatty acids in rat liver microsomes was studied with particular reference to the metabolism of linoleic acid. Five groups of weanling rats were fed diets containing 20% (w/w) peanut oil (PO), partially hydrogenated peanut oil (HPO), partially hydrogenated Norwegian capelin oil (HCO), partially hydrogenated herring oil (HHO), and rapeseed oil (RSO) for 10 weeks. The partially hydrogenated oils were supplemented with linoleic acid corresponding to 4.6 cal % in the diets. Accumulation of linoleic acid and reduced amount of total linoleic acid metabolites were observed in liver microsomal phospholipids from rats fed partially hydrogenated oils as compared to PO feeding. The most striking effects on the distribution of ω6-polyunsaturated fatty acids was obtained after feeding HHO, a marine oil with a moderate content of *trans* fatty acids in comparison with HPO but rich in isomers of eicosenoic and docosenoic acids. Liver microsomal Δ^6- as well as Δ^5-desaturase activities as measured in vitro were reduced in rats kept on HHO as compared to PO dietary treatment. The results obtained suggest that the dietary influence of partially hydrogenated marine oils on the metabolism of linoleic acid might be better related to the intake of isomeric eicosenoic and docosenoic acids than to the total intake of *trans* fatty acids.
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INTRODUCTION

Partially hydrogenated vegetable and marine oils are consumed in substantial amounts. These oils contain a wide range of positional isomers of mainly monounsaturated fatty acids with the double bond in *trans* as well as in *cis* configuration (1-3).

Animal studies have shown that dietary *trans* octadecenoic acids, which constitute the major part of the *trans* fatty acid content in partially hydrogenated vegetable oils, have the ability to exaggerate the signs and symptoms of essential fatty acid deficiency (4,5).

Positional *cis* and *trans* isomers of octadecenoic acids which are common constituents of partially hydrogenated vegetable oils have been reported to inhibit liver microsomal desaturation of 18:2ω6 and 20:3ω6 in vitro (6,7). These fatty acids may also be desaturated to some extent by liver microsomes to form various isomers of octadecadienoic acids in vitro (8-10). The inhibitory effect of dietary isomeric octadecenoic acids on the conversion of linoleic acid to arachidonic acid may, in part, explain the aggravation of symptoms of essential fatty acid deficiency reported after feeding partially hydrogenated oils (11).

In a recent work by Hill et al. (12), it was shown that feeding of rats with diets containing partially hydrogenated soybean oil together with reduced amounts of linoleic acid affected the metabolism of essential fatty acids with consequent changes in the pattern of polyunsaturated fatty acids of structural lipids in liver and heart. The suppression of linoleate metabolism observed was largely due to decreased Δ^6- and Δ^5-desaturase activities.

Aaes-Jørgensen and Hølmer studied the effects of partially hydrogenated vegetable and marine oils on growth and testis development in rat (13). They found that partially hydrogenated herring oil devoid of essential fatty acids was more efficient to stress essential fatty acid deficiency than partially hydrogenated peanut oil.

However, little is known about the effects of partially hydrogenated marine oils when an adequate amount of linoleic acid is supplied. Previously we studied the influence of some partially hydrogenated marine oils and peanut oil on the mitochondrial function and membrane phospholipid fatty acid composition of rat heart (14). In these studies, the amount of linoleic acid in diet was sufficient to avoid symptoms of essential fatty acid deficiency. Large amount of isomeric octadecenoic acids were incorporated into the phospholipids after feeding the partially hydrogenated oils. The amount of total ω6-polyunsaturated fatty acids in the mitochondrial phospholipids was not decreased, however.

In the present paper, the effect of dietary partially hydrogenated marine and vegetable oils on rat liver microsomal Δ^6- and Δ^5-desaturase activities was studied, using conditions similar to those in the previous work (14). The results obtained were compared to the distribution of ω6-polyunsaturated fatty acids in microsomal phospholipids.

EXPERIMENTAL PROCEDURE

Materials

[1-^{14}C]Palmitic acid ([1-^{14}C]16:0, sp act 59 mCi/-

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mmole), [1-^{14}C]linoleic acid ([1-^{14}C]18:2ω6, sp act 56 mCi/mmol), and [2-^{14}C]eicosatrienoic acid ([2-^{14}C]20:3ω6, sp act 55 mCi/mmol) were purchased from The Radiochemical Centre (Amersham, England), and were 99.0%, 99.8% and 99.2% radiochemically pure, respectively, as judged by high performance liquid chromatography (HPLC) and liquid radioactivity detection. The corresponding unlabeled fatty acids and palmitoleic acid (16:1ω7), γ-linolenic acid (18:3ω6), and arachidonic acid (20:4ω6) were obtained from Nu-Chek-Prep. (Elysian, MN). ATP, CoA, NADH, glutathione, and bovine serum albumin (fraction V, essentially fatty acid free) were purchased from Sigma Chemical Co. (St. Louis, MO). Methanolic sodium methoxide was obtained from Applied Science Europe B.V. (Oud-Beijerland, The Netherlands) and hydrogen chloride from AGA, Special Gas (Lidingö, Sweden). All other reagents and chemcals were purchased from Merck (Darmstadt, GFR).

Animals and Diets

Male weanling Sprague-Dawley rats, divided into 5 groups of 6 rats each, were fed a semisynthetic diet containing 20% (w/w) peanut oil (PO), partially hydrogenated peanut oil (HPO), partially hydrogenated Norwegian capelin oil (HCO), partially hydrogenated herring oil (HHO), and rapeseed oil (RSO) for 10 weeks. In order to prevent development of essential fatty acid deficiency in rats fed partially hydrogenated oils, the corresponding diets were supplemented with safflower oil (4.6 cal % linoleic acid in diets). The content of *trans* fatty acids, linoleic acid, eicosenoic acid (20:1), and docosenoic acid (22:1) in the experimental diets is summarized in Table 1. The complete composition of the dietary oils and the basal diet were as described previously (14,15). The rats were subjected to reversed lighting periods (light was automatically switched on at 6 P.M. and off at 6 A.M.). No differences in food consumption and only small differences in weight gain (less than 5%) were observed between the different groups of rats. The animals were fasted overnight prior to sacrifice by decapitation and exsanguination at about 9A.M. on the day of the experiment.

Preparation of Microsomes and Incubation Conditions

The livers were immediately removed and rinsed in ice cold homogenization solution consisting of 0.15 M KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.25 M sucrose, 1.5 mM glutathione, 0.05 M potassium phosphate buffer (pH 7.0) (7). The livers were homogenized in 4 volumes of homogenization solution in a Teflon pestle homogenizer. The homogenate was centrifuged at 20,000 × g for 10 min, and microsomes were recovered by centrifuging the supernatant obtained at 100,000 × g for 1 hr. The pellet was resuspended in the homogenization solution to a final concentration of ca. 3 mg of microsomal protein/ml as determined by Lowry et al. (16). All operations were carried out at 4 C.

The microsomes were assayed for Δ^5-, Δ^6-, and Δ^9-desaturase activities immediately after preparation mainly as described by Mahfouz and Holman (8). The incubation mixture consisted of 0.15 M KCl, 0.25 M sucrose, 5 mM ATP, 0.25 mM CoA, 1.0 mM NADH, 5 mM $MgCl_2$, 1.5 mM glutathione, 45 mM NaF, 0.5 mM nicotinamide, and 0.1 M phosphate buffer (pH 7.0). The effects of substrate concentration, microsomal protein concentration and incubation time was investigated for all the substrates used for desaturation study (*cf.* Fig. 1). In the standard procedure, ca. 1 mg of microsomal protein and 100 nmol of fatty acid substrates corresponding to 0.5 μCi were incubated for 20 min at 37C in air. The fatty acids were used as sodium salt/albumin complex (1 μg free fatty acid/11.5 μg bovine serum albumin) (7). The substitution of air for O_2 had no influence on the rate of conversion. The rate of conversion was always compensated for the corresponding value obtained from a blank incubation.

Analysis of Fatty Acids

The incubations were terminated by the addition

TABLE 1

The Content of *trans* Fatty Acids, Eicosenoic Acids (20:1), Docosenoic Acids (22:1), and Linoleic Acid (18:2ω6) as Weight Percent of Total Fatty Acids in Dietary Oils[a]

Dietary Oil	% *trans*	% 20:1	% 22:1	18:2ω6
PO	-	1.1	0.2	37.8
HPO + 4.6 cal % 18:2	44.2	1.0	0.3	11.3
HCO + 4.6 cal % 18:2	34.6	14.8	9.8	11.0
HHO + 4.6 cal % 18:2	24.1	16.8	23.3	11.5
RSO	-	9.7	43.1	15.1

[a]The complete composition of dietary oils is given in ref. 14.

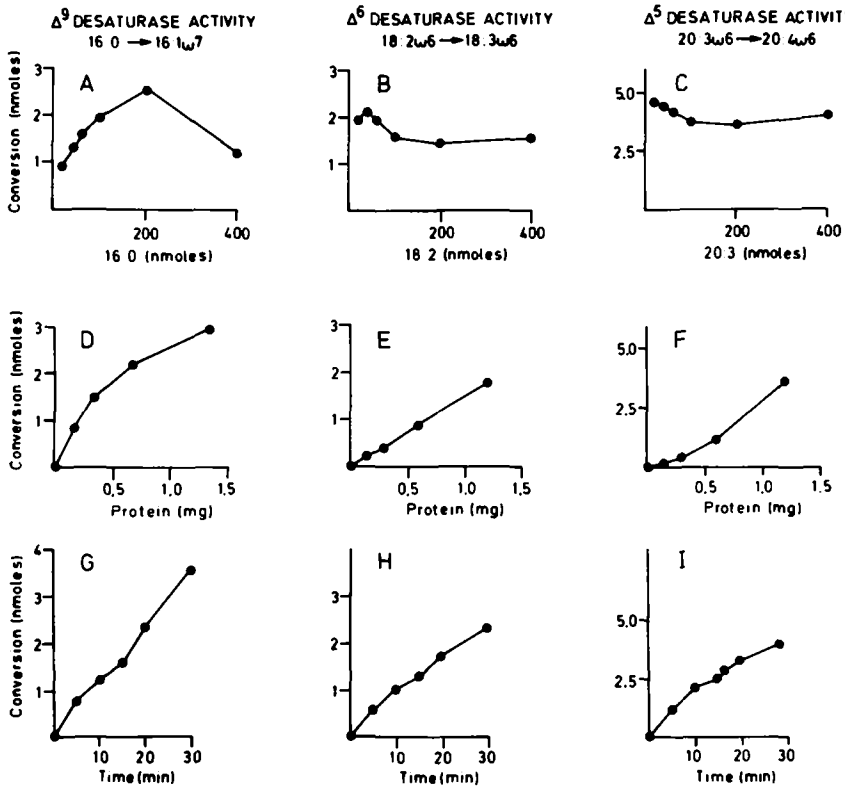

FIG. 1. Δ^9-, Δ^6-, and Δ^5-desaturase activities as a function of substrate concentration (A-C), microsomal protein concentration (D-F), and time of incubation (G-I). Incubations were carried out for 20 min (A-F) in the presence of 100 nmol of substrate (D-I), 1.35 mg microsomal protein (A,G) or 1.2 mg microsomal protein (B,C,H,I) as described in text.

of 1 ml of 5% HCl in methanol. A mixture of unlabeled 16:0 + 16:1ω7, 18:2ω6 + 18:3ω6, or 20:3ω6 + 20:4ω6 was added as carrier and marker substances and the lipids were extracted with chloroform/methanol (2:1, v/v). Fatty acid methyl esters were prepared by transesterification of the isolated lipid with methanolic sodium methoxide (0.5 M) followed by acidic esterification with methanolic hydrogen chloride (1 M) essentially as described by Carreau and Dubacq (17). The enzymatic conversion of the fatty acid substrates to the corresponding desaturation products were estimated by reversed-phase HPLC with liquid radioactivity detection. The fatty acid methyl esters obtained were separated using an Optilab 931 HSR1 high performance liquid chromatograph (Optilab, Vällingby, Sweden) equipped with a Lichroma 200×4.6 mm id stainless-steel column, slurry packed with reversed-phase material 5 μ Nucleosil C_{18} (Macherey-Nagel, Düren, GFR) and methanol/water (89:11, v/v) as mobile phase (3). The mass was monitored by an Interference Refractive Index Detector Multiref 902 and the radioactivity by a Berthold BF5026 liquid radioactivity detector (Laboratorium Prof. Dr. Berthold, Wildbad, GFR). Figure 2 shows a typical chromatogram of fatty acid methyl esters obtained after incubation with [2-^{14}C]20:3ω6 as substrate. The radioactivity corresponding to substrate and product were measured and the percent conversion were calculated. The sensitivity of the radioactivity detector (about 3% dynamic efficiency) did not permit accurate measurement of Δ^6- and Δ^9-desaturase activities. These activities were calculated after collection of the appropriate fractions and subsequent liquid scintillation counting.

In order to study the influence of the different dietary treatment on the microsomal phospholipid fatty acids, the microsomes were extracted according to Folch et al. (18) and the phospholipid fraction was isolated by silicic acid chromatography (19). After converting to methyl esters, the total fatty acid distribution of the phospholipids was determined by glass capillary gas chromatography using Silar-5CP as stationary phase (20) and *trans* fatty acids by HPLC (3).

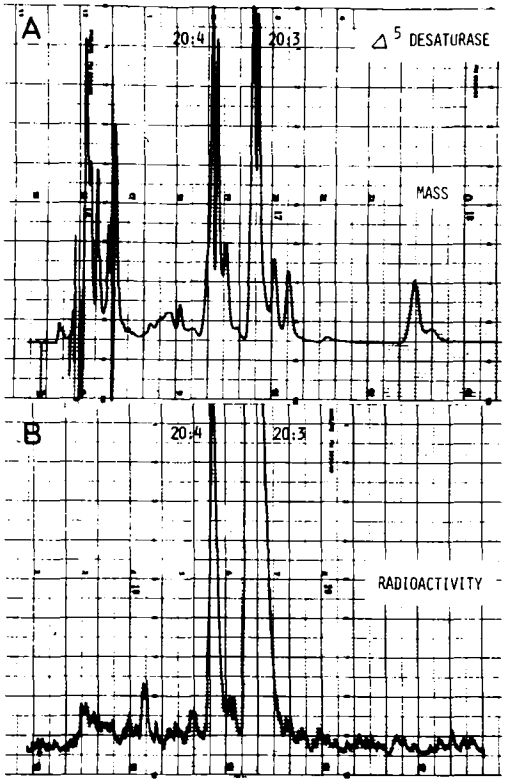

FIG. 2. HPLC registrations of fatty acid methyl esters obtained after incubation with [2-^{14}C]20:3ω6. (A) Mass registration; (B) radioactivity registration.

RESULTS

Separation of Substrates and Desaturation Products by HPLC

With respect to assay of fatty acid desaturase activity, the use of HPLC instead of gas liquid chromatography (GLC) for the separation of substrates and desaturation products has certain advantages. The separations can be performed at room temperature when using HPLC, thus subjecting the polyunsaturated fatty acids to very mild conditions. In the HPLC procedure used in this work, the recovery of ^{14}C-labeled 16:0, 18:2ω6, and 20:3ω6 after fraction collection was 99%, 98%, and 103%, respectively. When standard mixtures containing known amount of ^{14}C-labeled 20:3ω6 and 20:4ω6 were analyzed with the present technique, the results obtained were identical to the corresponding calculated values. Thus, there was no tendency to adsorption or breakdown of the fatty acid methyl esters under the conditions employed. On a reversed phase column, the more unsaturated desaturation products are eluted before the substrates (cf. Fig. 2). The coefficient of variation of the total assay was better than 4% as calculated from assay of 6 replicate incubations.

Effect of Substrate Concentration, Microsomal Protein Concentration and Incubation Time on Desaturase Activities

During incubation of liver microsomes with fatty acid substrates in the presence of ATP, CoA, Mg^{2+}, and NADH, the fatty acids are not only activated to the corresponding CoA derivatives with subsequent desaturation but are also incorporated together with desaturation products into glycerolipids (21), mainly into phospholipids. Thus, the incorporation of fatty acids into phospholipids are most likely to compete with the desaturation process. In view of the complexity of the system under study, it is important to find out the optimal conditions for the desaturase assays. The requirements of CoA, NADH, and ATP have been investigated elsewhere (9). In the present study, the effect of substrate concentration, microsomal protein concentration and incubation time was investigated for all the substrates used.

The results from this investigation are summarized in Figure 1. The conversion of 18:2ω6 to 18:3ω6 was linear with protein concentrations up to 1.2 mg and apparently linear with time up to 20 min at a substrate level of 100 nmol. Although the corresponding curves for the conversion of 20:3ω6 to 20:4ω6 have a tendency to nonlinearity, the enzyme seems to be saturated at a substrate level of 100 nmol. Thus, the conversion obtained during incubation of 100 nmol of substrate and ca. 1 mg of microsomal protein for 20 min would give satisfactory conditions for assay of liver microsomal Δ^5- and Δ^6-desaturase activities. The conversion of 16:0 to 16:1ω7 was not linear with protein concentration and the enzyme was not saturated at a substrate level of 100 nmol. The conversion was linear with incubation time, however. At a substrate level of 400 nmol, the conversion was inhibited. The desaturation rate of palmitoyl-CoA did not differ from that obtained for 16:0 under the conditions used. This observation rules out the possibility that the acyl-CoA synthetase activity may be limiting under the conditions employed (cf. ref. 22).

Effect of Dietary Oils on Fatty Acid Distribution in Liver Microsomal Phospholipids

The content of *trans* monounsaturated fatty acids and distribution of ω6-polyunsaturated fatty acids in liver microsomal phospholipids isolated from rats fed the different experimental diets are summarized in Table 2. Also, the total content of ω6-polyunsaturated fatty acids and 18:2ω6 metabolites are included in the table. The rats fed partially hydrogenated oils incorporated *trans* fatty

TABLE 2

Trans Fatty acids and ω6-Polyunsaturated Fatty Acids as Weight Percent of Total Fatty Acids in Liver Microsomal Phospholipids Isolated from Rats Fed Diets Containing 20% (w/w) PO, HPO+4.6 cal % 18:2, HCO+4.6 cal % 18:2, HHO+4.6 cal % 18:2, and RSO for 10 Weeks[a]

Fatty acid[b]	PO	HPO+4.6 cal % 18:2	HCO+4.6 cal % 18:2	HHO+4.6 cal % 18:2	RSO
18:1 *trans*	-	12.39±1.55	7.41±0.87	6.78±0.72	-
20:1 *trans*	-	-	0.25±0.12	0.26±0.13	-
18:2ω6	10.26±0.54[d]	11.82±0.59[g]	13.95±0.36[i]	14.02±0.60[h]	10.87±0.37[e]
18:3ω6	0.17±0.02	0.19±0.03	0.25±0.02[g]	0.23±0.03[i]	0.22±0.03[f]
20:2ω6[k]	0.54±0.09	0.41±0.05[f]	0.42±0.10[e]	0.59±0.10	1.01±0.13[g]
20:3ω6	0.66±0.09	1.31±0.10[h]	1.60±0.11[i]	2.08±0.23[j]	1.41±0.09[i]
20:4ω6	33.06±0.87	28.78±1.00[h]	27.56±0.82[h]	25.72±0.85[i]	22.97±0.36[i]
22:4ω6	0.70±0.04	0.41±0.04[i]	0.35±0.03[j]	0.24±0.02[j]	0.08±0.02[j]
22:5ω6	2.21±0.35	2.71±0.41[e]	2.31±0.15	1.16±0.20[g]	traces
Σω6 polyunsaturated fatty acids	47.60±0.41	45.63±0.80[g]	46.42±0.51[f]	44.07±0.80[h]	36.55±0.78[j]
Σω6 metabolites	37.34±0.61	33.82±1.04[g]	32.47±0.79[g]	30.05±0.81[i]	25.68±0.38[j]

[a]The partially hydrogenated diets were supplemented with safflower oil.
[b]The shorthand notation used for the fatty acids indicates chain length: number of double bonds. ω=First double bond position beginning from the hydrocarbon end; methylene interruption and *cis* geometry are assumed if not otherwise stated.
[c]The *trans* fatty acid content was not determined.
[d]Values are mean±standard deviation from 6 rats.
[e-j]Probability level as calculated according to Student's t-test; e,f,g,h, i, and j are significantly different from PO values: $p<0.05$, $p<0.01$, $p<0.001$, $p<0.0001$, $p<0.00001$, $p<0.000001$, respectively.
[k]20:3ω9 coincides with 20:2ω6 in the capillary GC under the conditions used.

acids mainly 18:1 *trans*, into the phospholipids. Small amount of 20:1 *trans* derived from the partially hydrogenated marine oils was also incorporated into the phospholipids, but no significant amount of 22:1 *trans*. The content of 18:2 isomers other than 18:2ω6 was found to be 0.18%, 0.34%, and 0.58% after feeding HPO, HCO, and HHO, respectively (not shown). The identity of these isomers was not established.

The polarity of the column used in the present work did not permit separation of 20:2ω6 and 20:3ω9. Thus, the presence of 20:3ω9 in the rat liver microsomal phospholipids obtained after the various dietary treatments cannot be excluded. However, in a recent study on myocardial phospholipids from rats fed the present diets, these two fatty acids could be distinguished in the capillary GC (14). The content of 20:2ω6 in the cardiac phospholipids showed a similar pattern after the different dietary treatments as was observed in this study on microsomal phospholipids and only small amount of 20:3ω9 (<0.1%) was found.

The liver microsomes of rats fed RSO contained considerably less ω6-polyunsaturated fatty acids than those of the other experimental groups. This was to some extent compensated for by higher amounts of eicosa- and docosapolyenoic acids with ω3-structure (not shown), most probably derived from 18:3ω3 in the RSO diet (8.2%) by desaturation and elongation. This observation is consistent with the higher affinity of the Δ^6-desaturase for 18:3ω3 as compared to 18:2ω6 reported by Brenner (21).

A small but significant decrease in the total content of ω6-polyunsaturated fatty acids in the microsomal phospholipids was obtained after feeding the partially hydrogenated oils (cf. Table 2). In comparison with the PO group of rats, the distribution of individual ω6-polyunsaturated fatty acids was altered after feeding partially hydrogenated oils. The content of 18:2ω6 and 20:3ω6, the substrates for the Δ^6- and Δ^5-desaturase, respectively, was significantly increased. The major product in the synthesis of ω6-polyunsaturated fatty acids, 20:4ω6, was significantly decreased. The most striking effect was observed after feeding HHO, a partially hydrogenated marine oil containing large amounts of isomeric eicosenoic acids (20:1) and docosenoic acids (22:1). After feeding HHO to rats, the content of 22:5ω6 was significantly lower in comparison with the corresponding amount in rats fed PO, HPO, and HCO. The low content of 22:4ω6 obtained in all groups kept on partially hydrogenated oils as compared to the group of rats fed PO is consistent with a disturbance in the chain elongation process of 20:4ω6 in these groups.

Effect of Dietary Oils on Liver Microsomal Desaturase Activities

The results from the in vitro measurements of the Δ^9-, Δ^6-, and Δ^5-desaturase activities in liver microsomes from rats fed the different experimental diets are presented in Table 3. The variations of the

TABLE 3

Δ^9-, Δ^6-, and Δ^5-Desaturase Activites in Liver Microsomes Isolated from Rats Fed Diets Containing 20% (w/w) PO, HPO + 4.6 cal % 18:2, HCO + 4.6 cal % 18:2, HHO + 4.6 cal % 18:2, and RSO for 10 Weeks[a]

HHO + 4.6 cal % 18:2	0.8 ± 0.5	1.7 ± 0.5[c]	3.0 ± 0.9[d]
RSO	1.2 ± 0.5[c]	3.4 ± 0.8	6.0 ± 2.6

[a]The partially hydrogenated oils were supplemented with safflower oil.
[b]Values are mean ± standard deviation from 6 rats.
[c,d]Probability level as calculated according to Student's t-test; c and d are significantly different from PO values: $p < 0.05$ and $p < 0.005$, respectively.

values obtained were considerably higher than those obtained for the fatty acid distribution in the microsomal phospholipids (cf. Table 2). Different circadian rhythms have been reported for the Δ^9- and the Δ^6-desaturase activities in mouse liver (23). In an attempt to reduce the influence of a possible diurnal change on the activities measured, the rats in the present study were subjected to controlled lighting conditions and were killed at a fixed time.

Although the Δ^9-desaturase activity could not be measured at saturated substrate level and is not directly involved in the metabolism of ω6-polyunsaturated fatty acids, it was included for comparison. The apparent Δ^9-desaturase activity in microsomes from animals fed HPO, HCO, and RSO was elevated as compared to the corresponding activity in the group fed PO. The dietary changes observed for the Δ^9-desaturase activity in the present study could to some extent be attributed the content of linoleic acid in the diets. This is in agreement with the observations made by Jeffcoat and James (24) that high amount of dietary linoleic acid have a tendency to decrease the Δ^9-desaturase activity in rat liver.

In contrast to the Δ^9-desaturase assay, the activities of the Δ^6- and Δ^5-desaturation enzymes were measured at saturated substrate levels under the conditions used. Generally, the maximal velocities obtained for the Δ^5-desaturase were higher than the corresponding activities of the Δ^6-desaturase. A tendency to decreased activity of the Δ^6-desaturase was observed in liver microsomes isolated from rats fed partially hydrogenated oils. Feeding HHO to the rats resulted in a significant decrease in Δ^6- as well as Δ^5-desaturase activities in comparison with PO feeding.

DISCUSSION

In the present work, the diets containing partially hydrogenated oils were supplemented with an adequate amount of linoleic acid and the rats did not show any clinical signs of essential fatty acid deficiency (cf. ref. 14). Although, the amount of 20:3ω9 in the liver phospholipids could not be determined with certainty, the sum of the content of 20:2ω6 and 20:3ω9 was low and did not show any tendency to increase after feeding partially hydrogenated oils. No other desaturation elongation products of 18:1 could be detected with the exception for the presence of small amount of 18:2 isomers other than 18:2ω6, possibly of dietary origin. However, the content of 20:3ω9 as an indicator of essential fatty acid deficiency should be used with caution when *trans* fatty acids are dietary constituents in view of the ability of *trans* fatty acids to affect the synthesis of 20:3ω9 (4). The significance of 20:3ω9 in this connection could also be questioned when diets containing linolenic acid are used in view of the fact that linolenic acid and its metabolites strongly suppress the synthesis of both 20:3ω9 and 20:4ω6 (25).

The small but significant decrease in total ω6-polyunsaturated fatty acids observed in the phospholipids after feeding partially hydrogenated oils (cf. Table 2) might be attributed the lower content of 18:2ω6 in the partially hydrogenated oil (11% 18:2ω6) as compared to PO (38% 18:2ω6). The possibility cannot be excluded that the large proportion of 18:1ω9 (42%) in PO could be inhibitory to the synthesis of ω6-polyunsaturated fatty acids if the concomitant proportion of 18:2ω6 was of the same magnitude as in the partially hydrogenated oils. However, Mohrhauer et al. (26) have shown that dietary oleic acid fed in proportion as high as 22 cal % of the diet did not significantly alter the conversion of 18:2ω6 to 20:4ω6 if 18:2ω6 was fed at or above the minimal dietary requirement of ca. 1 cal %. Thus, the accumulation of 18:2ω6 and the reduced content of total 18:2ω6 metabolites observed after feeding partially hydrogenated oils is consistent with an altered synthesis of ω6-polyunsaturated fatty acids in these groups of animals as compared to rats kept on PO.

The effects of the partially hydrogenated oils obtained in vivo on the distribution of ω6-polyunsaturated fatty acids in the microsomal phospholipids do to some extent reflect the effects on the microsomal desaturase activities obtained in vitro. The accumulation of 18:2ω6 and the decreased

content of 20:4ω6 observed in the phospholipids from rats fed partially hydrogenated oils as compared to rats fed PO are consistent with the tendency to decreased microsomal Δ^6-desaturase activities obtained in vitro. The significant decrease in Δ^6- as well as Δ^5-desaturase activities obtained in vitro after HHO treatment are in accord with the high content of 18:2ω6 and 20:3ω6 observed in the phospholipids.

Generally, the Δ^5-desaturase activities obtained in vitro were higher than those of the Δ^6-desaturase (cf. ref. 21). From this point of view, the Δ^6-desaturase is likely to be the main regulatory enzyme in the synthesis of polyunsaturated fatty acids. However, the measurement of the maximal velocities in vitro may not be representative for the metabolism in vivo. In fact, the significant accumulation of 18:2ω6 and 20:3ω6 observed in microsomal phospholipids after feeding partially hydrogenated oils might be indicative of a disturbed regulation of the biosynthesis of polyunsaturated fatty acids not only at the level of the first desaturation step represented by the Δ^6-desaturase but also at the intermediate step at the Δ^5-desaturation.

In a recent paper by Pollard et al. (27), it was shown that liver microsomal Δ^5-desaturase was capable to desaturate 20:3ω6 as well as certain isomers of eicosenoic acid to a significant degree as compared to corresponding isomers of octadecenoic acid. Although, the incubations were performed with nonsaturating substrate concentrations, the possibility cannot be excluded that isomers of eicosenoic acid derived from partially hydrogenated marine oils have the capability to compete with the natural substrate for the Δ^5-desaturase with concomitant decrease formation of arachidonic acid. In the present study, the dietary partially hydrogenated marine oils altered the metabolism of ω6-polyunsaturated fatty acids to a larger extent than dietary HPO. The most striking effect was observed after feeding HHO, a partially hydrogenated marine oil with a moderate content of *trans* fatty acids as compared to HPO but rich in isomers of eicosenoic and docosenoic acids. Furthermore, the reduced content of 22:5ω6 observed after feeding HHO might be explained in a similar way in that isomeric docosenoic acids act as competitive inhibitors on the conversion of 22:4ω6 to 22:5ω6 at the level of a Δ^4-desaturase. With regard to the RSO diet, any influence on the metabolism of ω6-polyunsaturated fatty acids attributed to eicosenoic and docosenoic acids would most probably be masked by the interaction of α-linolenic acid derived from the diet.

Acyl-CoA desaturases are membrane-bound enzymes and the importance of lipids for maximal activity of microsomal stearic acid Δ^9-desaturase has been shown by Jones et al. (28) and Holloway (29). A lipoprotein has been suggested by Brenner (21) to play a specific role in the desaturation process of both Δ^9- and Δ^6-desaturases. Pugh and Kates (30) have presented evidence for a direct desaturation of eicosatrienoyl lecithine at the Δ^5-position by rat liver microsomes. In view of these facts, it is possible that dietary long-chain monounsaturated fatty acid isomers not only might act as competitive inhibitors on the desaturation of natural fatty acid substrates but also may affect the physical properties and integrity of a lipoprotein structure essential for optimal desaturase activity.

However, it is also possible that the degradation of arachidonic acid might be under dietary control. Thus, the lower content of arachidonic acid observed after feeding partially hydrogenated marine oils might be the result of a preferential peroxisomal oxidation of arachidonic acid induced by the dietary regimes (31,32).

In conclusion, the results of the present study demonstrate that dietary partially hydrogenated oils supplied with an adequate amount of linoleic acid and in particular partially hydrogenated marine oils affect the metabolism of linoleic acid in rat liver microsomes in comparison with dietary peanut oil. It remains to be established, however, if these changes are of such a magnitude that they are able to affect membrane fluidity or availability of precursors for prostaglandin synthesis.

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Cholinephosphotransferase Activity in Human Platelets

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ABSTRACT

Disrupted human platelets possess a cholinephosphotransferase activity (EC 2.7.8.2) whose properties have been studied in this work. The labeling of choline glycerophospholipid (CGP) from radioactive cytidine-5'-diphosphate choline (CDP-choline) in vitro shows a maximum at pH 8.0 (using Hepes [4-(2-hydroxyethyl)-piperazine-1-ethane-2-sulfonic acid] as a buffer) and is stimulated by Mn^{2+}, Mg^{2+} and diacylglycerol. The enzymic activity is inhibited by Ca^{2+}. The dependence of human platelet cholinephosphotransferase upon CDP-choline concentration does not follow the Michaelis-Menten equation. CMP strongly inhibits the reaction. The functional implications of this newly discovered platelet activity are briefly considered.
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INTRODUCTION

Choline glycerophospholipid (CGP) represents ca. 40% of the total phospholipid of human platelets (1). This lipid shares with inositol phosphatide the property of releasing arachidonic acid upon platelet stimulation (2-4). For these reasons, the catabolic pathways of CGP in human platelets have received much attention and enzymic activities leading to the release of fatty acids have been particularly studied (5-9). On the other hand, biosynthetic pathways leading to CGP synthesis are less known.

Platelets have been shown to incorporate orthophosphoric acid and glycerol into lipids (10,11); in addition, some properties of the enzymes involved in the synthesis of ethanolamine and inositol glycerophospholipids have been studied (12,13). With regard to CGP, the acylation of the corresponding lyso-compound (14) and the methylation of ethanolamine glycerophospholipid (15-17) have been reported.

Contrary to ethanolaminephosphotransferase (13), cholinephosphotransferase activity (EC 2.7.8.2) in platelets has never been described. The reaction catalyzed by this enzyme transfers the phosphocholine moiety from cytidine-5'-diphosphate choline (CDP-choline) to 1,2-diradyl-*sn*-glycerol (diglyceride) and is reversible in some tissues (18-20). The reversal of cholinephosphotransferase, followed by diglyceride lipase, may represent a catabolic pathway able to release fatty acids from CGP (21). This pathway may, therefore, release fatty acids in platelets. Moreover, cholinephosphotransferase could participate in the synthesis of 1-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine (platelet activating factor, PAF) in platelets, as observed in other tissues (22).

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The aim of the present work is to ascertain whether the cholinephosphotransferase activity is present in human platelets, and to study its biochemical properties.

MATERIALS AND METHODS

Materials

Cytidine-5'-diphosphate-[methyl-^{14}C]-choline (radioactive CDP-choline, specific radioactivity 62 Ci/mol) was obtained from the Radiochemical Centre (Amersham, UK) and tested for radiochemical purity before use. Other reagents were purchased from the common commercial sources. Solvents were freshly distilled before use.

Preparation of Human Platetets

One hundred to 300 ml of human blood were obtained from healthy volunteers fasted for 12 hr and abstaining from any drugs for at least 2 weeks. Venous stasis was carefully avoided. The blood was mixed with 3.8% trisodium citrate (10:1, by vol) to prevent clotting. Platelet-rich plasma was obtained by centrifugation at 100 × g × 15 min and freed from residual erythrocytes by centrifuging 3 times at 1,000 × g × 15 sec. Platelets were pelleted by centrifugation at 1,000 × g × 20 min at room temperature, gently resuspended in 2 ml of isotonic Tris-HCl buffer (pH 7.6) containing 2 mM EDTA and washed twice with 0.32 M sucrose at 4C. Plasticware was always used during the described procedures.

The purity of the platelet preparation was assessed by determining the percentage of white and red blood cells by phase microscopy. The final suspension contained 0.05-0.25% erythrocytes and 0.0-0.04% leukocytes. Platelets, resuspended in 1 ml of

0.32 M sucrose were subsequently sonicated 4 times (10 sec, each time) by means of a MSE sonicating apparatus (100 W), keeping the samples in melting ice.

Disrupted platelets were immediately used or stored at -20C for not more than 2 weeks. Within this period, cholinephosphotransferase activity did not vary upon storage. Protein was determined according to Lowry et al. (23) using bovine serum albumin as a standard.

Assay of Cholinephosphotransferase Activity

Disrupted platelets (1-4 mg protein/ml) were incubated with radioactive CDP-choline in the conditions described in Results. The final volume of the incuation mixture was 0.3 ml, the incubation temperature 37C and the reaction was stopped by adding 4.6 ml of chloroform/methanol (2:1, by vol) to each sample.

Extraction and Analysis of Lipids

Lipids were extracted following described procedures (24). The final lipid extract was dried under vacuum, resuspended in chloroform/methanol (2:1, by vol) and quantitatively transferred to Silica Gel G plates (0.5 mm, thickness). Chloroform/methanol/water (65:25:4, by vol) was used as developing solvent. Spots were visualized with I_2 vapors and identified with pure reference standards. After I_2 sublimation, the spots corresponding to CGP were scraped off the plate and transferred to scintillation vials; 0.5 ml of ethanol/water (1:1, by vol) and 10 ml of Emulsifier Scintillator 299™ (Packard, Downers Grove, IL) were added to each sample before measuring radioactivity by means of a Packard Scintillator (Tri-Carb, mod. 3330), using an external standard to calculate efficiency.

Preparation of 1,2-diacyl-*sn*-glycerol

1,2-Diacyl-*sn*-glycerol (diglyceride) was prepared from soybean lecithin according to the method described by Binaglia et al. (25) and determined by gas chromatographic analysis (25). Diglyceride suspensions were prepared sonicating the lipid 3 times (1 min each time, MSE sonicating apparatus, 100 W) in a convenient volume of the buffer solutions described in Results and containing 0.5% Tween-20.

RESULTS

General.

Disrupted human platelets formed radioactive CGP upon incubation with labeled CDP-choline in various experimental conditions. Labeled CDP-choline was not hydrolyzed in our experimental conditions. No radioactive CGP was formed using platelets which had been boiled for 5 min. The rate of CGP formation was quite low (ca. 0.5 nmol × mg protein^{-1} × 30 min^{-1}) with endogenous diglyceride as substrate. Enzymic activity was, therefore, usually assayed in the presence of added exogenous diglyceride.

Effect of pH

The highest rates of CGP synthesis were obtained at pH 8.0 using Hepes (4-(2-hydroxyethyl)-piperazine-l-ethane-2-sulfonic acid) as a buffer (Fig. l). This is not probably the optimal pH for the cholinephosphotransferase reaction, since this activity shows a peak at pH 9.2 using glycine-NaOH as a buffer; however, this buffer seems to inhibit the reaction (Fig. l) and, for this reason, Hepes buffer at pH 8.0 was always used in the assays of cholinephosphotransferase activity.

The effects of pH variations were the same in various experimental conditions (concentration of CDP-choline from 0.29 to 1.0 mM; concentration of diglyceride from 0.79 to 4.0 mM).

Effect of Protein Concentration

Figure 2 shows the dependence of CGP formation on platelet protein concentration. The enzymic activity was linear with protein concentration from

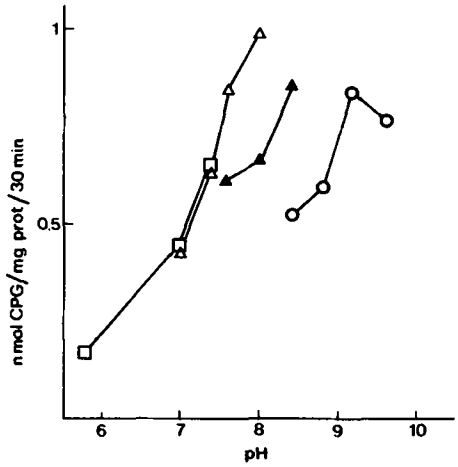

FIG. 1. The effect of pH on cholinephosphotransferase activity. The following buffers were used: Mops (3-(N-morpholino)-propanesulfonic acid): □--□--□ ; Hepes (4-(2-hydroxyethyl)-piperazine-l-ethane-2-sulfonic acid): △--△--△ ; Tris-HCl: ▲--▲--▲ and glycine-NaOH: ○--○--○. Incubation conditions: 0.29 mM labeled CDP-choline (specific radioactivity 5.8 Ci/mol), 60 mM buffer, 0.79 mM diglyceride, 0.04% Tween-20, 0.08 mM dithiothreitol (DTT), 15 mM NaF, 20 mM $MgCl_2$, 4.3 mg platelet protein/ml incubation medium. Final volume: 0.3 ml; incubation time: 30 min.

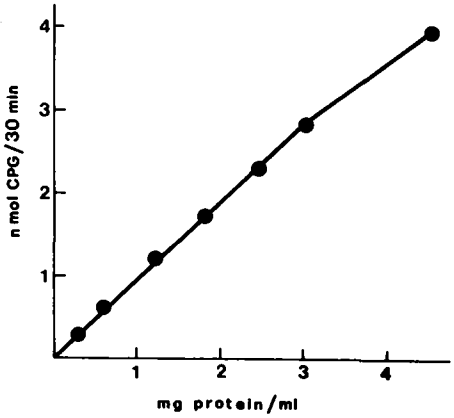

FIG. 2. The effect of protein concentration on cholinephosphotransferase activity. Incubation conditions: 0.25 mM labeled CDP-choline (specific radioactivity 0.741 Ci/mol), 60 mM Hepes (pH 8.0), 4.0 mM diglyceride, 0.04% Tween-20, 0.08 mM DTT, 15 mM NaF, 10 mM $MnCl_2$, platelet protein as indicated. Final volume: 0.3 ml; incubation time: 30 min.

0 to 3 mg protein × ml incubation mixture^{-1}, a deviation from linearity being observed only at 4.5 mg protein × ml incubation mixture^{-1} (Fig. 2).

Effect of Divalent Cations

Divalent cations (Mn^{2+} and Mg^{2+}) affected the reaction rate, as shown in Figure 3. Mg^{2+} (up to 40 mM) stimulated the incorporation of CDP-choline into CGP, whereas the presence of both 20 mM Mg^{2+} and 2 mM Ca^{2+} reduced the reaction rate by ca. 10 times, compared to 20 mM Mg^{2+} alone (result not shown in Fig. 3). Mn^{2+} also stimulated the transfer of phosphocholine from CDP-choline to lipid (Fig. 3). At low concentrations, Mn^{2+} was a better stimulator than Mg^{2+}, and therefore the former ion was used throughout this work. The effect of divalent cations remained substantially unmodified if the concentration of diglyceride was 0.79 mM instead of 4.0 mM.

Effect of Incubation Time

The amount of synthesized CGP increased with increasing incubation time (Fig. 4). However, the slope of lipid labeling depended on incubation times, probably as a result of the time required by the added diglyceride to interact with platelet membranes.

Effect of Diglyceride Concentration

The activity of cholinephosphotransferase depended upon the concentration of the diglyceride added to the incubation mixture (Fig. 5). However, the rate of increase was slow at low diglyceride

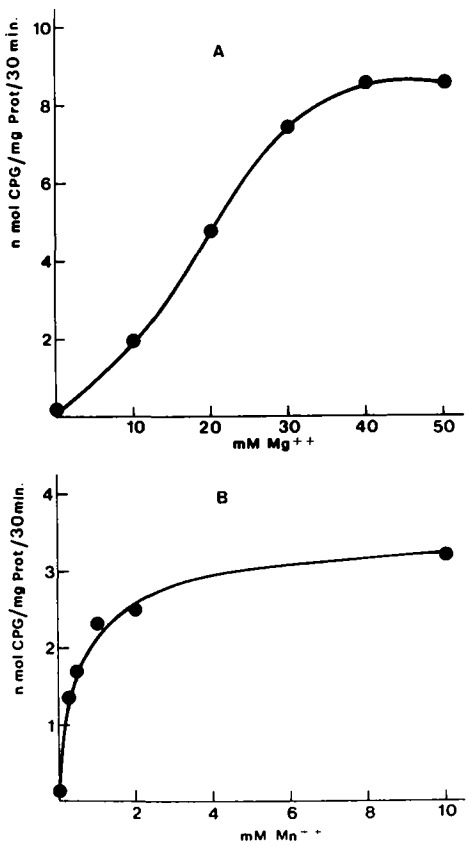

FIG. 3. The effect of divalent cations on cholinephosphotransferase activity. Incubation conditions: 0.25 mM labeled CDP-choline (specific radioactivity 0.741 Ci/mol), 60 mM Hepes (pH 8.0), 4.0 mM diglyceride, 0.04% Tween-20, 0.08 mM DTT, 15 mM NaF, 1.35 mg platelet protein/ml incubation medium, (A) $MgCl_2$ or (B) $MnCl_2$, at the indicated concentrations. Incubation volume: 0.3 ml; incubation time: 30 min.

concentrations, rising abruptly above 2 mM diglyceride (Fig. 5A). This may be the result of several factors, such as the physical state of the added lipid and/or the action of a diglyceride lipase (4).

The ratio diglyceride/detergent during the sonication of lipids appears to be important for enzymic activity (cf. Figs. 5A and 5B). Indeed, varying this ratio, an activation by diglyceride on CGP synthesis was already detectable at 0.5 mM diglyceride (Fig. 5B). Moreover, the addition of indomethacin (0.1 mg/ml incubation mixture), which is known to inhibit diglyceride lipase (26), increased the synthesis of CGP at low diglyceride concentrations (Fig. 6). Therefore, both the physical state of diglyceride suspensions and the activity of diglyceride lipase could explain the data reported in Figure 5A.

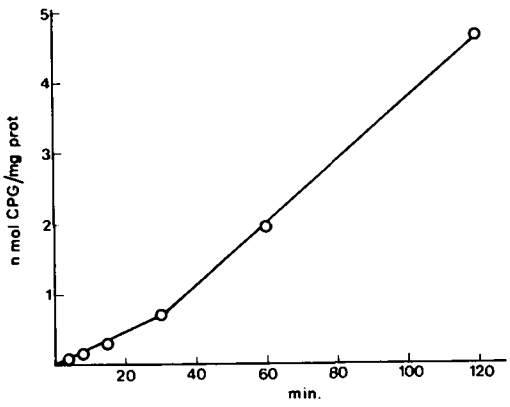

FIG. 4. The effect of the incubation time on CGP formation. Incubation conditions: 0.32 mM labeled CDP-choline (specific radioactivity 2.6 Ci/mol), 60 mM Hepes (pH 8.0), 0.84 mM diglyceride, 15 mM NaF, 0.04% Tween-20, 0.08 mM DTT, 10 mM $MnCl_2$, 2.8 mg platelet protein/ml incubation medium. Final volume: 0.3 ml. Similar results were obtained with 1 mM labeled CDP-choline and 4 mM diglyceride.

Effect of CDP-choline Concentrations

Three experiments, using different platelet preparations, have been performed to study the effect of CDP-choline concentration on the synthesis of CGP, both in the presence and in the absence of 1 mM CMP. The reaction did not follow the Michaelis-Menten equation (Fig. 7) using concentrations of CDP-choline ranging from 0.021 mM to 6.0 mM. The same behavior was observed after the addition of 1 mM CMP.

Kinetic constants at various concentrations have been tentatively evaluated by the direct linear plot method (27). From 0.021 mM to 0.28 mM CDP-choline, the apparent K_M was ca. 0.045 mM and the V_{max} was 0.25 nmol $\times$ min^{-1} $\times$ mg $protein^{-1}$. If CDP-choline ranged from 0.28 to 6.0 mM, the apparent K_M was about 0.47 mM and the V_{max} 0.65 nmol $\times$ min^{-1} $\times$ mg $protein^{-1}$. In both cases, 1 mM CMP inhibited the reaction. The ratio CMP/CDP-choline appeared to be important in this connection. Indeed, the greatest inhibition (ca. 80%) occurred when the ratio was higher than 10 whereas, at lower values, the inhibition decreased to 50%. A negative cooperativity effect could be liable for the anomalous kinetic behavior of cholinephosphotransferase; indeed, the Hill's coefficient was 0.52 (Fig. 8).

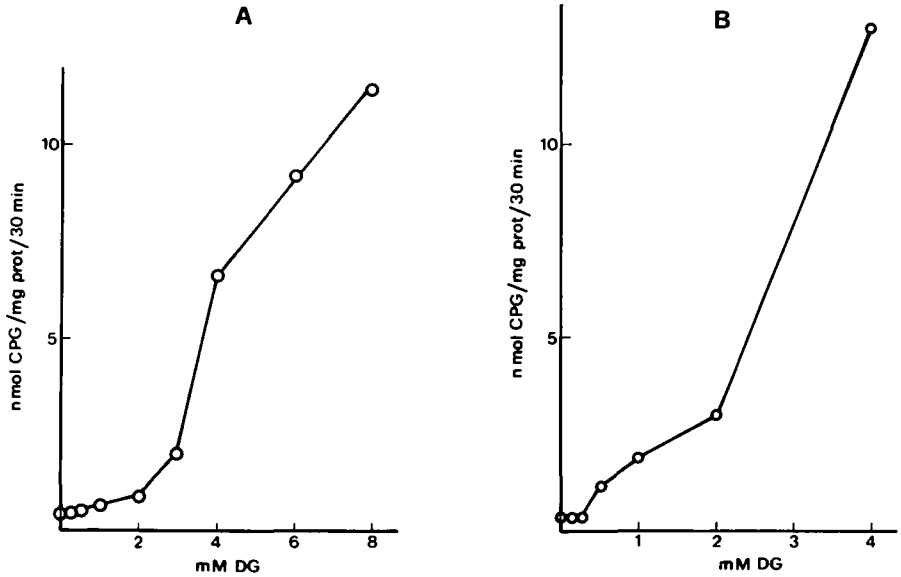

FIG. 5. The effect of diglyceride concentration on cholinephosphotransferase activity. (A) Incubation conditions: 0.39 mM labeled CDP-choline (specific radioactivity: 1.6 Ci/mol), 60 mM Hepes (pH 8.0), 10 mM $MnCl_2$, 0.04% Tween-20, 0.08 mM DTT, 15 mM NaF, 1.5 mg platelet protein/ml incubation medium, diglyceride, as indicated. Different diglyceride concentrations were obtained diluting the initial 48 mM diglyceride suspension (prepared as described in Materials and Methods), with convenient amounts of 0.5% Tween-20. (B) Incubation conditions: as in (A), except that different diglyceride concentrations were obtained by sonicating separately different amounts of diglyceride in the same volume of detergent solution.

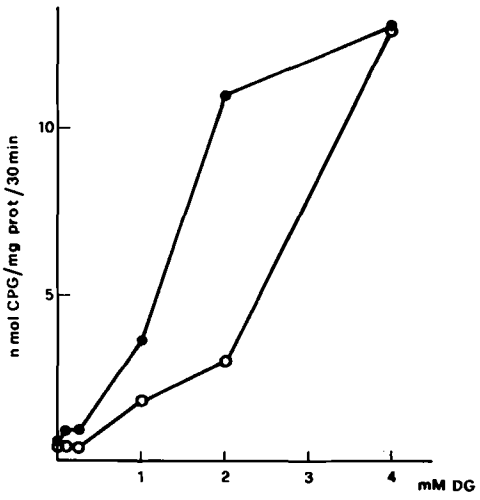

FIG. 6. The effect of indomethacin on CGP synthesis at different diglyceride concentrations. Incubation conditions: 1 mM labeled CDP-choline (specific radioactivity 0.82 Ci/mol), 60 mM Hepes (pH 8.0), 10 mM $MnCl_2$, 0.04% Tween-20, 0.08 mM DTT, 15 mM NaF, 1.1 mg platelet protein/ml incubation medium. Final volume: 0.3 ml; incubation time: 30 min. Diglyceride suspensions were prepared as described in Figure 5B. No indomethacin added: ○--○--○; 0.1 mg indomethacin/ml incubation medium: ●--●--●.

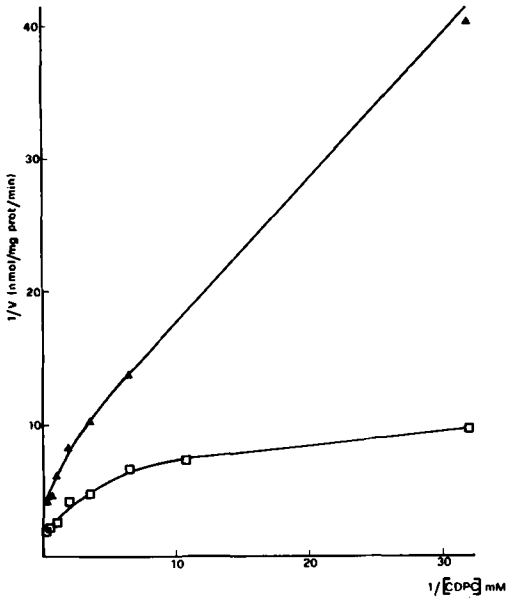

FIG. 7. The effect of CDP-choline concentration on choline phosphotransferase activity. Incubation conditions: 60 mM Hepes (pH 8.0), 0.08 mM DDT, 15 mM NaF, 10 mM $MnCl_2$, 4.0 mM diglyceride, 0.04% Tween-20, 1 mg platelet protein/ml incubation medium, labeled CDP-choline, as indicated. Final volume: 0.3 ml; incubation time: 30 min. No CMP added: □--□--□; 1 mM CMP: ▲--▲--▲.

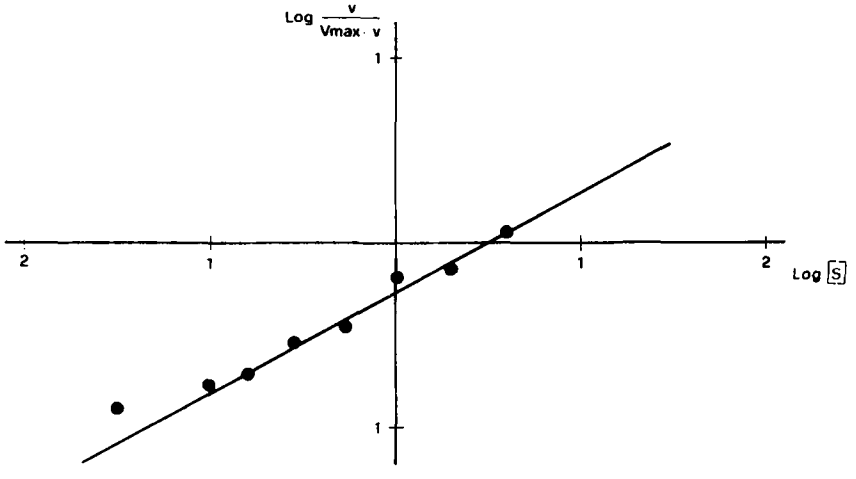

FIG. 8. Hill's plot for the activity of cholinephosphotransferase vs CDP-choline concentration. Incubation conditions: as in Figure 7. V_{max} has been calculated by extrapolating the Lineweaver-Burk plot. Hill's coefficient = 0.52; r = 0.99.

DISCUSSION

Various mechanisms seem to be involved in the renewal of platelet CGP. In fact, the exchange of CGP from plasma lipoproteins to platelets has been reported (28) in addition to interconversion reactions leading to CGP synthesis (14-17). A synthesis ex novo of CGP has been demonstrated in platelets (10,11,15), but the enzymes responsible for CGP biosynthesis have not been described.

In this work, human platelets have been shown to possess a cholinephosphotransferase activity (EC 2.7.8.2). The alkaline pH optimum, the activation by Mn^{2+} and Mg^{2+} and the inhibition produced by Ca^{2+} on human platelet cholinephotransferase are properties quite similar to those of the enzyme from other sources (29,30). In platelets, low concentrations of Mn^{2+} are a much more active stimulator than the corresponding concentrations of Mg^{2+}. From this point of view, the enzyme resembles ethanolaminephosphotransferase of human platelets (13) and of other tissues (31). On the other hand, the highest stimulation of human platelet cholinephosphotransferase is obtained with 1-2 mM Mn^{2+}, whereas 10 mM Mn^{2+} is required to produce the same effect on ethanolaminephosphotransferase (13).

The content of diglyceride in platelets is low (1), which can explain the small amount of activity found when no diglyceride is added to the incubation mixture. The time required by added exogenous diglyceride to interact with platelet membranes may explain the results shown in Figure 4. On the other hand, the effect of the addition of soybean diglyceride to the incubation mixture is rather complicated. Figures 5 and 6 show that both the method of preparing diglyceride suspensions and the activity of a diglyceride lipase (4) represent factors able to influence the stimulatory properties of diglyceride on the enzyme.

It was impossible, in our experimental conditions, to calculate both the K_M and the V_{max} for CDP-choline. Indeed, the enzymic activity did not follow the classical Michaelis-Menten equation; for this reason, the apparent K_M and V_{max} depended on the concentrations of CDP-choline. Increasing the concentration of the substrate, the V_{max} apparently increased 2- to 5-fold and the K_M 10-fold. This anomalous behavior can be due to a negative cooperative effect, although the presence of two or more enzymes possessing different kinetic properties cannot be ruled out. Further studies on platelet subfractions may help clarify this point.

The reaction is inhibited by 1 mM CMP at any CDP-choline concentration; however, the degree of inhibition depends on the ratio between the concentration of the substrate and that of the inhibitor. Because of the anomalous kinetic behavior of human platelet cholinephosphotransferase, it is impossible to state the type of inhibition exerted by CMP. On the other hand, the effect of CMP on the reaction rates might be connected with a possible reversibility of cholinephosphotransferase activity, as shown in other tissues (18-20).

CGP is an important source of free fatty acids in platelets, and phospholipases (5-9) can certainly account for this role of CGP; however, a reversal of cholinephosphotransferase activity followed by the action of a diglyceride lipase could also produce the arachidonic acid required for the synthesis of prostaglandin. The possibility that platelets possess two pathways (possibly differently regulated) for the release of free fatty acid from CGP is certainly interesting and should be investigated in future work. Moreover, cholinephosphotransferase could also be implied in the synthesis of PAF, which has been shown to be a powerful inducer of platelet aggregation (32).

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Biliary and Fecal Steroid Excretion in Rats Fed Partially Hydrogenated Soybean Oil

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ABSTRACT

Male Wistar rats were fed cholesterol-free or cholesterol-enriched diets containing partially hydrogenated soybean oil with different levels of *trans*-fatty acids or unhydrogenated soybean oil at the 10% level. The linoleic acid content of hydrogenated fat diets was adjusted to 3.6% of the total energy. Hydrogenated fat diets contained 29% and 41% *trans*-acids, mainly as *t*-18:1. *Trans*-fats exerted no untoward effects on growth parameters, but increased liver weight. Dietary hydrogenated fats influenced neither the concentration nor composition of biliary steroids, irrespective of the presence or absence of cholesterol in the diet. In rats fed a cholesterol-free diet, daily fecal output of neutral and acidic steroids was enhanced by hydrogenated fats and the magnitude of augmentation was proportional to the dietary level of *trans*-fatty acids. The increased fecal steroid excretion corresponded to an increase in total excreta. Hydrogenated fats also tended to enhance bile acid excretion when feeding a cholesterol-enriched diet. The results suggest that dietary *trans*-fatty acids, in relation to *cis*-polyunsaturated fatty acids, provoke demonstrable change in steroid homeodynamics.

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INTRODUCTION

Partially hydrogenated fats usually contain considerable amounts of *trans*-fatty acids. Recent studies of Canadian (1,2) and Japanese (3,4) margarines showed that most of the *trans*-isomers were monoenes and only small or negligible amounts of *trans*-dienes were presented. Thus, *trans*-isomers in average processed fats are composed primarily of *trans*-octadecenoic acid (*t*-18:1). A report submitted to the US Food and Drug Administration in 1976 concluded that hydrogenated soybean oil is not hazardous to the public so far as dietary levels that are now current or that might reasonably be expected in the future (5). An epidemiological survey, however, claimed a significant positive correlation between dietary *trans*-fatty acid components and cancer causation (6,7), though there are a number of criticisms against the concept (8-10).

Since dietary fat is thought to enhance the development of bowel tumors, presumably by stimulating production of bile acids (11,12), it is of considerable interest if dietary *trans*-fatty acid might affect the biliary and hence, fecal excretion of steroids. The relationship of *trans*-fatty acids in diets to the metabolism of cholesterol and bile acids is still not well understood (13,14).

In this report, the effects of two types of hydrogenated soybean oils containing different levels of *trans*-fatty acids on biliary and fecal steroid excretion as well as serum lipids and apolipoproteins in rats were compared with those of unhydrogenated soybean oil from which hydrogenated products were prepared.

MATERIALS AND METHODS

Animals and Diets

Specific pathogen-free male Wistar rats were obtained at about 4 weeks of age from Kyudo Co., Kumamoto, and were housed individually in stainless steel cages. After 4-5 days of acclimation in an air-conditioned room (20 C, RH 60%, Biotron) with alternating 12-hr periods of light (08.00-20.00 hr), the rats (weighing an average of 100 g and 106 g for the first and second experiments, respectively) were then divided into experimental groups with food and water available ad libitum. Two sets of studies were done with diets free of or containing cholesterol using soybean oil and two partially hydrogenated soybean oils as a fat source. The composition of the diet (15) is given in Table 1, together with the fatty acid composition. Hydrogenated fats were prepared in the presence of stabilized nickel catalyst at hydrogen pressure 3 kg/cm^2 and at 180-200 C. These fats contained α-tocopherol 100 ppm and citric acid 25 ppm. Hydrogenated fats are referred to in this paper as high *trans*-fat and low *trans*-fat, depending on the content of *trans*-isomers. When high *trans*-fat was used, 2% out of 10% was replaced with safflower oil to avert essential fatty acid deficiency; hydrogenated fat diets thus supplied 3.6% of the total energy as linoleic acid.

Food intake and body weight were measured every 2 days. A small volume of blood was withdrawn from the tail vein for analysis of serum cholesterol and apolipoproteins 1-2 days before bile collection. After feeding cholesterol-free diets for 32-36 days or cholesterol-containing diets for

TABLE 1

Composition of Diets

Ingredients[a]	Soybean oil diet (%)	Low *trans*-fat diet (%)	High *trans*-fat diet (%)
Casein	20	20	20
Soybean oil	10	-	-
Low *trans*-fat	-	10	-
High *trans*-fat	-	-	8
Safflower oil	-	-	2
Mineral mixture	4	4	4
Vitamin mixture (water soluble)	1	1	1
Choline chloride	0.15	0.15	0.15
Cellulose powder	2	2	2
Sucrose	62.85	62.85	62.85
Fatty acid composition (%)			
16:0 (1.00)[b]	10.2	10.1	10.4
18:0 (1.29)	4.5	5.5	12.2
t-18:1 (1.46)	-	22.9	40.6
c-18:1 (1.54)	26.3	36.8	16.8
ct-18:2 (1.79)	-	6.2	0.2
cc-18:2 (1.91)	54.2	18.0	17.8
ccc-18:3 (2.32)	4.7	1.0	-

[a]The diet contained fat soluble vitamins in 0.25 g corn oil/ 100 g (retinyl palmitate 400 IU, cholecalciferol 200 IU and DL-α-tocopheryl acetate 10 mg). Cholesterol (0.5%) was added at the expense of sucrose.

[b]The relative retention time in parenthesis. *ct*-18:2 includes *tc*-18:2. The relative retention time of *tt*-18:2 was 1.65.

25-28 days, the bile duct was cannulated under light ether anesthesia during 10.00-11.30 hr. Rats were kept in restraining cages and the bile was collected for 2 hr by monitoring the flow rate at 30-min intervals, and kept frozen until analyzed. The bile drained at the constant rate was used for steroid analysis. Rats were killed by decapitation immediately after termination of bile collection, blood was collected and the liver and epididymal adipose tissue excised. Prior to blood withdrawal from the tail vein, feces were collected for 2 days and lyophilized.

Lipid and Steroid Analyses

Blood sera obtained before bile duct cannulation were analyzed for cholesterol by the enzymatic method (Cholesterol C-Test, Wako Pure Chemicals Inc., Osaka) and for apolipoproteins A-I, B and E by immunoelectrophoresis (16). Lipids from livers and blood sera obtained after decapitation were extracted with a Folch's solvent (17) and analyzed for cholesterol, triglyceride and phospholipid as reported elsewhere (18). Biliary and fecal neutral steroids (19) and acidic steroids (20) were analyzed by gas-liquid chromatography (GLC). Neutral steroids were assayed as trimethylsilyl ether using 3% OV-17 on Gas-Chrom Q with a 5α-cholestane standard. Bile acids were assayed as hexafluoroisopropyl ester-trifluoroacetate derivative (21) using 2% QF-1 on Uniport HP and as methyl ester-trimethylsilyl ether derivative using 1.5% SE-30 on Chromosorb W AW DMCS with 5β-cholanic acid as a standard. Biliary bile acids were also determined by the enzymatic method as taurocholate standard (22).

Fatty acid compositions of the total lipid fraction of serum, liver and adipose tissue were determined by GLC using 10% DEGS (containing 1% H_3PO_4) on Uniport HP (3 mm × 2 m glass column). These packings were obtained from Gaskuro Kogyo Inc., Tokyo. Tissue lipids were also analyzed on packed column (15% OV-275 on Chromosorb P AW, 3 mm × 6 m glass column, Shimadzu Seisakusho, Kyoto) to separate *cis*- and *trans*-isomers (23,24). The results obtained with DEGS and OV-275 columns agreed reasonably well with each other (4). The GLC analyses were performed on Shimadzu Gas Chromatograph 4CMPF.

Statistical Analysis

Scheffe's analysis of variance (25) was used to evaluate the statistical significance with the probability level, 0.05.

RESULTS

Effects of Dietary Fats on Serum and Liver Lipids

In two sets of experiments, cholesterol-free and cholesterol-enriched diets, different dietary hydrog-

TABLE 2

Effects of Dietary Fats on Serum and Liver Lipids[a]

Lipids	Dietary fats		
	Soybean oil	Low *trans*-fat	High *trans*-fat
Cholesterol-free diet			
Serum[b]			
Cholesterol (mg/dl)	99.6 ±7.8	108 ±3.3	121 ±6.9
Apolipoproteins (mg/dl)			
Apo A-I	88.8 ±3.7	98.3 ±4.0	101 ±3.6
Apo B	15.9 ±0.9[c]	8.82±0.45[d]	12.2 ±0.6[c]
Apo E	48.9 ±3.0	59.2 ±4.5	54.2 ±4.0
Liver			
Weight (g/100 g body wt.)	3.69±0.01[c]	3.95±0.09[c,d]	4.24±0.13[d]
Cholesterol (mg/g)	2.58±0.11	2.34±0.15	2.37±0.10
Triglyceride (mg/g)	17.4 ±1.5[c]	13.7 ±0.8[c,d]	11.9 ±0.4[d]
Cholesterol-supplemented diet			
Serum[b]			
Cholesterol (mg/dl)	133 ±21.7[c]	197 ±24.1[c,d]	252 ±19.9[d]
Apolipoproteins (mg/dl)			
Apo A-I	85.5 ± 6.9	86.6 ± 4.2	93.9 ± 2.4
Apo B	12.4 ± 0.9	12.6 ± 1.1	13.2 ± 1.6
Apo E	24.6 ± 1.6[c]	31.0 ± 1.9[d]	25.7 ± 1.1[c,d]
Liver			
Weight (g/100 g body wt.)	5.06± 0.18[c]	5.43± 0.09[d]	5.32± 0.14[c]
Cholesterol (mg/g)	32.2 ± 4.6	27.1 ± 1.6	27.4 ± 2.2
Triglyceride (mg/g)	32.9 ± 6.2[c]	16.4 ± 1.3[d]	12.1 ± 0.7[d]

[a]Mean ± SE of 7-8 rats per group.
[b]Before bile drainage.
[c,d,e]Values in the same line not sharing the common superscript letters were significantly different at $P<0.05$.

enated fats showed no untoward effect on weight gain and food intake. The average body weight gain of rats fed cholesterol-free and cholesterol-containing diets was 6.7 g/day and 7.2 g/day, respectively. Food consumption was 17.5 g/day for the former and 17.2 g/day for the latter. As shown in Table 2, liver weight tended to increase on feeding hydrogenated fats, in particular on feeding a cholesterol-free diet.

In rats fed a cholesterol-free diet, the concentration of serum cholesterol was virtually the same among 3 groups. Cholesterol feeding considerably increased the level of serum cholesterol and it was most prominent when high *trans*-fat was fed. Serum triglyceride and phospholipid levels stayed in the same range among different groups in both trials, respectively (data not shown). No demonstrable difference due to *trans*-fat feeding was observed in serum apo A-I level. In rats fed a cholesterol-free diet, the serum apo E level was indistinguishable among 3 groups whereas apo B tended to decrease on feeding hydrogenated fats in relation to unhydrogenated fat. No such difference in apo B was observed in rats fed a cholesterol-containing diet, while the apo E level was slightly high in animals fed low *trans*-fat compared to those fed soybean oil.

Hepatic triglyceride decreased in rats fed hydrogenated fats both in cholesterol-free and more markedly in cholesterol-containing diets compared to the corresponding animals fed untreated soybean oil. Consequently, dietary cholesterol-dependent accumulation of triglyceride was evident only in rats fed soybean oil, though the magnitude of cholesterol deposition was apparently the same among different groups. No differences were observed on the phospholipid levels in two sets of experiments (data not shown).

Effects of Dietary Fats on Biliary Steroids

As shown in Table 3, in rats fed a cholesterol-free diet, dietary fat type did not alter the concentration of biliary cholesterol and bile acids, though there was an equivocal difference in the rate of bile flow. Cholesterol feeding considerably increased the concentration of cholesterol and bile acids in the bile, but no significant dietary fat-dependent alterations in these parameters were observed. Dietary fat type also did not alter biliary bile acid compositions in both experiments, though they were cognitively modified by cholesterol feeding. Main changes due to dietary cholesterol were the increase in the percentage of β-muricholic acid and the decrease in ω-muricholic acid.

Effects of Dietary Fats on Fecal Steroid Excretion

The data for fecal steroid excretion are summar-

TABLE 3

Effects of Dietary Fats on Biliary Steroids[a]

Biliary steroids	Dietary Fats		
	Soybean oil	Low *trans*-fat	High *trans*-fat
Cholesterol-free diet			
Bile flow (ml/hr)	0.84 ± 0.04[d,e]	0.69 ± 0.05[e]	0.95 ± 0.06[d]
Cholesterol (mg/ml)	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
Bile acids			
Concentration (mg/ml)[b]	9.97 ± 0.86	10.5 ± 0.8	9.48 ± 0.42
Composition (%)[c]			
Lithocholic	1.0 ± 0.3	0.3 ± 0.0	0.3 ± 0.0
Deoxycholic	2.0 ± 0.3	1.8 ± 0.3	1.9 ± 0.6
Chenodeoxycholic	4.6 ± 0.3	5.0 ± 0.7	4.0 ± 0.5
Cholic + α-muricholic	49.9 ± 3.7	47.5 ± 2.1	45.4 ± 2.4
12-ketolithocholic	11.2 ± 4.0	10.4 ± 0.4	11.0 ± 1.0
β-Muricholic	5.7 ± 1.0	8.2 ± 0.9	8.6 ± 3.1
ω-Muricholic	10.3 ± 2.1	10.3 ± 1.7	15.1 ± 2.0
Cholesterol-supplemented diet			
Bile flow (ml/hr)	0.74 ± 0.09	0.79 ± 0.06	0.75 ± 0.07
Cholesterol (mg/ml)	0.22 ± 0.02	0.18 ± 0.01	0.19 ± 0.01
Bile Acids			
Concentration (mg/ml)[b]	16.4 ± 1.9	16.4 ± 2.0	16.8 ± 2.3
Composition (%)[c]			
Lithocholic	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
Deoxycholic	2.4 ± 0.6	2.3 ± 0.6	2.6 ± 0.5
Chenodeoxycholic	4.3 ± 0.6	3.0 ± 0.7	2.3 ± 0.5
Cholic + α-muricholic	42.7 ± 2.0	39.7 ± 2.2	37.0 ± 1.7
12-Ketolithocholic	7.3 ± 0.4	6.9 ± 0.4	6.5 ± 0.7
β-Muricholic	14.3 ± 2.1	23.5 ± 2.5	22.9 ± 2.4
ω-Muricholic	3.3 ± 1.0	5.1 ± 1.6	7.6 ± 2.3

[a]Mean ± SE of 7-8 rats per group.
[b]As taurocholate.
[c]Excludes 3-4 unidentified bile acids.
[d,e]Values in the same line not sharing common superscript letters were significantly different at $P < 0.05$.

ized in Table 4. The daily neutral steroid excretion rate in rats fed a cholesterol-free diet, but not in those fed a cholesterol-containing diet, increased on feeding hydrogenated fats and the difference between unhydrogenated soybean oil and high *trans*-fat was significant. The increase was largely ascribed to an increase in the excreta and the concentration of fecal neutral steroids was comparable (8.1-9.3 mg/g dry feces). Hydrogenated fats raised the concentration (5.3 mg/g dry feces for unhydrogenated fat and 7.4-8.5 mg/g for hydrogenated fats, $P < 0.05$) and daily excretion of fecal acidic steroids significantly in rats fed a cholesterol-free diet. A similar extent of the increase in fecal excretion of acidic steroids was also observed in rats fed a cholesterol-containing diet, though not statistically significant. Semiquantitative analysis showed a slight increase in fecal total lipids (diethylether extractable) on hydrogenated fats particularly in a cholesterol-free diet, suggesting the difference in the rate of absorption of dietary fats.

Effects of Dietary Fats and Fatty Acid Composition of Tissue Lipids

In the experiment with a cholesterol-free diet, fatty acids from the serum total lipids of rats fed low and high *trans*-fats contained ca. 5% and 10% *t*-18:1, liver lipids 13% and 17%, and the adipose tissue, 18% and 25%, respectively. In rats fed a cholesterol-containing diet, both serum and liver lipids from rats fed hydrogenated fats contained similar amounts of *t*-18:1, 8% and 12% for fats low and high in *trans*-fatty acids and the adipose tissue contained 17% and 23%, respectively. In both experiments, the percentage of *cis*-18:2 and 20:4 was comparable between low and high *trans*-fat groups, respectively. No sign of essential fatty acid deficiency was recognized in rats fed hydrogenated fats from the fatty acid profile since virtually no 20:3*n*9 was detected.

DISCUSSION

The present study indicated that hydrogenated fats containing mainly *t*-18:1, compared with the unhydrogenated fat, altered the fecal output of steroids. The increase in fecal acidic steroid excretion was clearly demonstrated in the absence of dietary cholesterol. Though the difference was not statistically significant, a similar trend could also

TABLE 4

Effects of Dietary Fats on Fecal Steroid Excretion[a]

Fecal steroids	Dietary fats		
	Soybean oil	Low *trans* fat	High *trans*-fat
Cholesterol-free diet			
Feces (g/day)[b]	0.57 ± 0.04[d]	0.77 ± 0.04[e]	0.83 ± 0.05[e]
Neutral steroids (mg/day)			
Coprostanol	3.02 ± 0.46	4.01 ± 0.35	4.54 ± 0.56
Cholesterol	1.72 ± 0.14[d]	2.24 ± 0.16[e]	3.16 ± 0.18[e]
Total	4.74 ± 0.58[d]	6.25 ± 0.46[d,e]	7.70 ± 0.59[e]
Acidic steroids (mg/day)			
Lithocholic	0.29 ± 0.02[d]	0.47 ± 0.04[e]	0.46 ± 0.08[e]
Deoxycholic	0.60 ± 0.14	1.05 ± 0.14	0.98 ± 0.20
Cholic + α-muricholic	0.18 ± 0.18	0.40 ± 0.08	0.44 ± 0.10
12-Ketolithocholic	0.15 ± 0.02	0.24 ± 0.04	0.30 ± 0.06
β-Muricholic	0.41 ± 0.08	0.59 ± 0.13	1.22 ± 0.38
ω-Muricholic	1.13 ± 0.17[d]	2.78 ± 0.23[d,e]	3.30 ± 0.64[e]
Total[c]	3.00 ± 0.40[d]	5.69 ± 0.56[d,e]	7.05 ± 1.24[e]
Cholesterol-supplemented diet			
Feces (g/day)	0.72 ± 0.04[d]	0.95 ± 0.05[e]	0.88 ± 0.05[d,e]
Neutral steriods (mg/day)			
Coprostanol	14.3 ± 1.2	22.0 ± 2.0	14.7 ± 2.3
Cholesterol	29.5 ± 3.2	33.5 ± 2.3	24.9 ± 2.2
Total	43.7 ± 3.1[d,e]	55.6 ± 3.3[d]	39.8 ± 2.9[e]
Acidic steroids (mg/day)			
Lithocholic	3.60 ± 0.31[d]	6.10 ± 0.62[e]	5.79 ± 0.61[e,e]
Deoxycholic	2.39 ± 0.23[d]	3.93 ± 0.29[e]	2.82 ± 0.33[d,e]
Cholic + α-muricholic	3.27 ± 0.84	4.30 ± 1.60	3.47 ± 0.97
12-Ketolithocholic	0.78 ± 0.09	1.14 ± 0.22	1.41 ± 0.37
β-Muricholic	2.61 ± 1.30	6.51 ± 0.97	9.85 ± 3.50
ω-Muricholic	4.81 ± 1.20	5.42 ± 0.80	4.38 ± 0.61
Total[c]	17.6 ± 1.9	27.6 ± 2.7	28.0 ± 5.6

[a]Mean ± SE of 7-8 rats per group.
[b]Dry weight.
[c]Includes 1-2 unidentified bile acids.
[d,e]Values in the same line not sharing common superscript letters were significantly different at $P<0.05$.

be observed in the presence of dietary cholesterol. Neutral steroid excretion also increased in the absence of cholesterol in the diet. The enhanced fecal neutral steroid output was ascribed largely to the increase in the excreta, whereas as to bile acids both the concentration and daily output increased, suggesting a net increase in the fecal excretion in response to dietary *trans*-fatty acids.

The type of dietary fat is likely to influence the concentration and composition of biliary bile acids and hence those of the excreta. Polyunsaturated fats in relation to the less unsaturated or saturated counterparts are generally believed to enhance biliary bile acid excretion (26,27). This action is thought to be one of the mechanisms by which polyunsaturated fats exert the hypocholesterolemic effect. Studies of Reddy et al. (28) with rats were, however, unable to demonstrate the effect of dietary fat type on the biliary bile acid concentration and composition, and it was the level of dietary fats that determined fecal as well as biliary bile acid excretion. Thus, enhanced bile acid excretion due to partially hydrogenated fat feeding appears to represent a specific biological action of *trans*-fatty acid rather than the effect of the differences in the degree of unsaturation of dietary fat.

Since hydrogenated fats stimulated fecal excretion of steroids, particularly on feeding a cholesterol-free diet, without influencing the concentration, composition and secretion rate of biliary steroids, it is reasonable to consider that hydrogenated fat feeding results in the reduction of bile acid reabsorption from the lower intestine. Accordingly, it seems plausible that *trans*-fatty acid stimulates the bile acid production in the liver. In swine, however, the specific activity of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase and cholesterol-7α-hydroxylase was not influenced by dietary fat blend of various proportion of *trans*-, *cis*- and saturated fats (29). Ramesha et al. (26) showed an enhancement of several parameters for cholesterol and bile acid synthesis by safflower oil compared with hydrogenated vegetable oils, though no data for *trans*-fatty acid content of the latter were given. The exact mechanism for the *trans*-fat induced increase in fecal steroid excretion remains to be

clarified.

The concentration of serum apo B and E, but not apo A-I, was modified by partially hydrogenated fats in some occasions. This raises the possibility that *trans*-fats alter the metabolism of triglyceride-rich lipoproteins. Apo B and E are known to be secreted into the circulation as components of triglyceride-rich lipoproteins (30). At present, it is indeed difficult to predict from the observed data alone how *trans*-fats affect the metabolism of these lipoproteins, since the concentration of serum apolipoproteins is determined by the rates of entry into and disappearance out of the circulation. However, from the commonly observed decrease in the concentration of hepatic triglyceride, it seems that hydrogenated fats at least modify the dynamics of triglyceride metabolism. Moreover, the response to *trans*-fats of serum apolipoproteins was rather different in two experiments with and without cholesterol. Dietary cholesterol also affect the metabolism of apolipoproteins (31).

The finding that *trans*-fats affected the fecal excretion of bile acids is of interest in the light of postulated relationship between carcinogenesis in the colon and dietary fat (32,33). It has been hypothesized that a high fat diet not only changes the composition of bile acids but also modifies the activity of gut microflora which, in turn, produces tumor-promoting substances from bile acids in the lumen of the colon (11,12). Thus, partial hydrogenation may also modify the carcinogenic activity of fat (34). Though the rate of bacterial transformation of cholesterol to coprostanol was invariable in both experiments, there was a cognitive difference in the fecal bile acid composition in particular in rats fed a cholesterol-containing diet. Even considering the possible difference in the rate of reabsorption of individual bile acids, the results strongly suggest modification of gut microflora due to dietary hydrogenated fats.

Partial hydrogenation yields a wide range of both geometric and positional isomers of unsaturated fatty acids (35). It is thus of interest to examine what type of *trans*-isomer is most effective to augment steroid excretion (36). Since in the present studies we tentatively compared the effect of partially hydrogenated fats to that of polyunsaturated fat from which hydrogenated products were prepared, the explanation of the results obtained should be restricted to a considerable extent. However, the observed effects of hydrogenated fats are at least likely to reflect the specific biological action of *trans*-fatty acids.

Available information indicates that the effect of *trans*-fats on the fatty acid metabolism is modified by the type of dietary fat simultaneously ingested (29,37). More systemic studies utilizing various fats with comparable fatty acid compositions are, therefore, currently in progress in our laboratory. These studies will hopefully lead toward a better understanding of the overall effect of *trans*-fatty acids on various parameters of cholesterol and bile acid metabolism.

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Sterols of the Sponge *Tethya amamensis:* Occurrence of (24E)-24-Ethylidenecholesta-5,7-dienol, (24E)-24-Propylidenecholesta-5,7-dienol, and (24Z)-24-Propylidenecholesta-5,7-dienol

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ABSTRACT

The sponge *Tethya amamensis*, collected from Kagoshima Bay, Japan, contained at least 24 sterols, including Δ^5-sterols (82.2% of total sterols) and $\Delta^{5,7}$-sterols (17.8%). The predominant sterols were cholesterol (29.0%), cholesta-5,22-dienol (13.8%), 24-methylcholesta-5,22-dienol (10.9%), 24-methylenecholesterol (8.3%), 24-methylcholesta-5,7,22-trienol (6.8%), 24-ethylcholest-5-enol (6.1%), and isofucosterol (4.1%). Combined gas liquid chromatography-mass spectrometry suggested the presence of 3 uncommon sterols, (24E)-24-ethylidenecholesta-5,7-dienol, (24E)-24-propylidenecholesta-5,7-dienol, and (24Z)-24-propylidenecholesta-5,7-dienol as minor components. The sterols of *T. amamensis* also contained small amounts of 24-norcholesta-5,7,22-trienol and (24Z)-24-ethylidenecholesta-5,7-dienol.
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INTRODUCTION

Marine invertebrates contain complex mixtures of sterols, for example as reviewed by Goad (1,2) and Schmitz (3). In particular, primitive invertebrates such as sponges and coelenterates contain a variety of types of sterols which often include unusual sterols with unprecedented side-chain alkylation patterns (4-19) or unconventional steroid ring systems (3,20). We have previously shown the diversity of sterol components of Japanese sponges (21), indicating the presence of a new C_{28} sterol, 3β-hydroxymethyl-24-methylene-A-nor-5α-cholestane in *Hymeniacidon perlevis* (22).

As part of our studies on marine sterols, we have examined the sterols of the sponge *Tethya amamensis* (class Demospongiae, order Hadromerina), paying attention to minor components and $\Delta^{5,7}$-sterols. We report the sterol composition of this sponge which includes 3 uncommon $\Delta^{5,7}$-sterols with C_{29} or C_{30} carbon atoms.

MATERIALS AND METHODS

Gas liquid chromatography (GLC) was performed on a Shimadzu GC-3BF with a hydrogen flame ionization detector and a column (2 m × 3 mm id) with 1.5% OV-17 as the liquid phase, operated at 260 C (23). Combined GLC-mass spectrometry (GLC-MS) was conducted on a Japan Electron Optics JEOL-JMS-300 unit as described previously (21): ionizing energy, 70 eV or 22 eV; GLC column, 3.0% OV-1 (2 m × 2 mm id) at 285 C. Ultraviolet (UV) absorption spectra were measured in methanol. Column chromatography on alumina (Brockmann grade III) with hexane-benzene or 20% (w/w) $AgNO_3$-silicic acid with hexane-benzene were carried out as described previously (21,24).

The specimens of *T. amamensis*, a small, round sponge (3 cm in diameter) with a reddish-pink color, were collected at a depth of 1.0-2.0 m in Kagoshima Bay, Japan, during May 1980. Kagoshima Bay is located in the southern part of Japan and is not highly polluted. The sponges (5.6 kg in fresh weight) were chopped and extracted with acetone 4 times immediately after sampling. The sponges analyzed probably contained some symbiotic organisms, because their surface had many tubers (ca. 2 mm in diameter, 1 mm in height) partitioned with narrow grooves. The acetone extract was saponified with 10% ethanolic KOH for 24 hr at room temperature. Crude sterols were isolated from unsaponifiable matter by alumina column chromatography and acetylated with pyridine acetic anhydride (1:1) for 24 hr at room temperature. The steryl acetates so obtained (6.0 g) were chromatographed on $AgNO_3$-silicic acid (350 g) with 1200 ml each of hexane, 10, 15, 20, 25, 27, 29, 33, 36, 40, 45, 50, 55, 60, 70, 80% benzene in hexane, and benzene. Eighty-three fractions were collected and analyzed by GLC and/or GLC-MS to identify the steryl acetates of each fraction. The details of procedures for isolation and identification of sterols were described previously (24).

RESULTS

Preliminary GLC on 1.5% OV-17 of the steryl acetates from *T. amamensis* showed 8 peaks. The prominent peaks gave relative retention times (RRT) identical with those of cholesta-5,22-dienyl, cholesteryl, 24-methylcholesta-5,22-dienyl, 24-methylenecholesteryl, 24-ethylcholesteryl, and isofucosteryl acetates. The UV spectra of the steryl

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acetate mixture showed absorption maxima at 260, 272, and 282 nm, indicating the presence of $\Delta^{5,7}$-steryl acetates (17.8% of total steryl acetates). The steryl acetates were fractionated by argentation column chromatography. The subsequent GLC and GLC-MS analyses of the fractions from argentation column chromatography made possible the identification of the sterols, including the minor components. Conventional sterols were identified by direct comparison of RRT in GLC on 1.5% OV-17 and 3.0% OV-1 and mass spectra with those of authentic samples (24,25). In this text, RRT are relative to cholesteryl acetate in GLC on 1.5% OV-17 unless otherwise specified. Table 1 shows the sterol composition of *T. amamensis* determined in the present study.

Fractions 11-20, eluted with 25-27% benzene in hexane by argentation column chromatography, afforded cholesteryl (RRT 1.00), 24-methylcholest-5-enyl (RRT 1.28), 24-ethylcholest-5-enyl (RRT 1.59), and 24-ethylcholesta-5,22-dienyl (RRT 1.41) acetates. Fractions 21-32, eluted with 29-33% benzene in hexane, gave 24-methycholesta-5,22-dienyl (RRT 1.14), cholesta-5,22-dienyl (RRT 0.94), and 24-norcholesta-5,22-dienyl (RRT 0.67) acetates. Fractions 33-43, eluted with 36-40% benzene in hexane, yielded isofucosteryl acetate (RRT 1.76) as a prominent component and small amounts of fucosteryl (RRT 1.66), desmosteryl (RRT 1.20), unknown C_{29} diene steryl (RRT 1.59), and 2 C_{30} steryl (RRTs 1.99 and 2.12) acetates. These C_{26}, C_{27}, C_{28}, and C_{29} sterols have been widely found in various phyla of marine invertebrates (1,2).

The C_{30} steryl acetates gave RRT in GLC and mass spectra (22 eV) identical with those of authentic (24E)-24-propylidenecholest-5-enyl (**3a**, RRT 1.98) and (24Z)-24-propylidenecholest-5-enyl (**4a**, RRT 2.10) acetates (Fig. 1), respectively, which had been isolated from the oyster *Crassostrea virginica* (24). The hydrolysis of the C_{30} steryl acetates with 5% ethanolic KOH gave free sterols, **3a** and **4a**, the mass spectra (70 eV) of which were similar to each other. The mass spectra of **3a** showed a molecular (M^+) at m/e 426 (15%, relative intensity) and other prominent ions at m/e 408 (4%, M^+ − HOH), 314 (10%, M^+ − C-23 to C-30 − 1H), 299 (14%, m/e 314 − CH_3), 296 (14%, m/e 314 − HOH), 281 (12%, m/e 314 − CH_3 − HOH), 271 (6%, M^+-R-2H, R = side chain), 255 (2%, M^+-R-HOH), 229 (8%, M^+ − R − 27 − HO), and 213 (5%, M^+ − R − 42 − HOH). The GLC and GLC-MS data indicate that the 2 C_{30} sterols from *T. amamensis* are (24E)-24-propylidenecholest-5-enol and (24Z)-24-propylidenecholest-5-enol.

After 24-methylenecholesteryl acetate (RRT 1.33 was eluted with 45-50% benzene in hexane, further elution with 55-80% benzene in hexane (fractions 55-81) gave a number of $\Delta^{5,7}$-steryl acetates (Table 1). During the argentation column chromatography, however, some $\Delta^{5,7}$-steryl acetates seemed to decompose, since the yield of $\Delta^{5,7}$-steryl acetates was low as compared with the quantity determined by UV spectral analysis of total sterols. Fractions 55-69 afforded 24-methylcholesta-5,7,22-trienyl acetate (RRT 1.26) as the major component and small amounts of cholesta-5,7-dienyl (RRT 1.13), 24-methylcholesta-5,7-dienyl (RRT 1.44), 24-eth-

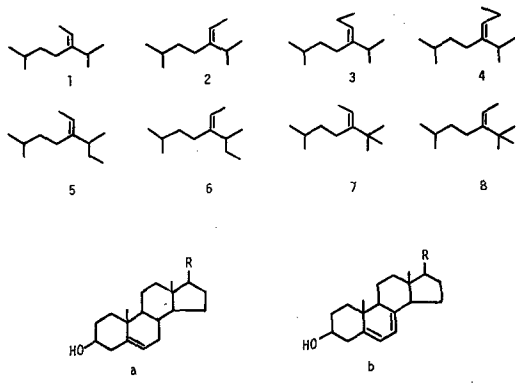

FIG. 1.

TABLE 1

Sterol Composition of the Sponge, *T. amamensis*

Sterol	RRT[a]	(%)
Δ^5-Sterols		
24-Norcholesta-5,22-dienol	0.77	1.4
Cholesta-5,22-dienol	0.94	13.8
Cholesterol	1.00	29.0
24-Methylcholesta-5,22-dienol	1.14	10.9
Desmosterol	1.20	0.7
24-Methylcholest-5-enol	1.28	2.8
24-Methylenecholesterol	1.33	8.3
24-Ethylcholesta-5,22-dienol	1.41	2.0
24-Ethylcholest-5-enol	1.59	6.1
Unknown C_{29} sterol	1.59	0.5
Fucosterol	1.66	1.1
Isofucosterol	1.76	4.1
(24E)-24-Propylidenecholest-5-enol	1.99	Trace
(24Z)-24-Propylidenecholest-5-enol	2.12	1.1
$\Delta^{5,7}$-Sterols[b]		
24-Norcholesta-5,7,22-trienol	0.77	1.4
Cholesta-5,7,22-trienol	1.06	2.6
Cholesta-5,7-dienol	1.13	1.8
24-Methylcholesta-5,7,22-trienol	1.26	6.8
24-Methylcholesta-5,7-dienol	1.44	0.6
24-Ethylcholesta-5,7,22-trienol	1.56	1.4
24-Ethylcholesta-5,7-dienol	1.77	1.1
(24E)-24-Ethylidenecholesta-5,7-dienol	1.88	1.3
(24Z)-24-Ethylidenecholesta-5,7-dienol	1.99	0.4
(24E)-24-Propylidenecholesta-5,7-dienol	2.22	Trace
(24Z)-24-Propylidenecholesta-5,7-dienol	2.38	0.4

[a]RRT in GLC on 1.5% OV-17 of sterols (as acetate derivatives).
[b]The quantity of $\Delta^{5,7}$-sterols was determined by UV spectrometry and GC.

ylcholesta-5,7-dienyl (RRT 1.77), cholesta-5,7,22-trienyl (RRT 1.06), and 24-ethylcholesta-5,7,22-trienyl (RRT 1.56) acetates. These $\Delta^{5,7}$-sterols have been widely encountered in molluscs (1,2,24) and sponges (26-28).

Fractions 69-72 contained a C_{26} steryl acetate along with small amounts of 24-methylcholesta-5,7,22-trienyl and cholesta-5,7,22-trienyl acetates. The C_{26} steryl acetate (RRT 0.77) was characterized as 24-norcholesta-5,7,22-trienyl acetate on the basis of mass spectral data (70 eV): m/e 410 (18%, M^+), 350 (100%, M^+ − AcOH), 335 (60%, M^+ − AcOH − CH_3), 309 (16%, M^+ − C-1 to C-3 − 1H − AcOH), 253 (95%), 251 (9%), 227 (20%), 226 (17%), 211 (45%), 157 (95%), 143 (90%), and 123 (52%). 24-Norcholesta-5,7,22-trienol has been isolated from the oyster *C. virginica* as a new sterol (25).

Fractions 73-81, eluted with 70-80% benzene in hexane, contained (24Z)-24-ethylidenecholesta-5,7-dienyl (**2b**, RRT 1.99) and 3 uncommon steryl acetates (**1b**, **3b**, and **4b**). The acetates of **1b** (RRT 1.88) and **2b** were slightly more polar in argentation column chromatography than those of **3b** (RRT 2.22) and **4b** (RRT 2.38). The mass spectrum of **2b** from *T. amamensis* was almost the same as that of (24Z)-24-ethylidenecholesta-5,7-dienol which has been isolated from the sponge *Dysidea herbacea* by Delseth et al. (27), as shown in Table 2. The component **1b** gave a mass spectrum similar to **2b** (Table 2). The mass spectrum of **1b** exhibited a molecular ion at m/e 410 corresponding to a C_{29} triene sterol and ions diagnostic for the $\Delta^{5,7}$-diunsaturated nucleus at m/e 351 due to the cleavage at C(1)-C(10) and C(3)-C(4) bonds plus one hydrogen transfer (29,30). The presence of the $\Delta^{5,7}$ bonds was also substantiated by the ions at m/e 128, 143, and 158 (31). The ions at m/e 312 due to the cleavage of C(22)-C(23) bond with one hydrogen transfer by McLafferty rearrangement (32), 294 (m/e 312 − HOH), and 279 (m/e 312 −HOH − CH_3), along with the ions at m/e 269, 253, and 211, were indicative of 24-ethylidene group. The RRT of **1b** acetate agreed with the calculated value (RRT 1.88) on the basis of the RRT of **1a**, **2a**, and **2b** acetates. Considering the above mentioned data on GLC and GLC-MS, the component **1b** was identified as (24E)-24-ethylidenecholesta-5,7-dienol which has not been isolated from natural products.

On the other hand, the acetates of **3b** (RRT 2.22) and **4b** (RRT 2.38) were identified as (24E)-24-propylidenecholesta-5,7-dienol and (24Z)-24-propylidenecholesta-5,7-dienyl acetates, respectively, on the basis of the RRT in GLC and mass spectral data. The RRT of **3b** and **4b** acetates almost agreed with the calculated values for **3b** (RRT 2.24) and **4b** (RRT 2.39), on the basis of the RRT of **1a**, **2a**, **3a**, **4a**, **1b**, and **2b** acetates. The mass spectra (22 eV) of **3b** and **4b** acetates were similar to each other. The mass spectrum of **3b** acetate gave a molecular ion at m/e 466 (10%) and other ions at m/e 406 (100%, M^+ − AcOH), 391 (40%, M^+ − AcOH − CH_3), 365 (4%, M^+ − C-1 to C-3 − 1H − AcOH), 294 (15%, MacLafferty rearrangement), 253 (9%), 226 (9%), 211 (13%), 158 (46%), and 143 (23%). Furthermore, the mass spectrum (70 eV) of **3b** gave a molecular ion at m/e 424 and other diagnostic ions at m/e 365 (M^+ − C-1 to C-3 − 1H), 312 (M^+ − C-23 to C-30 − 1H), 294 (m/e 312 − HOH), 279 (m/e 312 − HOH − CH_3), 157, and 143 for the sterol with $\Delta^{5,7,24,(28)}$ bonds (29-31), indicating similar cracking patterns with those of **1a** and **2a**. The mass spectrum of **3b** was similar to that of **4b**. This suggests that both

TABLE 2

Mass Spectral Data for the Sterols from the Sponge *T. amamensis*

Fragmentation	Sterol							
	1b		**2b**		**3b**		**4b**	
M^+	410	(100)*	410	(100)	424	(100)	424	(100)
$M^+ - CH_3$	395	(8)	395	(5)	409	(1)	409	(1)
M^+ − HOH	392	(16)	392	(26)	406	(11)	406	(14)
M^+ − HOH − CH_3	377	(75)	377	(86)	391	(79)	391	(86)
M^+ − C(1)-C(3) − 1H	351	(20)	351	(18)	365	(11)	365	(9)
M^+ − C(23)-C(29 or 30) − 1H	312	(3)	312	(5)	312	(5)	312	(6)
m/e 312 − HOH	294	(9)	294	(16)	294	(10)	294	(14)
m/e 312 − HOH − CH_3	279	(6)	279	(7)	279	(1)	279	(1)
M^+ − R; R = side chain	271	(11)	271	(8)	271	(8)	271	(4)
M^+ − R − 2H	269	(12)	269	(12)	269	(7)	269	(6)
M^+ − R − HOH	253	(16)	253	(22)	253	(22)	253	(31)
M^+ − R − 2H − HOH	251	(15)	251	(10)				
M^+ − R − 27 − HOH	226	(6)	226	(8)	226	(6)	226	(8)
M^+ − R − 42 − HOH	211	(18)	211	(23)	211	(8)	211	(10)
Other ions	157	(25)	157	(30)	157	(18)	157	(27)
	143	(36)	143	(51)	143	(34)	143	(55)

*Relative intensity (%).

compounds have a similar structure. Kokke and coworkers (8,13) have shown that the C_{30} diene sterols such as stelliferasterol, strongylosterol, verongulasterol, and 24-isopropenylcholesterol afforded mass spectra qualitatively identical but quantitatively slightly different from each other, indicating that all these sterols gave the 2 MacLafferty rearrangement peaks at m/e 314 and 328 which are indicative of the presence of Δ^{25}-bonds (12,33). The absence of a m/e 326 peak, corresponding to m/e 328 peaks in the mass spectra of **3b** and **4b**, excluded the possibilities of the side-chain structures like the 4 C_{30} sterols mentioned above. The side-chain structures of **5**, **6**, **7**, and **8** are also plausible alternatives for **3** and **4**. Although the failure to isolate sufficient amounts of **3b** and **4b** did not allow definative determination of the side-chain structures by nuclear magnetic resonance (NMR) spectrometry, the occurrence of various $\Delta^{5,7}$-sterols with the same side chains as those of Δ^5-sterols in *T. amamensis* suggests that the components **3b** and **4b** possibly have the same side chains as **3a** and **4a**, respectively. Therefore, **3b** and **4b** were tentatively identified as (24E)-24-propylidenecholesta-5,7-dienol and (24Z)-24-propylidenecholesta-5,7-dienol, respectively. The 2 C_{30} sterols have not been isolated from natural sources.

DISCUSSION

The sponge *T. amamensis* contained considerably large amounts of $\Delta^{5,7}$-sterols with C_{26} to C_{30} carbon atoms in addition to the Δ^5-sterols occurring commonly in marine environments. $\Delta^{5,7}$-Sterols are the major sterols in some microorganisms such as yeast and protozea, but they are generally present as only minor components in marine invertebrates except for some molluscan species (1,2). However, $\Delta^{5,7}$-sterols have been found as the principal sterols in the sponges belonging to the order Dictioceratida such as *Spongia nitens* (26), *Spongia officinalis* (26), *Ircinia muscarum* (26), *Ircinia spinosula* (26), *Dysidea avara* (26), *Dysidea herbacea* (27), *Axinella cannabini* (26), and *Axinella acuta* (26). The characteristic sterol compositions of the order Dictioceratida have been of interest from the viewpoint of chemotaxonomy. De Rosa et al. (26) have proposed that the family Spongidea can be discriminated from other sponges by the occurrence of large amounts of $\Delta^{5,7}$-sterols. It seems impossible to generalize on this criterion, however, as shown by the present study of *T. amamensis* (order Hadromerina) and by the work on *Biemma fortis* (order Poecilosclerida) by Delseth et al. (28).

During the present study, we have detected 3 uncommon sterols, (24E)-24-ethylidenecholesta-5,7-dienol (**1b**), (24E)-24-propylidenecholesta-5,7-dienol (**3b**), and (24Z)-24-propylidenecholesta-5,7-dienol (**4b**) in *T. amamensis*. The Δ^5-sterols with the same side chains as the above $\Delta^{5,7}$-sterols have been found in several marine invertebrates. (24E)-24-Ethylidenecholest-5-enol occurs widely in marine invertebrates among the minor components (1,2). (24Z)-24-Propylidenecholest-5-enol (**4a**) was first isolated from the scallop *Placopecten magellanicus* (34), and later from the sponges *Tethya aurantia* (35) and *Petrosia ficiformis* (17). Its 24E-isomer (**3a**) also occurs in the same scallop (36), the oyster, *C. virginica* (24), and the sponge *P. ficiformis* (17) as a minor component. Interestingly, Rohmer et al. (37) have demonstrated that a cultured Chrysophyte of the Sarcinocrisis group contained **4a** as the major sterol. The present study gives no clear answer of the origin of **1b**, **3b**, and **4b**. We assume that these sterols are possibly derived from either de novo synthesis or from the modification of corresponding Δ^5-sterols, **1a**, **3a**, and **4a** by the symbiotic organisms such as algae and fungi. The cooccurrence of Δ^5-sterols and $\Delta^{5,7}$-sterols with the same side chains in *T. amamensis* (Table 1) may give a little support to the latter assumption.

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Lipid Deterioration: β-Carotene Destruction and Oxygen Evolution in a System Containing Lactoperoxidase, Hydrogen Peroxide and Halides

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ABSTRACT

A model system containing lactoperoxidase/H_2O_2/halide decomposed β-carotene in a reaction greatly affected by the concentration of H_2O_2. The optimal concentrations of H_2O_2 for activation of iodide and bromide were 2 mM and 10 μM, respectively. The oxidation of chloride by a lactoperoxidase, using β-carotene destruction as a sensitive method to determine the activity of the enzyme, is reported herein. In the presence of optimal amounts of H_2O_2, the rate of β-carotene destruction increases slowly until a critical concentration of the halides, followed by a rapid increase in the rate when halide concentrations were furthere increased. A lactoperoxidase/H_2O_2/iodide and/or bromide system generates oxygen in the presence of high H_2O_2 and halide concentrations. β-Carotene inhibited the evolution of oxygen. A possible mechanism of β-carotene destruction and triplet unexcited oxygen evolution by a lactoperoxidase/H_2O_2/halide system are proposed.
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INTRODUCTION

The peroxidase catalyzed peroxidation of halides is established as being an important step in the biosynthesis of the hormone thyroxine (1,2) and in biological defense mechanisms (3-5). More recently, these peroxidase systems have also been shown to be toxic to tumor cells (6-8).

Singlet oxygen, a reactive excited oxygen, has frequently been postulated as the principal microbiocidal agent, primarily on the basis of experiments with singlet oxygen trapping agents and chemiluminescence of these systems (9,10). Piatt and O'Brien (10) found a great similarity between the activity of a lactoperoxidase (LPO)/H_2O_2/Br^- system and that published previously by Rosen and Klebanoff (9) for the chemiluminescence of myeloperoxidase (MPO). Recently, it has been demonstrated that most $^1O_2$ trapping agents are not $^1O_2$ specific and can react with a variety of oxidants including HOCl (11-13) and that the chemiluminescence derived from peroxidase/H_2O_2/halide system is not specific to singlet oxygen. Therefore, the postulation that singlet O_2 is involved in initiating the killing process must now be questioned.

Early studies postulated that the MPO/H_2O_2/Cl^- system could generate HOCl (14,15); however, the presence of this compound and chlorine were only recently confirmed (16). The HOCl or halide equivalents are now believed to be the microbiocidal agent (17,18) derived from activated neutrophils in the MPO/H_2O_2/Cl^- system, or $OSCN^-$ derived from the LPO/H_2O_2/SCN^- system (19,20).

Peroxidase catalyzed peroxidation of halogens has been recognized also as a valuable tool for the study of proteins, their cellular location, and metabolism. In several studies, it has been shown that LPO catalyzed iodination of intact cells labels not only surface proteins, but also several classes of membrane lipids (21-23).

The interaction of active halogen compounds with lipids may be of general biological significance since there are many peroxidases with the capacity to peroxidize halogens, e.g., peroxidase from salivary, mammary, thyroid glands and ooctyes, and the MPO which is produced by neutrophils and eosinophils (2).

The mechanism of peroxidase catalysis can be accommodated within the classic concepts of Chance (24) and George (25). This implies that the initial steps of the reaction invariably involve oxidation of peroxidase by peroxides and that the resulting derivative (compound I) is the oxidant of the halide ions. Such a reaction may result either in the formation of free halogens, hypohalous acids, halogenation of the enzyme apoprotein, decarboxylation of proteins, oxidation of reducing compounds and SH groups (2,26) or oxygen evolution (10).

Although the peroxidase generated compound I oxidizes halides at different rates (I^- > Br^- > Cl^-), compound I formed by different peroxidases may have different redox potentials. All peroxidases studied were found to catalyze the oxidation of iodide; MPO oxidized chloride, while horseradish peroxidase and LPO were never observed to oxidize chloride, and none of these enzymes was capable of oxidizing fluoride (2)

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In our previous study (27), we developed a sensitive method to determine the cooxidation of β-carotene by an activated halide using myeloperoxidase from fish leukocytes. The sensitivity of the method is in the range of one nmol of β-carotene.

In this study, we demonstrated the facile cooxidation of β-carotene by a LPO/H_2O_2/halide (I^-, Br^-, Cl^-) model system and determined some characteristics of the reaction.

MATERIALS AND METHODS

Materials

Hydrogen peroxide (30%), sodium chloride, potassium bromide, sodium acetate, and the potassium mono- and diphosphate were purchased from Mallinckrodt (St. Louis, MO). The LPO (60-80 Sigma units/mg), β-carotene, pyrogallol, purpurogallin and sodium iodide were from Sigma Chemical Co. (St. Louis, MO).

Methods

Carotene destruction. The discoloration of β-carotene was used to determine the cooxidation reaction resulting from the oxidation of halide ions by the LPO/H_2O_2 complex. The oxidative equivalents derived from the halides could cooxidize other molecules. We developed a method based on the destruction of β-carotene, (28).

Briefly, the technique consists of monitoring the decrease in absorbance at 460 nm in a cuvette containing the enzyme system. The sample contained 1.5 ml of buffered carotene solubilized using Tween-20, 0.1-0.4 ml active fractions (enzyme, H_2O_2 and halides), and distilled water to a final volume of 2.0 ml. The concentrations of the initial reaction mixture were: β-carotene, 14 μM; Tween 20, 0.05%; and sodium acetate buffer, 0.1 M. The sample in the control cuvette contained all the reagents except β-carotene. The initial linear rate of decrease in absorbance was computed from a recorder tracing and converted into the rate of carotene disappearance in nmol/min.

Protein concentration was determined by the Lowry procedure (29) using bovine serum albumin as standard.

The LPO concentration was calculated from an extinction coefficient of $E^{mM}_{412nm} = 114$ (30).

Oxygen evolution. Oxygen generation from a reaction mixture containing similar concentrations of reactants as the model system for β-carotene destruction was monitored with a Clark-type oxygen electrode at 25 C in a reaction vessel of 2.0 ml capacity. The initial rate of oxygen evolution was recorded and converted into nmol O_2 evolved.

RESULTS

The rate of β-carotene destruction by the LPO/H_2O_2/halide system depended on the concentration of H_2O_2 but varied with each halide. The optimal concentration of H_2O_2 for activation of LPO in the presence of I^-, Br^- and Cl^- were 2 mM, 5 and 10 μM, respectively. The maximal rate of β-carotene destruction for every halide was determined at concentrations of hydrogen peroxide that were neither limiting nor inhibitory (Fig. 1).

The dependence of LPO activity on the concentration of halide ions is shown in Figure 2. It was found that, in the presence of the optimal amount of H_2O_2, the rate of β-carotene destruction was first order at low concentration of the halide (2-5 μM), followed by a rapid increase in the rate when halide concentration was further increased (10-50 μM).

The rate of β-carotene destruction was found to have a pH optimum of 5.0 in the presence of iodide and 4.0 in the presence of Br^- and Cl^- ions (Fig. 3).

The effect of LPO concentration in the presence of optimal concentration of H_2O_2 and halides on β-carotene destruction is shown in Figure 4.

The enzyme preferentially oxidized iodide and was significantly less effective with bromide and chloride. However, in the presence of 1 mM tryptophan, β-carotene destruction by the LPO/H_2O_2/chloride system increased almost 16-fold. This particular effect was observed only with chloride ions. Other amino acids were tested as activators or inhibitors of the reaction of LPO/H_2O_2/NaCl. Serine stimulated β-carotene destruction almost 3-fold, and only cysteine, glutathione, methionine, or histidine inhibited β-carotene destruction (data not shown). Cysteine and gluthathione produced a total inhibition for a very short time (induction period), which depended on the concentration of both of these compounds in the reaction mixture (Kanner and Kinsella, in preparation). β-Carotene destruction by the LPO/H_2O_2/halide system was not inhibited by antioxidants such as BHT or BHA.

In the presence of relatively high amounts of H_2O_2 and I^- or Br^- ions LPO generated oxygen. The rate of oxygen evolution in the presence of these two halides is shown in Figures 5 and 6. It was found that only iodide and bromide stimulated the production of oxygen. No oxygen evolution was obtained during the interaction of LPO system in the presence of chloride.

β-Carotene inhibited and even eliminated the evolution of oxygen. This inhibition was less affected in a system containing iodide than in those containing bromide ion (Figs. 5 and 6).

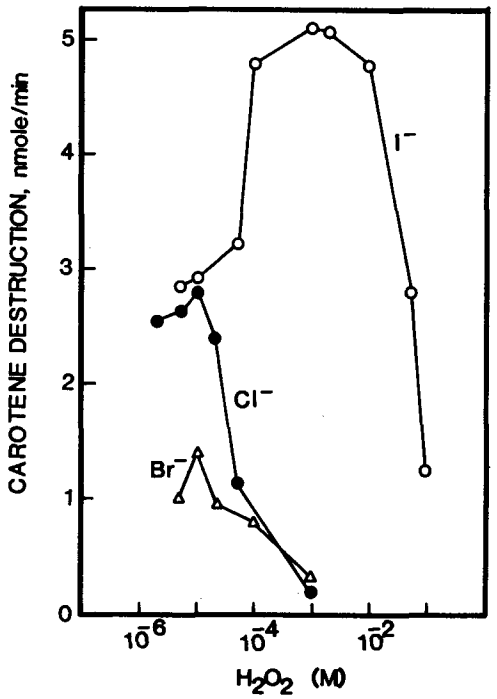

FIG. 1. The effect of H_2O_2 concentration on β-carotene destruction by a LPO/H_2O_2/halide system. Reaction mixture contained LPO (160 nM), iodide (5 μM), bromide (5 μM) or chloride 225 mM, β-carotene (14 μM), Tween 20 0.05% in 2 ml acetate buffer (0.1 M), pH 4.0 at 25 C., (n = 3).

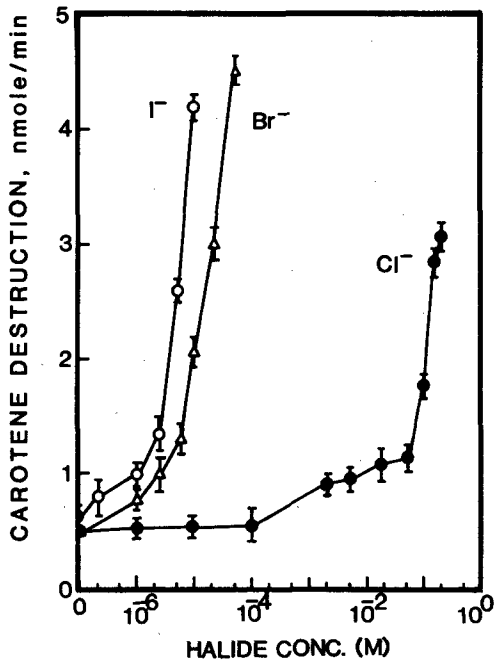

FIG. 2. The effect of halide concentration on β-carotene destruction by a LPO/H_2O_2/halide system. Reaction mixture contained LPO (180 nM), H_2O_2 (for iodide 100 μM; for bromide and chloride 6.2 μM), β-carotene and other conditions as Figure 1. Error I bars denote standard deviation (n = 3).

DISCUSSION

The LPO/H_2O_2/halide system cooxidizes β-carotene in a reaction strongly affected by the concentration of H_2O_2. The optimal concentrations of H_2O_2 were high (i.e., 2 mM) with iodide ions and low with chloride and bromide (10 μM).

It has not previously been reported that LPO oxidizes chloride ions. These results were obtained by using a sensitive method to identify the destruction of the chromaphore, e.g., less than 1 nmol of β-carotene destruction was enough to identify significant differences (in 2-ml reaction mixture). The optimization of the system, especially with respect to H_2O_2 concentration, enabled us to observe the condition under which such oxidations occurred. The decrease in the activity of the peroxidase at high H_2O_2 concentration was previously observed using MPO (9,10,15,27,31) or LPO (32,33). This may be caused by the generation of the peroxidase compound III (34,35) which is almost 1/300- as active as compound II (36). It is also possible that compound III is inactivated more rapidly by the halide oxidizing species. The inactivation of MPO by the byproducts of its reaction with Cl^- have been observed by others (37,38).

In the presence of the optimal amount of H_2O_2, the rate of β-carotene destruction was first order for low concentrations of the halides followed by a rapid increase in the rate when halide concentration further increased. These results could be explained from the data presented by Naskalski (37) and Matheson et al. (38), in which high concentrations of the halide prevented autoinactivation of the enzyme. In our system, increasing halide concentration not only increased the rate of the reaction but may also have prevented the inhibition of the enzyme by byproducts, such as HOCl.

The rate of β-carotene destruction showed a pH optimum of 5.0 with iodide and maximal activity of 4.0 in the presence of Br^- and Cl^- ions. The decrease in activity accompanying changing pH was low with iodide and chloride; but much greater in the presence of bromide, when pH was shifted from pH 4.0 to 5.0. Several researchers reported that the optimal pH for halide oxidation by MPO or LPO is between 4.0 and 4.5 (9,10). However, other researchers found that the optimal pH for the cytotoxic effect of MPO is in the range of 6.5-8.0 (8,39,40). At

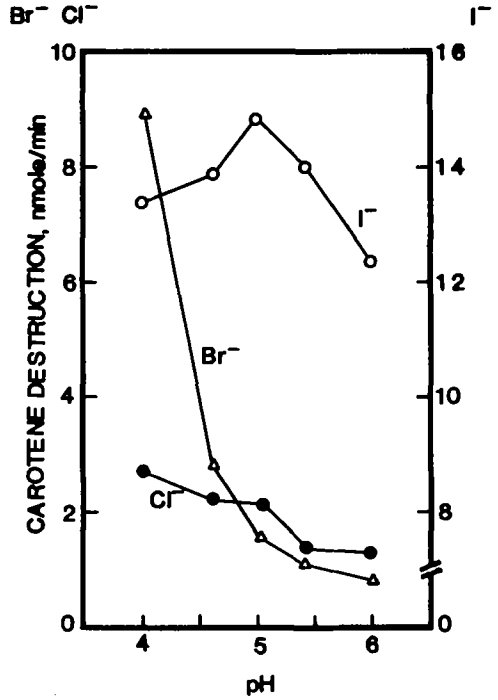

FIG. 3. The effect of pH on β-carotene destruction by LPO/H_2O_2/halide system. Reaction mixture contained LPO (145 nM), iodide (25 μM), bromide (25 μM), chloride (225 mM), H_2O_2 (for iodide and bromide 100 μM, for chloride 10 μM), β-carotene and other conditions as in Figure 1.

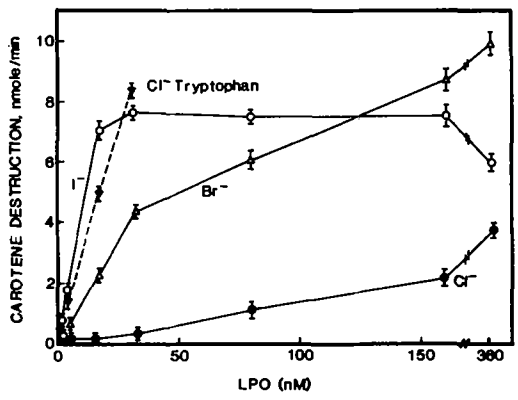

FIG. 4. The effect of LPO on β-carotene destruction by LPO/H_2O_2/halide system. The reaction mixture contained iodide (25 μM), bromide (25 μM), chloride (225 mM), tryptophan (1 mM), and H_2O_2 (for iodide and bromide 15 μM; for chloride 5 μM), β-carotene and other conditions as Figure 1. Error I bars denote standard deviation (n = 3).

low H_2O_2 concentrations (39) and at high concentration of the halides (38,40), the optimum pH shifted to a more alkaline pH.

The LPO/H_2O_2/Br^- system generated oxygen (10). In our model system the evolution of oxygen was observed in the presence of high H_2O_2 concentration and with iodide and bromide only. No evolution of oxygen occurred during the interation of LPO system with chloride ions. β-Carotene inhibited the evolution of oxygen and this inhibition was less with iodide than with bromide ions.

We conclude that one of the possibilities is that the inhibition is produced by the action of β-carotene with halide radicals or hypohalites, which prevented the interaction of these compounds with H_2O_2 and the subsequent generation of O_2. More recently, it was reported that chloroperoxidase catalyzed the peroxidation of chloride and bromide ion to molecular chlorine and bromine (34). The same results were published by Virion et al. (34) and Harrison and Schultz (16) for tyroid peroxidase and MPO, respectively. The mechanism of interaction of β-carotene with halide radicals has been discussed (27).

Following several studies with chemiluminescence, singlet oxygen quenchers or trapping agents (9,10,41), the evolved oxygen was tentatively identified as singlet oxygen.

The mechanism for the formation of $^1O_2$ was based in the action of hypochlorite, which is formed during the enzymatic reactions with H_2O_2 (16).

$$H_2O_2 + Cl^- \xrightarrow{\text{peroxidase}} {}^-OCl + H_2O \quad [1]$$

$$^-OCl + H_2O_2 \longrightarrow H_2O + Cl^- + {}^1O_2 \quad [2]$$

Recently, it has been realized that most of the $^1O_2$ traps are nonspecific and can react with a variety of oxidants, including HOCl (11-13).

LPO/H_2O_2/halide system emits light at the range of 433 nm, but this is not specific for singlet oxygen (10) and H_2O_2/Cl_2 does not produce chemiluminescence in acid media in the range of $^1O_2$ emission (42).

β-Carotene acts as an efficient singlet oxygen quencher in nonaqueous solution without itself being oxidized (43). If singlet oxygen was generated in our model system and β-carotene acts as a quencher, then the evolution of oxygen should not be inhibited and even should increase. However, we found that β-carotene inhibited oxygen evolution and during this reaction the β-carotene molecule was destroyed. It was also shown that β-carotene in micellar water dispersion failed to quench singlet oxygen (44).

Our data support the previous conclusion

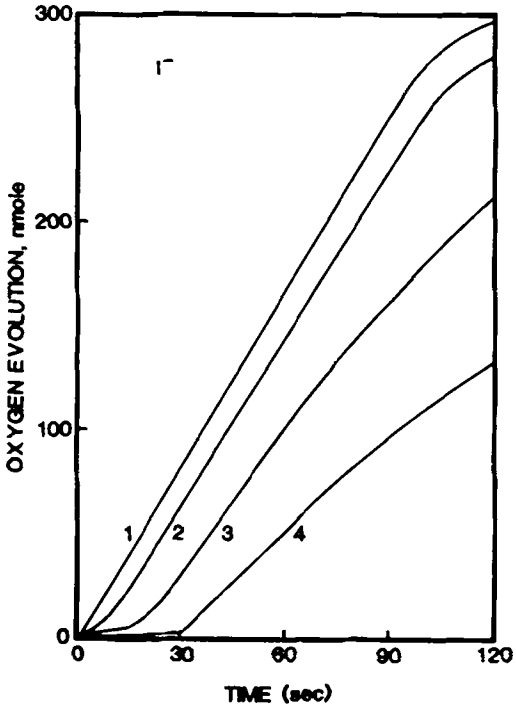

FIG. 5. Oxygen evolution by a LPO/H_2O_2/iodide system. I-control; 2, 3, 4, in the presence of β-carotene 3.25, 6.5 and 13.0 μM, respectively. Reaction mixture contained LPO (205 nM), H_2O_2 (50 μM), iodide (50 μM), Tween 20, 0.05% in 2 ml acetate buffer 0.1 M, pH 4.5 at 25 C.

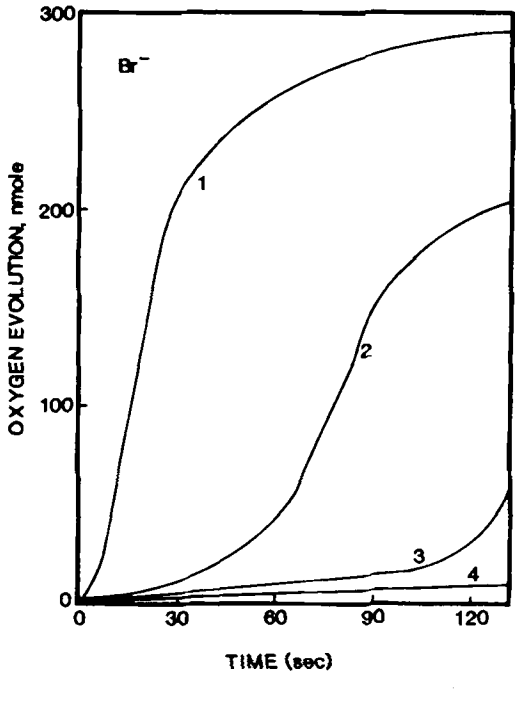

FIG. 6. Oxygen evolution by a LPO/H_2O_2/bromide system. I-control; 2, 3, 4 in the presence of β-carotene of 3.25, 6.5 and 13.0 μM, respectively. Reaction mixture contained LPO (205 nM), H_2O_2 (500 μM), bromide (500 μM), Tween 20 0.05% in 2 ml acetate buffer 0.1 M pH 4.5 at 25 C.

that, in the LPO/H_2O_2/halide system, β-carotene behaves as a halide ($X^{\cdot}$) quencher or trapper (27) and by this activity it prevents the interaction of halide radicals with a second molecule of H_2O_2.

We propose that the evolution of triplet unexcited oxygen could be generated in our model system by the following reactions:

$$LPO + H_2O_2 + 2X^- \xrightarrow{2H^+} LPO + 2H_2O + 2X^{\cdot} \quad [3]$$

$$2X^{\cdot} + 2H_2O_2 \rightarrow 2HO_2^{\cdot} + 2X^- \quad [4]$$

$$HO_2^{\cdot} + HO_2^{\cdot} \rightarrow H_2O_2 + O_2 \quad [5]$$

(LPO = lactoperoxidase; X- = halide ion or $X^{\cdot}$ = halide radical; $HO_2^{\cdot}$ = perhydroxyl radical).

The stoichiometry of this pathway is in agreement with the results published by Piatt and O' Brien (10) in which the reaction generated 0.5 mol oxygen/mol of H_2O_2 consumed. It is well known that chlorine radicals, molecular chlorine or hypochlorous acid can oxidize H_2O_2 (42). This one electron oxidation by a chlorine radical could initiate perhydroxyl radicals which by dismutation will produce triplet oxygen and H_2O_2 (45). We showed that the peroxidation of chloride ion occurred only in the presence of very low amounts of H_2O_2 where the oxygen O_2 evolved was very low. Increasing the concentration of the H_2O_2 inhibited the reaction. Both of these observations could explain the inability of the system to produce oxygen in the presence of chloride ion.

This study shows by using a sensitive method of β-carotene destruction, that LPO could oxidize the chloride ion. β-Carotene could be destroyed very rapidly by the activated halide. The capacity of β-carotene to inhibit oxygen evolution during the reaction seems to arise from its ability to quench or trap chlorine or other activated halide radicals and thereby prevent their interaction with other functional molecules.

Krinsky (46) showed that a mutant microorganism devoid of carotenes was rapidly killed by polymorphonuclear leukocytes, unlike the wild-type strain containing carotenoids. Our work has shown that β-carotene seems to be a very efficient halide quencher. The methods we used are sensitive enough to detect destruction of one double bond; however, based on the high unsaturation of β-carotene (11 double bonds and 8 allylic hydrogens), we hypothesize

that more than one site is available for radical attack. If this hypothesis is true, then it seems that carotenoids and vitamin A may function, in vivo, as protectors against free radical destruction of membranes.

Using this system, (LPO/H_2O_2/halides) under optimal conditions, we have demonstrated the cooxidation of linoleate, which will be described in a subsequent paper (47).

ACKNOWLEDGMENTS

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Initiation of Lipid Peroxidation by a Peroxidase/Hydrogen Peroxide/Halide System

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ABSTRACT

A lactoperoxidase/H_2O_2/halide system caused the initiation of linoleate peroxidation as indicated by diene conjugation. Coupled lipid peroxidation was accelerated by iodide, chloride and bromide ions at pH 4.0 and 6.2. No peroxidation occurred in the presence of H_2O_2 or lactoperoxidase alone. The rate of linoleate peroxidation by lactoperoxidase in the presence of chloride depended on the concentration of H_2O_2. Linoleate peroxidation by the enzymatic system was inhibited by high concentration of H_2O_2 by methionine, tryptophan and BHT. Oxygen was absorbed during peroxidation and the major products were the 13-hydroperoxides. The mechanisms of the initiation of lipid peroxidation by a peroxidase/H_2O_2/halide system are discussed.
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INTRODUCTION

Oxidation of polyunsaturated fatty acids in living tissues causes membrane damage and cellular death (1). Lipid peroxidation has been implicated in mammalian cell injury caused by chemicals such as CCl_4, various oxidants, hemolytic agents, and air pollutants such as NO_2 (2).

The oxidation of lipids in foods is one of the most important and complex deteriorative reactions. These reactions develop rancid off-flavors, cause destruction of vitamins and pigments, reduction of the biological value of proteins, and can ultimately result in accumulation of compounds toxic to mammalian cells (3).

With regard to lipid oxidation, one of the most important questions concerns the source of the primary radicals that initiate peroxidation in vivo and autoxidation in vitro.

The initiation of lipid peroxidation by reactive oxygen species has been reported by several authors (4-8) and reviewed by Tien et al. (9). Recently, it was stated that the perhydroxide radical ($HO_2^{\cdot}$) acts as an efficient chain initiator in forming the allylic linoleic acid radical (10). The mechanisms by which the NO_2 radical initiates lipid peroxidation were presented recently (11).

During phagocytosis, phagocytic cells generate powerful oxidizing agents by the partial reduction of oxygen to superoxide radical and hydrogen peroxide (12-14). Evidence for the formation of the hydroxyl radical has also been presented (15,16). Those oxygen species have been implicated as the microbicidal agents in phagocytosis. The microbicidal activity of H_2O_2 was found to be considerably increased by myeloperoxidase (MPO) and a halide (17-19) by a mechanism which involved chlorine and hypochlorite as the killing factors (14,20).

MPO has many properties similar to those of lactoperoxidase (21-23) and we showed that both MPO and lactoperoxidase (LPO) cause β-carotene destruction, probably via an initial addition mechanism (24,25).

During phagocytosis, lipid peroxidation occurs (26,27) possibly via a free radical mechanism generated by the peroxidase/hydrogen peroxide (H_2O_2) system. In vitro, a peroxidase/H_2O_2/halide system rapidly degrades β-carotene (25). In the present paper, we report the capacity of this system to cause peroxidation of linoleic acid.

MATERIALS AND METHODS

Materials

Hydrogen peroxide (30%), sodium chloride, potassium bromide, sodium acetate, acetic acid, tetrahydrofuran and acetonitrile were purchased from Mallinckrodt (St. Louis, MO). The LPO (60-80 units/mg), lipoxygenase (type I from soybean containing 150,000 units/mg) linoleic acid (18:2), sodium iodide, Tween 20, butylated hydroxytoluene (BHT), tryptophan and methionine were from Sigma Chemical Co. (St. Louis, MO). Ethyl acetate and hexane were

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purchased from Fisher Scientific Co. (Fair Lawn, NJ) and Silica Gel 60H from E. Merck (Darmstadt, West Germany).

Methods

LPO/H_2O_2/halide model system was previously described fully (25). A typical assay contained 0.1 ml of enzyme (50-500 nM LPO), 0.1 ml H_2O_2, 0.1 ml halide solution in 1.7 ml of sodium acetate buffer (0.05 M) and incubated at 25 C.

Linoleate diene conjugation. Linoleic acid (18:2) was purified according to a modification of the method developed by Gardner (28). The linoleic acid (18 mg) was dissolved in hexane containing 1% acetic acid, layered on the top of a mini column (0.5 x 5.0 cm packed with Silica Gel 60 H), and eluted with 7 ml of the same solvent system. The fraction eluted from the column, i.e., pure 18:2, was reduced to dryness under vacuum at 20 C.

The assay of linoleate diene conjugation was done as described (29). The reaction mixture contained 18:2 (200 μM) and Tween-20, (0.005%), in a 0.1 M buffer acetate at pH 4.0 and 6.2. The initial rate of increase in absorbance was measured with a Cary DB-19 UV-visible spectrophotometer, computed from a recorder tracing and converted into nmol of conjugated dienes using a molar extinction coefficient of 25,250 at 233 nm (30). The results are the mean of triplicates and, in the figures, the error I bars deonte standard deviation.

Oxygen absorption. Oxygen uptake from a reaction mixture containing similar concentration of reactants as the model system for linoleate diene conjugation was monitored with a Clark-type oxygen electrode at 25 C in a reaction vessel of 2.0 ml capacity. The initial rate of oxygen uptake was recorded and converted into nmol O_2.

The LPO concentration was calculated from an extinction coefficient of $E^{mM}_{412nm} = 114$ (31).

Separation of acylhydroperoxides by high pressure liquid chromatography (HPLC). Aqueous dispersions of linoleate were incubated in the presence of various prooxidants. After incubation, these were prepared for HPLC by extraction with 1.5 vol of ethyl acetate containing 0.005% BHT. Each system (2 ml) was extracted twice. After each addition of ethyl acetate, the mixture was mixed in a thermolyne specimix (Supelco, Inc., Bellefonte, PA) for 10 min. The aqueous and organic solvent layers were separated by centrifugation at 7000 rpm for 5 min. The top ethyl acetate layer was transferred to scintillation vials and evaporated to dryness by nitrogen at room temperature (32). The lipids were redissolved in 1 ml of tetrahydrofuran/acetonitrile/H_2O (25:35:45, vol/vol) and 10 μl from this solution was injected onto the HPLC column. The fatty acid hydroperoxides were separated by HPLC (Waters Associates Inc., Millford, MA) using 30 cm x 40 mm μ-Bondpak's packed fatty acid (Waters Associates) column. The hydroperoxides were eluted with a mobile phase identical to the solvent mixture used for dissolving the lipids. The flow rate was 1 ml/min. The conjugated dienes separated from the column were detected at 233 nm with a Waters UV detector sensitive to 0.002 difference in absorbance; the response was recorded continuously.

RESULTS

LPO/H_2O_2/halide system initiated linoleate diene conjugation. This was obtained in the presence of iodide, chloride and bromide ions at both pH 4.0 and 6.2 (Fig. 1). The optimal conditions for activation of the system were as found previously when studying β-carotene destruction by the same system (25). No peroxidation occurred when H_2O_2 or the enzyme was omitted. A significantly lower rate and extent of peroxidation (ca. 25%) were obtained when the system contained the enzyme and hydrogen peroxide but lacked the halide. The rate of linoleate peroxidation by LPO in the presence of chloride ions was dependent on the concentration of H_2O_2. The optimal rate of peroxidation was found between 5 and 10 μM H_2O_2. Increasing the concentration of H_2O_2 to 500 μM strongly inhibited the oxidation (Fig. 2).

The rate of linoleate peroxidation increased with increasing enzyme concentration up to 250 nM (Fig. 3). Conjugation of 18:2 by the LPO/H_2O_2/Cl^- system was inhibited in the presence of 20 μM of methionine, tryptophan or BHT, by 50, 80 and 90%, respectively (Fig. 4). Lipid peroxidation was inhibited also by other amino acids like alanine, glycine and valine, but only at concentrations above 1 mM.

During conjugation in the system containing LPO, H_2O_2 and chloride ions, a significant absorption of oxygen occurred. After an induction period of 2 min the level of oxygen decreased by 60 nmol during 10 min. The enzyme in the presence of only H_2O_2 also generated oxygen but to a lower extent. A similar effect was found during the formation of conjugated dienes. No absorption of oxygen occurred in the other controls (Fig. 5).

HPLC was performed to separate and identify the conjugated diene compounds formed after autoxidation of pure linoleic acid, of

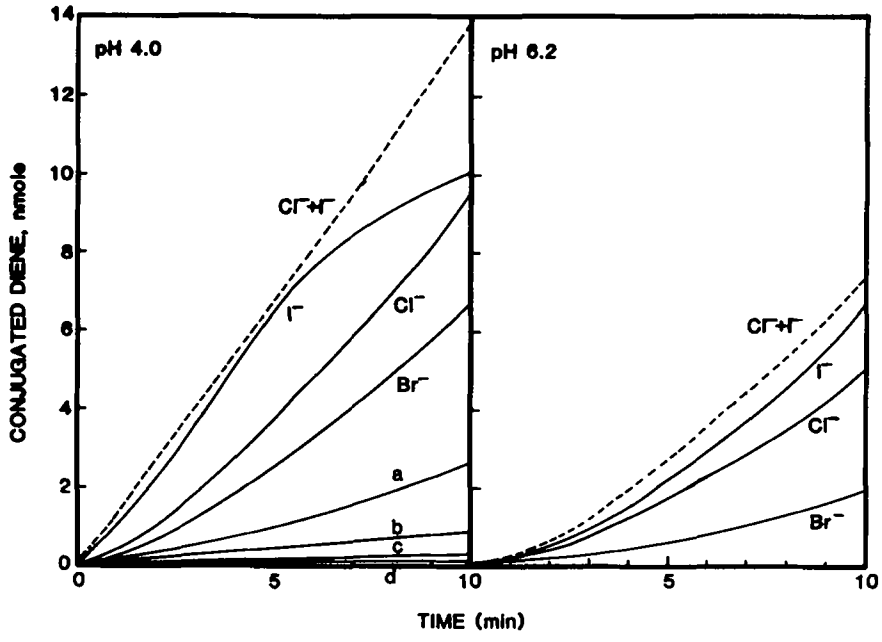


FIG. 1. Diene conjugation of linoleate by an LPO/H_2O_2/halide system. (a) LPO and H_2O_2; (b) LPO; (c) LPO and halides; (d) H_2O_2 or halides. Reaction mixture contained LPO (150 nM), iodide (50 μM), bromide (500 μM) and chloride (225 mM), H_2O_2 concentration for iodide and bromide (200 μM) and for chloride (10 μM), linoleic acid (200 μM), Tween-20 0.005% in 2 ml of 0.1 M buffer acetate, pH 4.0 or 6.2.

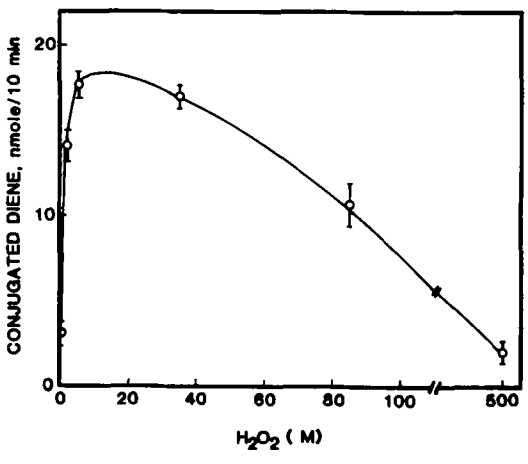


FIG. 2. The effect of H_2O_2 concentration on diene conjugation of linoleate by an LPO/H_2O_2/Cl^- system. The reaction mixture contained LPO (220 nM), chloride (225 mM), linoleic acid (200 μM), Tween-20 0.005% in 2 ml of 0.1 M buffer acetate, pH 4.0 or 6.2. Error I bars denotes standard deviation (n = 3).

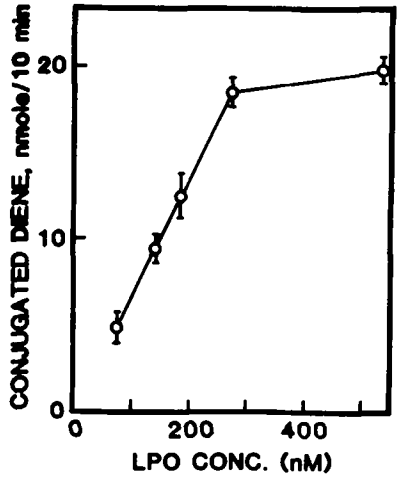


FIG. 3. The effect of LPO concentration on linoleate diene conjugation of linoleate by an LPO/H_2O_2/Cl^- system. The reaction mixture contained H_2O_2 (10 μM), chloride (225 mM), linoleic acid (200 μM), Tween-20 0.005% in 2 ml of 0.1 M buffer acetate, pH 4.0. Error I bars denotes standard deviation (n =3).

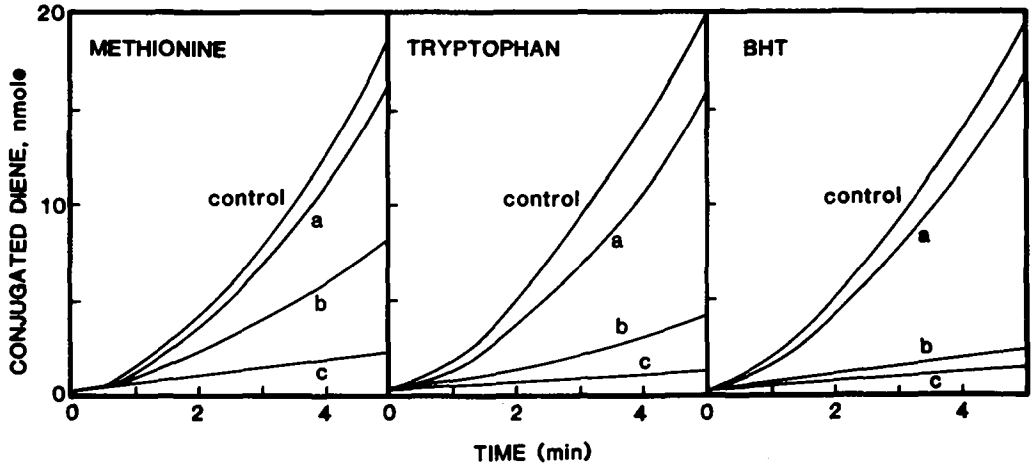


FIG. 4. Diene conjugation of linoleate by an LPO/H_2O_2/Cl^- system in the presence of tryptophan and BHT. The reaction mixture contained LPO (320 nM), H_2O_2 (15 μM), chloride (225 mM), methionine, tryptophan and BHT were (a) 2 μM, (b) 20 μM; (c) 200 μM, linoleic acid (200 μM), Tween-20 0.005% in 2 ml of 0.1 M buffer acetate, pH 4.0.

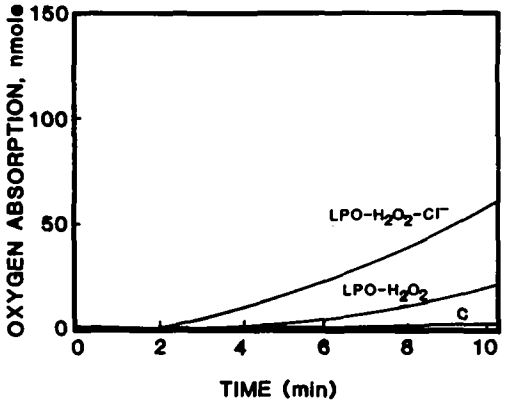


FIG. 5. Oxygen absorption in the presence of LPO/H_2O_2/Cl^- system and linoleate. The reaction mixture contained LPO (220 nM), H_2O_2 (10 μM), chloride (225 nM), linoleic acid (200 μM), Tween-20 0.005% in 2 ml of 0.1 M buffer acetate, pH 4.0; (c) controls of LPO/H_2O_2/Cl^-; LPO/Cl^-, H_2O_2 and Cl^-, or the same controls without linoleate.

aqueous linoleate dispersions, and of the system containing linoleate activated by LPO/H_2O_2/Cl^- or lipoxygenase at 25 C (Fig. 6). The separation of the conjugated dienes by HPLC revealed that autoxidation of bulk linoleic acid resulted in one major peak, the retention time (8.5 min) of which was the same as that of the peroxidized product formed in the system activated by the LPO. Soybean lipoxygenase type A preponderantly generates *cis-trans* 13-hydroperoxide (28). The freshly dispersed aqueous linoleate solution showed only traces of a peak that coincided with the 9-hydroperoxide. This peak increased significantly after and incubation of the solution at 25 C for 2 hr. After prolonged incubation, 2 additional peaks were obtained, one with a retention time corresponding to the 13-hydroperoxide (13-ROOH). The LPO/H_2O_2/Cl^- system resulted in the formation of mainly the 13-hydroperoxide (and another minor peak with a retention time of 7 min). This minor peak could be a *trans-trans* isomer of the 13-hydroperoxide (33). Increasing incubation time to 15 min resulted in the generation of a peak with a retention time similar to that formed during the incubation of the control aqueous linoleate solution, i.e., 13-ROOH (Figs. 6A and B).

DISCUSSION

In an earlier study, we showed very rapid destruction of β-carotene by a fish leukocyte MPO/H_2O_2/halide system (24). To test the hypothesis that during the phagocytosis process, an MPO/H_2O_2/halide could initiate lipid peroxidation, we performed experiments with LPO which behaves like MPO (23,34,35) and is available commercially as a pure enzyme. LPO can peroxidize chloride ions (25), an activity which was known to be unique for MPO and chloroperoxidase (21,36).

LPO in the presence of optimal concentration of H_2O_2 and halides cooxidized linoleate to conjugated diene compounds. The highest activity was found for iodide followed by chloride and bromide. The cooxidation of linoleate decreased almost 30-50% when the pH of the system was changed from 4.0 to 6.2. A

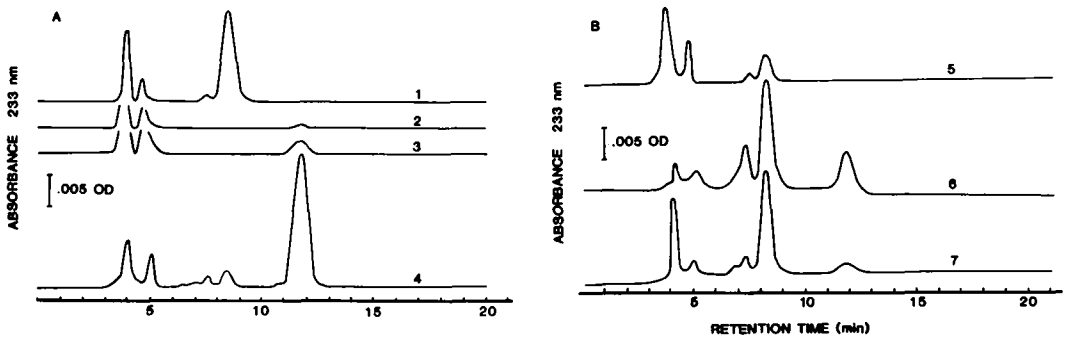


FIG. 6. HPLC of diene conjugated compounds derived from the interaction of LPO/H_2O_2/Cl^- and other prooxidants with linoleic acid. A (1) pure linoleic acid (0.5 nmol) incubated at room temperature for 24 hr; (2) aqueous linoleate dispersion, 0 time; (3) linoleate aqueous dispersion incubated for 30 min; (4) linoleate aqueous dispersion incubated for 2 hr; (5) linoleate aqueous solution in the presence of LPO/H_2O_2/Cl^- incubated for 5 min; (6) the same as (5) after incubation for 15 min; (7) lipoxygenase soybean type 1 (100 units, incubated for 3 min with linoleate. The first two peaks on the chromatograms are artifacts of the solvent system.

similar effect of the pH on this system was found and discussed in our previous study (25) and also by other researchers studying LPO and MPO halogenation (23,34).

The destruction of β-carotene by MPO-like enzyme from fish and by LPO (24,25) was highly sensitive to H_2O_2 concentration. The decrease in activity by those enzymes in the presence of high H_2O_2 concentration was also shown by others (23,24,35,37). In this study, we found that lipid peroxidation by LPO/chloride is activated by very low concentrations of H_2O_2 and inhibited by high concentrations. The optimal H_2O_2 concentration for increasing diene conjugation was the same as caused β-carotene destruction.

LPO/H_2O_2/halide system produced oxygen (23,25). The evolution of oxygen was found only in the presence of iodide and bromide ions when H_2O_2 was at a high concentration. Evolution of oxygen did not occur in the presence of chloride, probably because of the inactivation of the enzyme at high H_2O_2 concentrations (25).

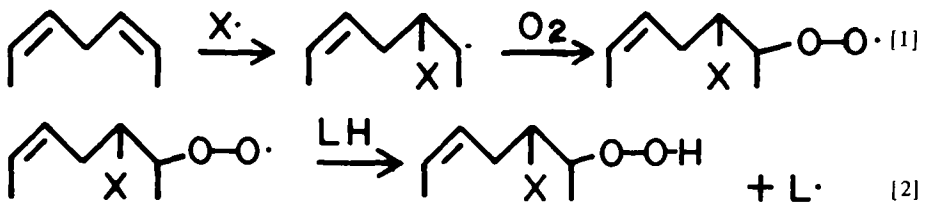
In the present study, oxygen was absorbed during the interaction of LPO/H_2O_2/chloride system with linoleate. Conceivably, this oxygen could interact with alkyl radicals and produce the conjugated dienes observed in our model system.

The diene conjugation of linoleate by LPO/H_2O_2/Cl^- was inhibited at low concentrations of BHT, tryptophan and methionine. In contrast, β-carotene destruction by the same system was not inhibited by BHT even at high concentrations, and tryptophan actually enhanced the reaction. These results reflect the differences on the mechanisms by which these two molecules, β-carotene and linoleic acid, are altered by the LPO/H_2O_2/halide system. It seems that β-carotene was destroyed by the enzymatic system via an addition reaction, whereas linoleic acid was peroxidized via an abstraction reaction, a reaction which was inhibited by BHT, tryptophan and methionine. It is known that amino acids interact with chlorine or HOCl derived from the MPO/H_2O_2/Cl^- system via reactions which deaminate and decarboxylate the amino acids (38). These interactions could obstruct hydrogen abstraction from the lipid substrate.

Extensive research has been undertaken to identify the primary free radical which initiates lipid peroxidation (4-6,9-11). We found evidence that chlorine or activated chlorine (or other halides), oxidized compounds that are generated during the reaction of a peroxidase/H_2O_2/Cl^- system (36,39), can initiate lipid peroxidation. These results are supported by the observation that, during enzymatic iodination of membrane proteins, lipids are also peroxidized (40).

Lipid peroxidation is known to be mediated by a free radical chain reaction. Initiation is thought to occur when linoleic acid reacts with an oxidizing compound which abstracts an allylic hydrogen from carbon-11 to produce a pentadienyl radical. This intermediate radical reacts at both ends with dioxygen to produce a mixture of conjugated 9- and 13-dienes (41).

However, there is another possibility by which a free radical (e.g., NO_2 [11,42]) will interact with the electrons of the double bond (Scheme 1, reaction 1). This addition reaction forms a free radical which can interact with other radicals or with oxygen to form a peroxy



SCHEME 1. x· = halogen radical; LH = 18:2

radical (11).

The peroxy radical generated could then initiate an allylic hydrogen abstraction from linoleate which can form an alkyl radical and a hydroperoxide (Scheme 1, reaction 2). Both of these compounds could then initiate lipid peroxidation. Diene conjugated compounds, however, can be formed in this reaction only after allylic hydrogen abstraction.

The increase in the diene conjugation of linoleate reflects hydrogen abstraction during the interaction of the LPO/H_2O_2/halide system with linoleate. Possibly this hydrogen abstraction could result from direct interaction of a halide radical with the allylic hydrogen or by an indirect interaction of a peroxy radical (ROO·) (derived by an addition reaction on the double bond by a halide radical and oxygen [reactions 1 and 2]) with linoleate.

Compounds such as N-chlorosuccinimide 43) and N-bromosuccinimide (44), which can serve as a source of chlorine and bromide in low concentrations, can abstract an allylic hydrogen from alkenes. The activation energy for the addition of chlorine or bromine and especially iodine to a double bond is lower than that of the corresponding hydrogen abstraction step. However, at very low concentrations, substitution is the dominant reaction (45-47). The allylic radical thus formed is not appreciably reversible.

Recently, Pryor and Lightsey (11) reported that the mechanisms by which NO_2 reacts with cyclohexane or unsaturated fatty acid was dependent on the concentration of the radical. At very low concentration of NO_2, allylic hydrogen abstraction was the dominant reaction and, in the presence of oxygen, NO_2 initiated peroxidation of polyunsaturated fatty acids.

The absorption of oxygen by our enzyme system reflected the formation of hydroperoxides. Thus, another possible mechanism of initiation of linoleate oxidation could have involved singlet oxygen. Such a reaction should produce the 9, 10, 12 and 13 hydroperoxides of 18:2 in a ratio of 2:1:1:2, respectively, from linoleic acid (48); however, we did not obtain this pattern of hydroperoxides (Fig. 6).

Originally, Allen et al. (49) hypothesized that the antimicrobial effect resulting from the action of MPO/H_2O_2/halide was singlet oxygen ($^1O_2$). During recent years, many attempts have been made to prove this hypothesis (1-3,6,50). However, there is growing evidence against the generation of $^1O_2$ by the MPO/ or LPO/H_2O_2/halide systems (20,51,52). Our conclusion from the earlier reports (24,25) are in agreement with those which question the generation of $^1O_2$ in this system. The pattern of isohydroperoxides from the reaction of the LPO/H_2O_2/Cl^- system with linoleate gave peaks with retention times similar to those produced by lipoxygenase, i.e., mainly the 13-hydroperoxide. We failed to get the pattern suggested by Frankel (48) and hence, it appears that singlet oxygen was not involved.

Thus, it appears that the peroxidation of linoleate by a peroxidase/H_2O_2/halide system is via a mechanism of hydrogen abstraction and/or radical addition which in the presence of oxygen form the conjugated dienes and hydroperoxides.

MPO, H_2O_2 and halides form a potent antimicrobial system which is operative in the phagocytic cells, e.g., neutrophils. Lipid peroxidation apparently occurs during phagocytosis (26,27). Conceivably, under certain conditions, e.g., injury and infection, MPO and H_2O_2 may be released from the leukocytes (17) and initiate peroxidation of lipids in continguous tissues and thereby disrupt normal functions. We recently observed that mammalian MPO could cause linoleate peroxidation in vitro. Further research to establish the nature of the radicals or activated species (Cl· or HOCl) which may initiate lipid peroxidation in vivo or in vitro is warranted.

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Effect of the Plasticizer Di(2-Ethylhexyl) Adipate (Dioctyladipate, DOA) on Lipid Metabolism in the Rat: I. Inhibition of Cholesterolgenesis and Modification of Phospholipid Synthesis

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ABSTRACT

DOA (di[2-ethylhexyl]adipate, dioctyladipate), a plasticizer used in the manufacture of polyvinylchloride plastic products, has been considered as a suitable substitute for di(2-ethylhexyl)phthalate (DEHP) in some applications. In the present studies, hepatic lipid metabolism was examined in liver mince preparations from rats fed 0.5% or 1.0% DOA in the diet for 2 weeks. By studying patterns of lipid synthesis from [^{14}C]acetate, [^{14}C]oleate, [^{14}C]mevalonate, and [^{14}C]octanoate, it was concluded that DOA feeding inhibits hepatic cholesterolgenesis and alters the pattern of phospholipids synthesized by the liver. DOA also exerted a cholesterol-lowering effect at the 1% level but did not affect plasma triglyceride levels. The results suggest that the biological effects of DOA in the rat are similar to those produced by DEHP.

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INTRODUCTION

Di(2-ethylhexyl)phthalate (DEHP) is the most widely used commercial plasticizer, both domestically (USA) and worldwide, with global production of ca. 4×10^9 lb annually (1). In recent years, there has been rising concern over the safety of DEHP. This concern emanates from a wide variety of biological studies which indicate that DEHP is mutagenic (2), teratogenic (3,4), displays cytoxicity (5,6), alters lipid metabolism (7-10), inhibits various enzymes (11-13), and alters tissue ultrastructure (14). Additionally, DEHP contaminates virtually all ecosystems (1) and has been found in human tissue (15-17) and in the food supply of man (18-20). In view of what has been stated above, there has been an interest in using less toxic substitutes for DEHP in certain applications such as in the production of polyvinylchloride plastic medical devices (e.g., plastic blood storage bags, catheters, etc.). Di(2-ethylhexyl)adipate, also known as dioctyladipate or DOA, is a plasticizer that is gaining attention as a DEHP-substitute in certain applications. Since we have previously shown that DEHP is an inhibitor of hepatic cholesterolgenesis in the rat and is capable of modifying other aspects of rodent lipid metabolism (7-10,21,22), it seemed important to study the effects of DOA feeding on lipid metabolism in the rat. The studies presented here indicate that DOA shares similar biological properties with DEHP in that DOA is an inhibitor of hepatic cholesterolgenesis, possesses plasma cholesterol-lowering activity, and modifies hepatic phospholipid metabolism.

MATERIALS AND METHODS

Male rats (Upjohn:TUC(SD)spf) weighing 150-160 g were individually housed with free access to food and water. The animals received either a stock diet (Purina Laboratory Chow) or the stock diet supplemented with 0.5% or 1.0% (w/w) di(2-ethylhexyl)adipate (Eastman Chemical Products, Kingsport, TN). The DOA was mixed into the diets dissolved in diethylether (7); the diets were then placed under an exhaust hood and stirred in order to permit the ether to evaporate. The rats were killed between 9 A.M. and 10 A.M. and liver minces were prepared (9) for incubation in 3.5 ml Krebs-Ringer bicarbonate buffer (pH 7.4) which contained either [1-^{14}C]oleate (sodium salt, sp act 56.0 Ci/mol), [1-^{14}C]acetate (sodium salt, sp act 56.0 Ci/mol), [1-^{14}C]octanoate (sodium salt, sp act 25.1 Ci/mol) or [2-^{14}C]DL-mevalonic acid (dibenzylethylene diamine salt, sp act 50.1 Ci/mol). The labeled precursors were obtained from New England Nuclear Corp., Boston, MA, and were present in the incubation mixtures at levels from 1.1 μCi to 3.0 μCi; levels used in a particular experiment are given in the table footnotes.

The tissue minces were incubated for 90 min at 37 C. After the incubation period, the tissues were either homogenized in chloroform/methanol as previously described (7) and the lipid extracts washed according to Folch et al. (23), or KOH and ethyl alcohol were added to the incubation mixtures to give final concentrations of 11% and 82%, respectively. In the latter case, hydrolysis of the tissue and saponification of

the lipids took place at 60 C for 60 min (8) and was followed by extraction of the nonsaponifiable lipids (sterols and squalene) with *n*-hexane (8). In some experiments, chloroform/methanol extracts and *n*-hexane extracts were fractionated by thin layer chromatography on Silica Gel G-coated glass plates in a solvent system consisting of *n*-hexane/diethyl ether/acetic acid (146:50:4) (7,8). The various lipids were visualized under UV light after spraying the chromatoplates with rhodamine 6G (0.05% in ethanol) (7,8). The lipid bands were scraped from the plates into vials containing scintillation fluid (Liquifluor, New England Nuclear Corp., Boston, MA) and assayed for radioactivity in a liquid scintillation spectrometer (Packard Tri-Carb, Model 3375, Packard Instruments, Downers Grove, IL). Quench corrections were made by the external standardization method. In experiments using [1-^{14}C]oleate, aliquots of the chloroform/methanol extracts of the tissues were also chromatographed in the system of Skipski et al. (24) in order to fractionate the tissue phospholipids. Since this system does not readily separate phosphatidylserine from phosphatidylinositol, these 2 fractions were combined for analysis. In some experiments, digitonin-precipitable sterols (sterols possessing a 3β-hydroxy group) were obtained from extracts of nonsaponifiable lipids (25) and assayed for radioactivity (25).

Plasma cholesterol and plasma triglycerides were measured by automated methods (26,27).

RESULTS AND DISCUSSION

The ability of rat liver minces to synthesize squalene and sterols from [^{14}C]acetate and [^{14}C]mevalonate was affected by feeding DOA at a level of 1.0% in the diet (Table 1). The incorporation of [^{14}C]acetate into cholesterol was reduced 62% ($p < 0.001$) in livers from the DOA-fed rats and paralleled a decrease (67%, $p < 0.001$) in [^{14}C]acetate incorporation into the sterol-precursor, squalene. In similar studies conducted with [^{14}C]mevalonate, no significant ($p > 0.05$) change in the formation of labeled cholesterol (or digitonin-precipitable sterols) was observed. There was, however, a significant decrease (32%, $p < 0.02$) in the incorporation of [^{14}C]mevalonate into squalene. The strong inhibition of squalene and cholesterol synthesis from [^{14}C]acetate in DOA-fed rats and the failure of DOA feeding to affect cholesterolgenesis from [^{14}C]mevalonate suggests that DOA affects sterol biosynthesis at a site or sites prior to the formation of mevalonic acid. This being the case, the partial inhibition of [^{14}C]mevalonate incorporation into squalene observed in livers of the DOA-fed rats may be explained if one or more of the enzymes between mevalonate and squalene is substrate-induced and, hence, diminishes in activity as endogenous mevalonate synthesis is reduced. It appears that squalene synthesis from mevalonate is adequate to sustain sterol synthesis in DOA-fed rats despite a 32% decline in squalene production. These results are similar

TABLE 1

Effect of Feeding 1.0% DOA on the Formation of Nonsaponifiable Lipids from [^{14}C]Acetate and [^{14}C]Mevalonate in Minces from Rat Liver[a]

	[^{14}C]Acetate		[^{14}C]Mevalonate	
	Control	DOA	Control	DOA
	(dpm/g wet wt)			
Cholesterol	555 ± 55[b]	210 ± 30[c]	32605 ± 2660	31850 ± 2780
Squalene	725 ± 85	240 ± 35[c]	74120 ± 6640	50180 ± 2660[d]
Digitonin-precipitable sterols	–	–	34785 ± 2175	35365 ± 2610

[a]Rats were fed either Purina Chow alone (control) or supplemented with DOA for 2 weeks. Liver minces (500 mg) were incubated for 90 min at 37 C in a total volume of 3.5 ml Krebs-Ringer bicarbonate buffer, pH 7.4, containing either 2 μCi DL-mevalonic [2-^{14}C]acid, or 3 μCi [1-^{14}C]acetic acid. The samples were hydrolyzed and saponified as described under Methods and the nonsaponifiable lipids (sterols, squalene) were extracted with *n*-hexane. One portion of the extracts was evaporated under N_2 and applied to Silica Gel G-coated plates in a small amount of chloroform/methanol (2:1, v/v) to isolate cholesterol and squalene as described under Methods; another portion of the extracts was evaporated, redissolved in acetone/ethanol (1:1, v/v), and treated with digitonin in order to precipitate the digitonin-precipitable sterols.

[b]Values are means ± SEM of 8 animals per group in the [^{14}C]acetate studies and of 6 animals per group in the [^{14}C]mevalonate studies.

[c,d]Significantly different from control values by Student's independent t-test (c, $p < 0.001$; d, $p < 0.01$).

to our previous observations with DEHP-feeding in the rat where it was observed that inhibition of cholesterolgenesis from [^{14}C]-acetate occurred about one week before significant inhibition of cholesterol synthesis from labeled mevalonate occurred. It seems likely that the rats fed 1% DOA in this study would have demonstrated inhibition of cholesterolgenesis from mevalonate if continued beyond 2 weeks on the diet (8,9,21).

The feeding of lower levels (0.5%) of DOA for 2 weeks also resulted in statistically significant inhibition of [^{14}C] acetate ($p < 0.02$) and [^{14}C] mevalonate ($p < 0.01$) incorporation into squalene by liver minces (Table 2). Cholesterol synthesis from [^{14}C] acetate tended to be reduced as well but the reduction was not statistically significant suggesting that the feeding of 0.5% DOA for 2 weeks yielded a threshold response with respect to sterol synthesis that reflected the dose of DOA and the duration of exposure. Just as was observed with feeding 1% DOA, 0.5% DOA feeding did not affect the incorporation of [^{14}C] mevalonate into cholesterol (Table 2).

Feeding of 1.0% DOA was also associated with a decrease in plasma cholesterol from 76 mg/dl to 66 mg/dl ($p < 0.02$, Table 3); alterations in plasma cholesterol did not occur at the 0.5% level of DOA (Table 3). These results are similar to those observed in DEHP-fed rats where reductions in hepatic cholesterolgenesis were also accompanied by lower circulating levels of plasma cholesterol (21). Although it is tempting to consider that the reductions in plasma cholesterol reflect the reduction in hepatic cholesterolgenesis, it is possible that other mechanisms such as increased lipoprotein clearance could be involved.

One potential problem in using labeled acetate to measure sterol synthesis involves the possibility of underestimating sterolgenesis under conditions in which an administered compound can be a source of acetyl units which dilute the specific activity of the acetate pool. In the case of the rat, DOA is metabolized in the gut and other tissues to yield the monoester, adipic acid (28), and presumably 2-ethylhexanol (28,29). Whereas 2-ethylhexanol is unlikely to generate acetate (29), there is evidence that adipate can undergo β-oxidation to some extent (28,30) and might, therefore, contribute to the endogenous acetate pool. For this reason, liver minces were prepared from 1% DOA-fed rats as in Table 1 and incubated with 2 μCi [1-^{14}C] octanoate which readily penetrates the mitochondria (31). The rapid generation of acetylCoA from the exogenous octanoate reduces the relative contribution of endogenous sources of acetate to sterol synthesis (31). In these experiments, the incorporation of [^{14}C] octanoate into the total nonsaponifiable lipid fraction was reduced ca. 50% in tissues from the DOA-fed rats (dpm/g wet wt: control, 17175±4050 [n=8] vs 1% DOA, 8725±

TABLE 3

Effect of Feeding DOA on Plasma Cholesterol and Triglyceride Levels in the Rat[a]

Dietary level of DOA	Plasma lipid levels (mg/dl)	
	Cholesterol	Triglyceride
0 (control)	76 ± 2[b]	126 ± 12
0.5%	71 ± 3	147 ± 16
1.0%	66 ± 3[c]	109 ± 10

[a]Rats were fed Purina Chow alone (control) or supplemented with 0.5% and 1.0% DOA for 2 weeks. Total cholesterol and total triglyceride values were determined on heparinized plasma derived from blood that was drawn by cardiac puncture.

[b]Values are means ± SEM of values from 8 animals/group.

[c]Significantly different from the control value ($p < 0.02$) by Student's independent t-test.

TABLE 2

Effect of Feeding 0.5% DOA on the Formation of Nonsaponifiable Lipids from [^{14}C] Acetate and [^{14}C] Mevalonate in Minces from Rat Liver[a]

	[^{14}C] Acetate		[^{14}C] Mevalonate	
	Control	DOA	Control	DOA
	(dpm/g wet wt)			
Cholesterol	2510 ± 785[b]	1470 ± 260	18615 ± 2180	14590 ± 775
Squalene	835 ± 215	170 ± 35[c]	26270 ± 2835	14500 ± 590[d]

[a]Same as footnote to Table 1, with the exception that 0.5% DOA was fed and DL-mevalonic [2-^{14}C] acid was present at a level of 1.1 μCi/incubation.

[b]Values are means ± SEM of 6 animals per group in all experiments.

[c,d]Significantly different from control values by Student's independent t-test (c, $p < 0.02$; d, $p < 0.01$).

1375 [n=8]). These data further support the contention that DOA feeding results in inhibition of sterol synthesis.

In rats fed 1% DOA for 2 weeks, the incorporation of [^{14}C] oleate and [^{14}C] acetate into liver phospholipids, triglycerides and steryl esters were not significantly affected (Table 4). The fact that free fatty acid formation from [^{14}C] acetate was also unaffected (Table 4) offers additional evidence against a significant expansion of the endogenous acetate pool arising from DOA metabolism in the tissue. Fractionation of the phospholipids (Table 5), however, indicated that there was a change in the pattern of the phospholipids synthesized. In livers from the DOA-fed rats, the percentage distribution of [^{14}C] oleate and [^{14}C] acetate incorporated into phosphatidylcholine fell from 43.0% to 35.8% ($p < 0.01$), and from 32.9% to 19.1% ($p < 0.05$), respectively (Table 5). The percentage distribution of [^{14}C] acetate into sphingomyelin was also reduced below control values with DOA feeding (6.8% vs 2.8%, $p < 0.05$), whereas the percentage of [^{14}C] acetate appearing in the phosphatidylethanolamine fraction increased from 37.9% to 53.3% ($p < 0.01$). These effects of DOA on rat liver phospholipids closely parallel the effects that were observed in livers of rats fed 0.5-1.0% DEHP (7,32).

In conclusion, the biological effects of DOA in the rat as they relate to lipid metabolism are strikingly similar to the effects observed with DEHP in the rat. These effects include: (a) inhibition of hepatic cholesterolgenesis; (b) reduction of plasma cholesterol; and (c) modification of hepatic phospholipid synthesis.

The studies presented here suggest that further biological evaluation of DOA is warranted before it becomes widely established as a substitute for DEHP as a plasticizer in synthetic products.

TABLE 4

Effect of 1% DOA Feeding on the Incorporation of [^{14}C] Oleate and [^{14}C] Acetate into Lipids in Minces from Rat Liver[a]

	[^{14}C] Oleate			[^{14}C] Acetate		
	Phospholipids	Triglycerides	Steryl esters	Phospholipids	Free fatty acids	Triglycerides
	(dpm/g wet wt)					
Control	74120 ± 8000[b]	219170 ± 22055	7880 ± 1660	1295 ± 195	1975 ± 575	990 ± 140
DOA	58265 ± 2695	174570 ± 11940	5275 ± 405	1025 ± 85	2320 ± 475	630 ± 55

[a]Rats were fed either Purina Chow alone (control) or supplemented with 1.0% DOA for 2 weeks. Liver minces (500 mg) were incubated for 90 min at 37 C in 3.5 ml Krebs-Ringer bicarbonate buffer, pH 7.4, containing either 2 μCi [1-^{14}C] oleic acid or 2 μCi [1-^{14}C] acetic acid. After incubation, tissue lipids were extracted and fractionated by thin layer chromatography as described under Methods, and assayed for radioactivity.

[b]Values are means ± SEM of 4 animals per group.

TABLE 5

Effect of 1.0% DOA Feeding on the Percentage Distribution of [^{14}C] Oleate and [^{14}C] Acetate into Individual Phospholipid Classes in Minces from Rat Liver[a]

		Lyso	Sph	PC	PS+PI	PE
[^{14}C] Oleate	Control	1.0 ± 0.1[b]	1.0 ± 0.1	43.0 ± 1.1	18.5 ± 0.7	36.6 ± 0.8
	DOA	1.1 ± 0.1	0.9 ± 0.1	35.8 ± 1.6[c]	21.4 ± 1.3	40.9 ± 2.7
[^{14}C] Acetate	Control	5.9 ± 1.4	6.8 ± 1.2	32.9 ± 5.4	16.6 ± 2.0	37.9 ± 2.6
	DOA	4.4 ± 0.9	2.8 ± 0.8[d]	19.1 ± 1.4[d]	20.3 ± 2.0	53.3 ± 2.5[c]

[a]See footnote to Table 4. Phospholipids were fractionated according to the method of Skipski et al. (24). Lyso, lysolecithin; Sph, sphingomyelin; PC, phosphatidylcholine; PS+PI, phosphatidylserine plus phosphatidylinositol; PE, phosphatidylethanolamine.

[b]Values are the mean ± SEM of 4 animals/group.

[c,d]Significantly different from control values by Student's independent t-test (c, $p < 0.01$; d, $p < 0.05$).

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Polycyclic Hydrocarbon and Polychlorinated Biphenyl Solubilization in Aqueous Solutions of Mixed Micelles

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ABSTRACT

To determine the physiochemical behavior of xenobiotic hydrocarbons in simulated intestinal content, we examined the partition of 7,12-dimethylbenzanthracene (DMBA), 3-methyl cholanthrene (MC), benzo(a)pyrene, and a polychlorinated biphenyl compound (PCB, Aroclor 1242) between an emulsified oil phase and a mixed micellar solution. In a mixed lipid-bile salt system, negligible amounts of hydrocarbon were present in aqueous solution below the critical micellar concentration (CMC) of sodium taurocholate. Once the CMC was obtained, the 4 hydrocarbons exhibited nearly identical partitions from the lipid into the micellar system which was enhanced by increased concentrations of bile salt, reduction of triglyceride concentration and the formation of mixed rather than pure bile salt micelles. The partition of DMBA and MC into micelles was optimized by long-chain monounsaturated oleic acid and monooleoylglycerol as compared to their octanoic or linoleic counterparts. Linoleic acid and monolinoleoylglycerol maximized the partition of PCB from the oil into the micellar phase. In a mixed micellar system excluding an oil phase and an excess of DMBA, a molar saturation ratio (mol hydrocarbon:mol bile salt) was calculated by regression analysis to be 0.162. This indicates that more than one molecule of hydrocarbon is solubilized per mixed micelle and that the aqueous solubilization of hydrocarbon may be attributed to true micellar solubilization.
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Many toxic substances in the environment, including certain well recognized carcinogens, are highly lipophilic. Some of these substances exist in significant amounts in the food chain (1, 2). Thus, the gastrointestinal tract is directly exposed to these compounds and also serves as a major portal of entry to the organism. It is established that the absorption of trace lipid nutrients such as sterols and the fat-soluble vitamins is intimately dependent on the normal processes of fat digestion and absorption and that bile salts, which play a facilitatory role in triglyceride absorption, are obligatory for absorption of these trace lipids (3). It seems likely that lipophilic xenobiotics will be handled in a similar fashion to trace nutrient lipids in the early stages of assimilation by the small intestine. In order to study factors governing the in vivo intestinal absorption of hydrocarbon carcinogens and polychlorinated biphenyls, an initial study of the behavior of selected examples of these compounds in model intestinal content was carried out using mixtures of bile salt, triacylglycerols, monoacylglycerols, fatty acids and phospholipids.

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Abbreviations: 7,12-dimethylbenzanthracene (DMBA); 3-methylcholanthrene (MC), benzo(a)pyrene (BaP); polychlorinated biphenyls (PCB); polycyclic aromatic hydrocarbons (PAH); critical micellar concentration (CMC).

MATERIALS AND EXPERIMENTAL METHODS

All reagents used were of the highest purity available and were used as such. Sources were as follows: 7,12-dimethylbenzanthracene (DMBA), 3-methylcholanthrene (MC), benzo(a)pyrene (BaP), oleic (*cis*-9-octadecenoic) acid and sodium taurocholate were purchased from Sigma Chemical Co., St. Louis, MO. Trioleoylglycerol, 1-monooleoylglycerol and the 1-monoacylglycerols, triacylglycerols and fatty acids of linoleic (*cis,cis*-9,12-octadecadienoic) acid and of octanoic acid were obtained from NuChek Prep Co., Elysian, MN, and were greater than 99% pure as confirmed by gas liquid chromatography (GLC). Phosphatidylcholine (1,2-diacyl-*sn*-glycero-3-phosphocholine) from egg was purchased from Serdary Research Laboratories, London, Ontario. Aroclor 1242 (PCB), an isomeric mixture of polychlorinated biphenyls containing 42% chlorine by weight, was obtained from Foxboro Canada Inc., LaSalle, Quebec. (G-^{3}H)-7,12-dimethylbenzanthracene and (G-^{3}H)-3-methylcholanthrene were purchased from Amersham Corp., Oakville, Ontario and (1,3,6-^{3}H)-benzo(a)pyrene and (U-^{14}C)-polychlorinated biphenyls (Aroclor 1242) were obtained from New England Nuclear Canada Ltd., Lachine, Quebec.

A. Preparation of Micellar Solutions

The lipids in heptane were combined with phosphatidylcholine in chloroform and the hydrocarbon (DMBA, MC, BaP or PCB) and its

radiolabeled tracer in toluene. The solvents were evaporated under a steady stream of nitrogen to yield an oil droplet containing the hydrocarbon. Buffered sodium taurocholate solution and a 0.1 M sodium phosphate buffer (pH 6.3, 0.15 M with respect to sodium) were added to give final volumes of 3 ml test mixtures. Specific activity of radiolabeled tracer in each flask was 0.5 μ Ci ^{3}H or 0.13 μ Ci ^{14}C per 3.0 ml. The biphasic mixture in each flask was then sonicated for exactly 1 min with a Sonifier Cell Disrupter (Model W185, Branson Sonic Power Co.) equipped with a standard microtip at power output 3 to yield an emulsion which was then incubated at 37C for 30 min. Samples (50 μl) were removed for scintillation counting. The emulsions were then centrifuged in a Beckman type 75Ti rotor at 106,000 x g_{av} (40,000 rpm) in a Beckman L2-65B Ultracentrifuge for 18 hr at ambient temperature to produce a supernatant oily phase and a clear aqueous infranatant bile salt solution. This latter phase was carefully drained and analyzed for solubilized hydrocarbon by liquid scintillation counting.

The liquid scintillation counting procedure entailed combining appropriate amounts of emulsion or micellar solution with 10 ml Aquasol-2 Cocktail (New England Nuclear). They were then subjected to direct scintillation counting in a Beckman LS 8100 Scintillation Counter. Quenching was corrected by means of an external standard to allow counts per minute to be converted into absolute activity.

B. Determination of Sodium Taurocholate Micellar Solubilization Capacity

Appropriate volumes of a 0.1 M sodium taurocholate solution and 0.1 M phosphate buffer (pH 6.3) were added to the lipids in each flask to give final lipid concentrations of 7.5 mM oleic acid, 2.5 mM monooleoylglycerol, 1.13 mM trioleoylglycerol, 0.68 mM phosphatidylcholine, 100 μ M hydrocarbon (BaP, MC, DMBA or PCB) and final sodium taurocholate concentrations of 0, 1, 2, 3, 4, 5, 6, 8, 10 and 12 mM. Subsequent procedures were as outlined in Section A.

Micellar:oil ratios of the hydrocarbon were calculated as

$$\underset{\text{infranatant}}{DPM_{aqueous}}/(\underset{\text{before centrifugation}}{DPM_{emulsion}} - \underset{\text{infranatant}}{DMP_{aqueous}})$$

for each bile salt concentration studied and represent the mean ± SEM for at least 4 replicate experiments. Data were also calculated as nmol hydrocarbon solubilized in the micellar phase per bile salt concentration. The slope of the straight portion of the resulting plot, above the inflection point, was calculated by regression analysis and used to determine a partition ratio (mol solute solubilized in aqueous phase/mol bile salt) for the system.

C. Effect of Simulated Triacylglycerol Hydrolysis on Micellar:Oil Partition of Hydrocarbon

Oleic acid, trioleoylglycerol and monooleoylglycerol were combined to give final concentrations as follows: trioleoylglycerol 20, 18, 16, 15, 10 and 5 mM, monooleoylglycerol concentrations corresponded to (20 mM – trioleoylglycerol concentration), and oleic acid concentrations were twice that of the monooleoylglycerol. Sodium taurocholate, phosphatidylcholine and DMBA concentrations were held constant at 12 mM, 0.68 mM, and 100 μ M, respectively. Conditions and procedures were as outlined in Section A. Micellar:oil ratios and percentages of hydrocarbon solubilized at each stage of simulated triacylglycerol hydrolysis were calculated and represent the means ± SEM for 4 replicate experiments.

D. Determination of Ability of Fatty Acid Saturation and Chain Length to Influence Hydrocarbon Oil-Aqueous Partition

The monoacylglycerol, triacylglycerol and fatty acid of either the octanoic (8:0), oleic (18:1) or linoleic (18:2) series with phosphatidylcholine were the lipids used. In this series of experiments, the amount of triacylglycerol was increased from 1.13 mM to 5 mM to give a more definite lipid supernatant phase. Fixed final concentrations of fatty acid (7.5 mM), monoacylglycerol (2.5 mM), phosphatidylcholine (0.68 mM) and hydrocarbon (MC, DMBA or PCB; 100 μM) were usedwith conditions and procedures as detailed in Section A. Micellar: oil ratios and percentage of hydrocarbon (DMBA, MC, PCB) solubilized in the aqueous phase were calculated and represent the means ± SEM for 8 replicate experiments.

E. Determination of a Molar Saturation Ratio for DMBA

Appropriate volumes of an 18 mM sodium taurocholate mixed micellar solution containing 7.5 mM oleic acid, 2.5 mM monooleoylglycerol, and 0.68 mM phosphatidylcholine were added in combination with 0.1 M sodium phosphate buffer (pH 6.3, 0.15 m Na^{+}) to total 2.5 ml in flasks containing 10 mg crystalline DMBA and its radiolabeled tracer. Final sodium taurocholate concentrations ranged to 15 mM. The flasks were then sealed and sonicated in an ultrasonic water bath (Cole Parmer) for 1 hr at ambient temperature. They then were shaken in the dark for 18 hr at 37 C and subsequently centrifuged for 30 min at 3,000 rpm (Sorvall

GLC-2 Benchtop Centrifuge). The supernatant solution was then filtered through Whatman 42 filter paper to retain crystalline DMBA. Aliquots of 100 μl of the micellar solutions were removed for liquid scintillation counting to determine solubilized hydrocarbon.

Statistical analysis for all experiments was performed by Student's t-Test.

RESULTS

The micellar:oil ratio is indicative of the ability of a typical bile salt, sodium taurocholate, to influence the partition of DMBA, BaP, MC and PCB from an oil phase into a micellar phase. The 4 hydrocarbons exhibit similar micellar:oil ratios which are dependent on bile salt concentration. When no bile salt was present in the system, the average micellar:oil ratio for the hydrocarbons was 0.005 ± 0.002. At 4, 8 and 12 mM sodium taurocholate, the micellar:oil ratios were 0.053 ± 0.007, 0.6 ± 0.1 and 4.3 ± 1.2, respectively. The difference in micellar:oil ratio between 0 and 12 mM sodium taurocholate, the latter, a physiological concentration of bile salt, represents nearly a 1,000-fold increase in partition of hydrocarbon from the lipid into the aqueous phase.

Below 4 mM sodium taurocholate, there are insignificant quantities of hydrocarbon present in the aqueous phase. In the absence of bile salt, only an average of 0.45 ± 0.22% of the 300 nmoles of hydrocarbon present in the system is found in the aqueous phase. This value increases to 5.2 ± 0.6% at 4 mM, 37 ± 5% at 8 mM and 79 ± 4% at 12 mM sodium taurocholate.

This ability of bile salt to enhance the solubility of the representative polycyclic aromatic hydrocarbons and polychlorinated biphenyl is shown in Figure 1. At 4 mM sodium taurocholate, there occurs an inflection point below which very little hydrocarbon is found. Above this 4 mM value, which is the apparent critical micellar concentration (CMC) for our system, the amount of hydrocarbon solubilized in the aqueous phase is directly proportional to the amount of bile salt present. Determination of the slopes of these curves above the inflection points by regression analysis allows calculation of a partition ratio for each compound in this system (mol solute solubilized in the mixed micellar phase/mol bile salt). These values were determined by regression analysis to be DMBA 0.0096, MC 0.010, BaP 0.010, PCB 0.0094.

The influence of the degree of simulated triacylglycerol hydrolysis on the oil:micellar partition of DMBA is depicted in Figure 2. When no monooleoylglycerol or oleic acid was included in the system (i.e., 20 mM trioleoylglycerol), 4.9 ± 0.4% of the DMBA was located in the micellar infranatant. The partition of DMBA into the aqueous phase from the oil phase progressed as the trioleoylglycerol was gradually replaced by monooleoylglycerol and oleic acid. Maximal micellar solubilization occurred at 40% "hydrolysis" (i.e., 12 mM trioleoylglycerol, 8 mM monooleoylglycerol, 16 mM oleic acid) where 13 ± 2% of DMBA had partitioned into the aqueous phase. After this 40% hydrolysis point, the amount of hydrocarbon present in the micellar phase decreased to 10 ± 1% at 50% "hydrolysis" and 0.9 ± 0.1% at 75% "hydrolysis" (5 mM tri-, 15 mM monooleoylglycerol, 30 mM oleic acid). When the amount of fatty acid was fixed at 7.5 mM and monooleoylglycerol fixed at 2.5 mM but only the quantity of trioleolyglycerol varied, the percentage of hydrocarbon present in the micellar phase was inversely related to the quantity of trioleoylglycerol (Table 1).

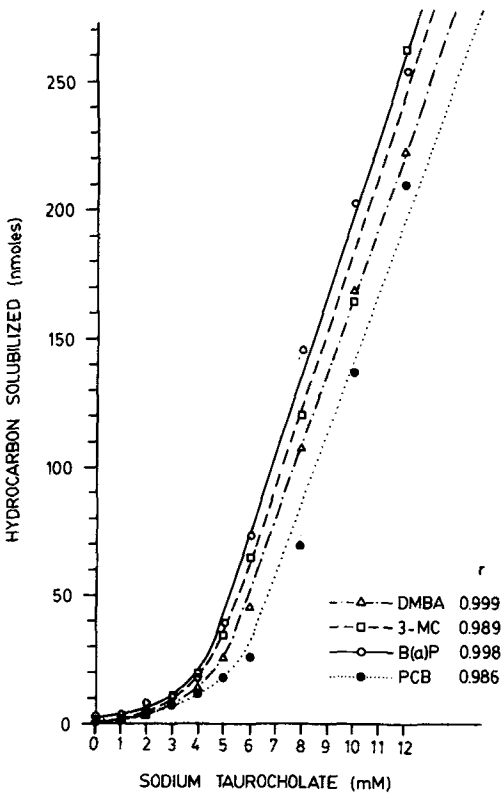

FIG. 1. Effect of bile salt concentration on the aqueous solubilization of DMBA, MC, BaP and a PCB in a 2-phase system composed of supernatant lipid phase and clear micellar infranatant phase. Solubility expressed as nmoles hydrocarbon solubilized in 3.0 ml of a sodium taurocholate solution at specified concentrations (n=4). Composition of mixtures as given in Methods, Section B.

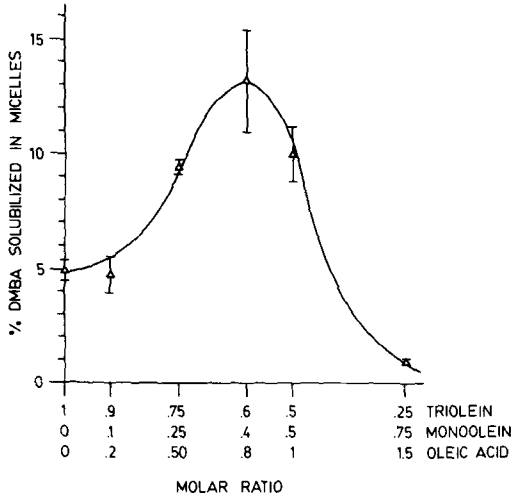

FIG. 2. Influence of simulated triacylglycerol hydrolysis on the partition of DMBA from a lipid into a mixed bile salt micellar solution. Partition is expressed as percentage of DMBA (± SEM) present in an aqueous bile salt micellar solution (n=4). Composition of mixtures as given in Methods, Section C.

TABLE 1

Effect of Triacylglycerol Concentration on the Partition of DMBA, MC, PCB (Aroclor 1242) between an Oil Phase and a Mixed Micellar Solution[a]

Trioleoylglycerol concentration (mM)	Percentage of 300 nmol of hydrocarbon solubilized in 3.0 ml aqueous phase		
	DMBA	MC	PCB
1.13	74 ± 4	87 ± 4	71 ± 7
5.00	21 ± 3	20 ± 1	25 ± 2

[a]DMBA, MC and a PCB were each combined with 2.50 mM monooleoylglycerol, 7.50 mM oleic acid, 0.68 mM phosphatidylcholine and either 1.13 mM or 5.0 mM trioleoylglycerol in a 0.1 M sodium phosphate buffer solution (pH 6.3, 0.15 m Na^+) containing 12 mM sodium taurocholate. (n = 4).

The effect of fatty acid saturation and chain length on the oil:micellar partition of DMBA, MC and PCB was investigated using the long-chain monounsaturated oleic acid and its 1-mono- and triacylglycerol, the long-chain diunsaturated linoleic acid, its 1-mono- and triacylglycerol and the medium-chain saturated octanoic acid and its 1-mono- and triacylglycerol. The abilities of the various lipid classes to influence the micellar partitions of the hydrocarbons are reflected in Figure 3. DMBA was solubilized in the aqueous phase of the octanoic system in amounts only 55% of that solublized

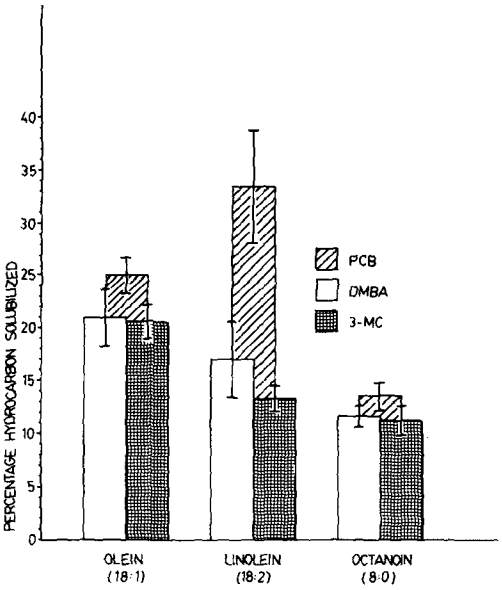

FIG. 3. Influence of fatty acid saturation and chain length on the partition of DMBA, MC and a PCB from a lipid into a bile salt micellar phase. Partition expressed as percentage of hydrocarbon (± SEM) present in aqueous solution (n=8). Composition of mixtures as given in Methods, Section D.

when the oleic system was used ($p<0.005$). Differences in the aqueous solubilization of DMBA between the linoleic system and the oleic or octanoic systems were not statistically significant ($p>0.05$). Likewise, MC solublization in the aqueous phase with the medium-chain system was only 54% of that solubilized in the aqueous phase of the oleic system ($p<0.0005$). There was no significant difference in the amount of MC solubilized between the linoleic and octanoic system ($p>0.05$), but the oleic system exhibited significantly more ($p<0.0025$) hydrocarbon in the aqueous, micellar phase than did the linoleic system.

Likewise for the PCB, the amount of hydrocarbon present in the micellar phase with the octanoic system was 54% ($p<0.0025$) of that seen with the oleic system. However, the linoleic system was able to increase the amount of PCB present in the micellar solution by 146% ($p<0.0005$) over that seen with the medium-chain octanoic series and by 34% ($0.05<p<0.10$) over that seen with the long-chain monounsaturated oleic acid system.

Using a mixed lipid-bile solution system and an excess of DMBA as described in Methods, Section E, a molar saturation ratio (mol hydrocarbon/mol bile salt) was calculated by regression analysis to 0.162 for DMBA in the mixed micelles when no lipid phase was present.

DISCUSSION

In the gastrointestinal tract, ingested lipids, nutrient or nonnutrient, exist in an aqueous environment. Since lipid absorption is passive, sufficient quantities of lipid in monomolecular solution must exist close to the enterocyte membrane to create the diffusion gradient responsible for absorption. Patton (4) has suggested a scheme of fat digestion which provides a continuous hydrocarbon domain for the transfer of lipids from the lumen to the enterocyte. A nondispersible mixed triacylglycerol phase is hydrolyzed by the action of pancreatic lipase to monoacylglycerol and fatty acid. These form a viscous isotropic phase which is subsequently solubilized by bile salts, producing an aqueous micellar solution. Trace lipids are able to flow from the bulk dietary oil into the viscous isotropic phase to be eventually solubilized in the hydrophobic interiors of the mixed micelles. The micelles serve to carry the products of fat digestion and trace lipids through the aqueous phase to the absorbing enterocyte.

Although it might be assumed that lipophilic xenobiotics such as PAH and PCB in intestinal content may behave in a similar fashion to trace nutrient lipids, there have been few studies to examine their physiochemical behavior in the intestinal situation. Early studies by Ekwall and associates (5,6) showed several PAH carcinogens to undergo aqueous solubilization in the presence of high concentrations of bile salt (in excess of 100 mM). Norman (7) demonstrated MC solubilization in solutions of conjugated and unconjugated bile salts beginning at 12 mM.

During fat digestion, intestinal lipids partition between an oil phase containing mainly higher glycerides and a bile salt micellar phase containing monoacylglycerol and fatty acid (8). Since bile salt lipid micelles are generally thought to be of great importance as a medium for delivery of trace nutrient lipids to the absorptive epithelial cells of the small intestine, and since similar considerations probably apply to lipophilic xenobiotics, factors governing the distribution of PAH and PCB between an oil phase and an aqueous phase were examined. Due to the high lipid solubility of these compounds, they are probably ingested in association with dietary fats. PCB are deposited in the fatty tissue of fish (2), are excreted in breast milk (9) and residues have been detected in human adipose tissue (10). It has been suggested that a major origin of PAH in food is by the pyrolysis of fat. PAH content of charcoal-broiled meat is at least partially dependent on the fat content (11). Once these hydrophobic compounds are ingested in dietary fat, their absorption probably depends on those systems which disperse and absorb nutrient lipid. Our simulated intestinal system was modeled after that used by El-Gorab and Underwood (12) at physiological pH (6.3) and contains concentrations of fatty acid, monoacylglycerol, triacylglycerol, phospholipid and sodium ion likely to be found postprandially in the small intestine. Although the majority of monoacylglycerol present in the luminal contents of the small bowel after a meal is in the form of 2-monoacylglycerol, it is expected that the use of 1-monoacylglycerol would not alter the physiochemical behavior of the system.

The mixed micellar system, which was generated progressively from a mixed lipid phase by increasing the concentration of bile salt (Fig. 1), efficiently transferred PAH and PCB compounds from an oil to an aqueous phase. The addition of 12 mM sodium taurocholate into the lipid/buffer system increased the partition of hydrocarbon from the oil into the aqueous phase by a factor of ca. 1,000. The pattern of hydrocarbon solubilization as bile salt concentrations increase (Fig. 1) with a sharp inflection point at ca. 4 mM sodium taurocholate suggests that aqueous solubilization of hydrocarbon depends on bile salt micelle formation. Only trace amounts of the 4 hydrocarbons were detected in the aqueous phase below the apparent CMC of sodium taurocholate (4 mM) in the system studied. Above this value, the solubilization of each hydrocarbon was directly proportional to the bile salt concentration. As these compounds exhibit like partition ratios in this in vitro system, it is probable that they have similar behavior in intestinal content.

For true micellar solubilization, it is necessary for each micelle to contain at least one molecule of solute. It is accepted that bile salt micelles are composed of ca. 15 bile salt molecules at 20 C and 0.15 M sodium chloride (13). In pure bile salt micelles, Norman (7) found that ca. 1,000 molecules of bile salt were required to solubilize 3 molecules of MC. Ekwall et al. (6) obtained similar results for DMBA in pure bile salt solutions. For these reasons, Carey and Small (13) concluded that these compounds are not true micellar solutes in pure bile salt solution. In the lipid mixture employed in this study, calculated partition ratios indicate that 100 molecules of bile salt in the presence of fatty acid and monoacylglycerol are required to cause one PAH molecule to partition from the oil into the aqueous phase. Partition ratios are a measure of relative affinity of the hydrocarbon for the lipid phase vs the hydrophilic core of the micelle and, alone, this data does not prove that "true" micellar solubilization is occurring.

To determine the capacity of mixed micelles

to solubilize a PAH (DMBA) in the absence of a competing lipid phase, a molar saturation ratio was determined utilizing an excess of hydrocarbon and solutions with increasing concentrations of mixed sodium taurocholate micelles. Our apparent saturation ratio for DMBA was calculated (mol DMBA solubilized/mol bile salt) to be 0.162, which corresponds to ca. 6.2 molecules of sodium taurocholate in the presence of oleic acid, monooleoylglycerol and phosphatidylcholine being necessary to promote the aqueous solubilization of 1 molecule of DMBA. Given the inflection point from Figure 1, which occurs at the estimated 3.1 mM CMC of sodium taurocholate (13), and the observation that ca. 6 molecules of bile salt can solubilize one molecule of hydrocarbon when allowed to form mixed fatty acid-monoacylglycerol-bile salt micelles, it appears that a "true"micellar solubilization is occurring, and the principle of at least one molecule of solute per micelle is maintained.

As DMBA, MC, BaP and PCB demonstrated similar behaviors in the bile salt-lipid mixture, as shown by their partition ratios, one compound (DMBA) was selected to determine the influence of simulated triacylglycerol hydrolysis on the oil-aqueous partition of a hydrocarbon (Fig. 2). When both fatty acid and monoacylglycerol were excluded from the system, and triacylglycerol and phosphatidylcholine were the only lipids present, ca. 5% of the 100 μ M DMBA in the system were present in the aqueous phase. As "hydrolysis" proceeded, increasing quantities of DMBA partitioned into the aqueous phase, reaching a maximum of 13% of the hydrocarbon in the aqueous phase at 40% "hydrolysis." It appears that the increased micellar volume, due to expansion of the phospholipid-bile salt micelles with monoacylglycerol and fatty acid, is responsible for an increased partition of hydrocarbon into the aqueous phase of the system. Beyond 40% "hydrolysis," the hydrocarbon favors the lipid phase. This is particularly evident at 75% hydrolysis, where it appears that the ability of bile salt micelles to solubilize fatty acid and monoacylglycerol has been exceeded, and a substantial oil phase is visible following ultracentrifugation. This expanded oil phase, composed of triacylglycerol, monoacylglycerol and fatty acid in molar proportions of 5:10:20, is able to retain greater than 99% of the hydrocarbon. In a normal physiological situation, a constant reduction of the oil phase would occur as the products of triacylglycerol hydrolysis would be rapidly absorbed by the enterocytes and would not contribute to expansion of the oil phase. From Table 1, it is evident that depletion of the triacylglycerol content at fixed fatty acid and monoacylglycerol concentrations will significantly promote the partition of PAH and PCB from an oil into an aqueous micellar solution. Triacylglycerol hydrolysis, therefore, is necessary for the appreciable partition of hydrocarbon from an oil phase into an aqueous dispersible bile salt solution.

In 1967, Borgström (14) demonstrated that cholesterol partitioned more in favor of an aqueous phase when the fatty acid species was oleic rather than linoleic acid. This is consistent with our observations regarding the effect of saturation on the partition of DMBA and MC between the oil and aqueous phases (Fig. 3). Conversely, PCB appeared to favor aqueous solubilization when the polyunsaturated rather than the monounsaturated fatty acid and monoacylglycerol were present. These contradictory observations, in spite of otherwise similar behaviors in the simulated intestinal system, are difficult to interpret at present. If a true micellar solubilization is occurring, the differences might be explained by stereochemical "fit" into the lipid cores of the mixed micelles. It is likely that various lipids expand the micellar core to different degrees. The PAH studied might have a better "fit" when the oleic lipids are incorporated into the micelles rather than linoleic acid and monolinoleoylglycerol. The PCB compound might be better accommodated in the mixed micelles when linoleic rather than oleic acid and monooleoylglycerol are present.

Fatty acids and monoacylglycerols of medium-chain length lipids form micelles with a poorer capacity to solubilize the hydrocarbons than the corresponding long-chain lipids (Fig. 3). Similarly, Takahashi and Underwood (15) found that aqueous solubilization of α-tocopherol was increased when lipids of medium-chain length were incorporated into a bile salt micellar system but that this effect was 3- to 7-fold less than for a corresponding long-chain mixture. If the hydrocarbons do undergo solubilization in the lipid cores of the mixed bile salt micelles, then perhaps greater expansion of this hydrophobic interior by long-chain lipids is responsible for the observed differences.

From the foregoing data, it can be supposed that PAH and PCB following ingestion will participate in the same digestive and dispersive processes as trace nutrient lipids in the intestinal lumen. Because we have demonstrated a requirement for mixed bile salt micelles to promote the solubilization of hydrocarbon in an aqueous environment simulating intestinal lumenal conditions, it is probable that mixed micelles are the vehicles responsible for delivery of hydrocarbon to the absorptive epithelial cells of the small bowel. The nondiscriminating "hydrocarbon continuum" necessary for the efficient ab-

sorption of essential micronutrients, such as fat soluble vitamins, may also serve to compromise the health of the organism by the delivery of lipid toxins to the absorptive cell.

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Relative Contribution of the Main Tissues and Organs to Body Fatty Acid Synthesis in the Rat

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ABSTRACT

Tritiated water was used to measure the rate of fatty acid synthesis in the main tissues and organs of 7-week old Wistar male rats in order to determine the relative contribution of each tissue to body fatty acid synthesis. We reached the following conclusions: (a) the liver is the main site of fatty acid synthesis, it alone synthesizes 42% of the newly synthesized fatty acids in the body. (b) The dissectable white adipose tissues synthesize 27% of the fatty acids in the body. This group of tissues is heterogeneous because the mesenteric adipose tissue alone contains 40% of the labeled fatty acids present in the white adipose tissues. (c) Besides the intestines, organs other than the liver play a negligible role (2% of the total) in fatty acid synthesis. (d) The skin contributes 7% of the body fatty acid synthesis. (e) The rest of the carcass, essentially composed of the musculature and the skeleton, contributes 18% of body fatty acid synthesis and accounts for 33% of the extrahepatic tissue fatty acid synthesis.
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For a long time, the relative contribution of the main tissues and organs to fatty acid synthesis in the body has been difficult to assess, due to methodological problems. A method was needed for measuring the total rate of fatty acid synthesis in each tissue independently of the origin of the carbon atoms incorporated into the fatty acids. The measurement of the incorporation of the ^3H of ^3H_2O into fatty acids meets this need (1,2). Using this tracer, liver contribution to body synthesis of fatty acids was estimated as 10-50% in rats (3-5) and mice (6-9), depending on the nutritional conditions.

By measuring the rate of fatty acid synthesis in the main tissues and organs of the mouse, it has been determined that the muscles are the main site of fat synthesis (some data 8,9). On the contrary, in rat, the role of the principal extrahepatic tissues and organs in fatty acid synthesis is still not well known; only the white adipose tissues have been widely studied (4,5, 9-11). These studies reported (a) that, at equal weight, the adipose tissues synthesized less fat than the liver (4,5,9-11); and (b) that they contributed little to body fatty acid synthesis, at least in nonobese rats (4). Consequently, it seems that tissues other than the liver and white adipose tissues play an important part in lipogenesis in rats. In this study, we wished to determine accurately the main sites of fat synthesis in the rat by measuring the rate of the incorporation of the ^3H of ^3H_2O into fatty acids in many tissues and organs.

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METHODS

Animals and Diets

We used 7-week old Wistar male rats having a mean live weight of 228 g. From weaning at the age of 21 days, they are fed a semisynthetic diet containing 0.3% fat and 18% protein. The composition of the diet and the daily food intake of the animals has already been published (12). With this low-fat diet, we created the best conditions for observing de novo fatty acid synthesis (13,14).

The rats were reared in a room on a 12 hr dark/12 hr light cycle at a temperature of 22 C. The dark period extended from 3 A.M. to 3 P.M., so that when the animals were killed at 9-10 A.M., fat synthesis was at its highest in the tissues and organs (15).

Many studies have shown that catecholamines induce or stimulate lipolysis in different tissues (liver, muscle, adipose tissues). It is quite probable that simultaneously they check or inhibit lipogenesis. Therefore, when rats are stressed by handling and injection of ^3H_2O, causing increased catecholamine secretion, the synthesis of tissue and organ lipids studied may be affected. To keep the influence of this putative factor at a minimum, the rats used in this study were handled every day; moreover, intraperitoneal injection of ^3H_2O was chosen, as it is almost painless, easy and quick to practise, and therefore least perturbing for the animals.

In vivo Measurement of Fatty Acid Synthesis

For measuring the rate of ^3H incorporation

into the fatty acids of the main tissues and organs, each animal was given one intraperitoneal injection of ^3H_2O (2.5 mCi in 0.4 ml of a 0.9% solution of NaCl), immediately returned to its cage, and killed by decapitation 10 min later.

We chose to leave a 10-min interval between the time the radioactive tracer was injected and the time the animals were killed; this choice was based on the following observations: (a) ^3H incorporation into the fatty acids of the tissues studied (liver, adipose tissues and rest of carcass) varied almost linearly in time for at least 60 min after injection (16), proving that ^3H specific radioactivity of water remained constant in each of these tissues during that time. These conditions are characteristic of the technique of radioactive tracer perfusion. (b) Moreover, ^3H specific radioactivity of plasma water became very similar to mean ^3H specific activity of body water (obtained by lyophilization) in the 3 min following injection and remained that way for at least 120 min (Fig. 1). This let us presume that ^3H specific radioactivity of water could be considered to be uniform in all tissues during this interval of 3-120 min. (c) As to the question of fatty acid exchange among the tissues, the profile of ^3H specific radioactivity in plasma total fatty acids in relation to time (Fig. 2) shows that the export of labeled fatty acids into blood was minimal during the 10 min following tracer injection and would not modify the estimate of the amounts of lipids synthesized in situ in each tissue. After 15 min, the quantity of plasma labeled fatty acids increased rapidly, making possible important exchange among the different tissues.

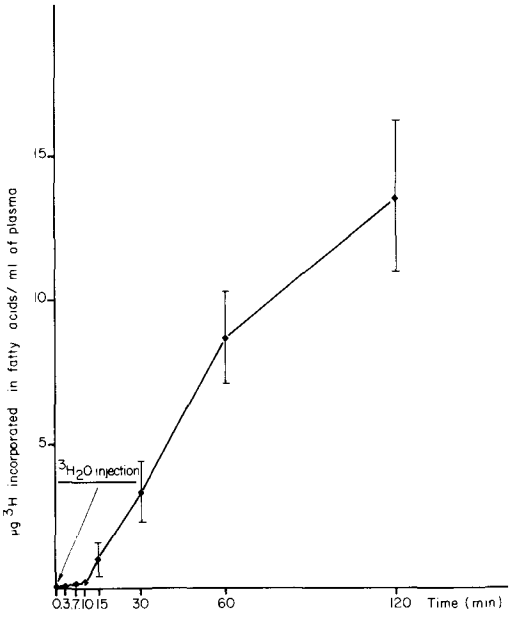

FIG. 2. Kinetics of specific total fatty acid radioactivity in plasma. Lots of 6-8 rats were decapitated immediately, 3, 7, 10, 15, 30, 60 and 120 min after intraperitoneal injection of a single dose (2.5 mCi/animal) of tritiated water. Specific fatty acid radioactivity is shown in μg of ^3H incorporated into fatty acids/ml of plasma at each time. Each point is the mean ± SD of 6-8 assays.

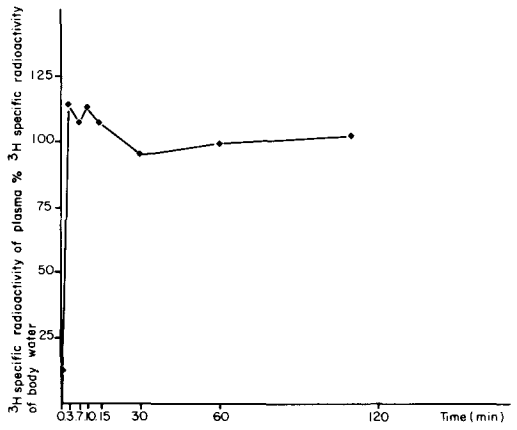

FIG. 1. Time-course of specific ^3H radioactivity in plasma water relative to ^3H radioactivity in body water. Lots of 6-8 rats were decapitated immediately, 3, 7, 10, 15, 30, 60 and 120 min after intraperitoneal injection of a single dose (2.5 mCi/animal) of tritiated water. Specific radioactivity of plasma and body water was determined by direct counting of 25 μl of water in scintillating liquid. The specific radioactivity is shown in dpm/1 μg of ^3H at each time.

Finally, considering all data, the chosen 10-minute interval appeared to be sufficient to obtain uniform distribution of ^3H_2O in the rat organism and linearity of ^3H incorporation into fatty acids over tissue. However, it appeared as well that it is not advisable to exceed this time lapse by too much, to avoid a risk of intercellular exchange.

Sampling of Tissues and Organs

After bleeding, the animal was skinned. The liver, heart, kidneys, lungs, pancreas, thymus, stomach, small and large intestines, brain and testes were dissected out and weighed. Depending on their anatomical location, the white adipose tissues were divided into 2 groups: 3 inner tissues (mesenteric, perirenal, epididymal)

and 3 outer tissues (dorsal subcutaneous and inguinal, other adipose tissues). "Other adipose tissues" included the whole of the cover fat remaining after the dorsal subcutaneous and inguinal adipose tissues were taken. The rest of the carcass included the musculature and the skeleton. The tissues and organs were immersed in liquid nitrogen as soon as they were dissected.

Measurement of ^3H Incorporation into Fatty Acids

The skin and carcass were ground under nitrogen stream. Except for the white adipose tissues, the tissues, organs, skin and rest of the carcass were freeze-dried to eliminate most of the tritiated water. The total lipids were extracted from tissue or organ lyophilisates and from the fresh adipose tissues using the method of Folch et al. (17). The lower phase (chloroform) was thoroughly washed in distilled water to avoid any ^3H_2O contamination of the fat. The weight and fat content of the liver, skin, adipose tissues and the rest of the carcass were enough to permit individual measurement of the specific radioactivity of the fats. For all the other tissues and organs, the fat was extracted from pools containing equal quantities of dry matter taken from the same organ of each animal.

After chloroform evaporation, we saponified 100-500 mg of the total lipid extract in an alcoholic solution of 10% potassium hydroxide (w/w). The nonsaponifiable lipid was extracted by hexane and discarded. After acidification, the fatty acids were extracted by 3 × 50 ml of hexane. The 3 extracts were grouped and the hexane evaporated. The fatty acids were dissolved in 4 ml of hexane and transferred quantitatively into counting vials. After 8 ml of scintillation fluid (Unisolve I Koch Light) was added, the radioactivity of each vial was measured in a "liquid scintillation counter." The quench corrections of each measurement was using the channel ratio. Specific radioactivity of the water ^3H was measured by counting 25 µl of plasma. The specific radioactivity of the water ^3H of all the organs and tissues were presumed to equal that of the plasma water.

Expression of Results

The rate of fatty acid synthesis in each tissue and organ was expressed in µg of ^3H atom incorporated into the fatty acids/10 min/g of fresh tissue or per whole tissue. The results were computed using the ratio:

$$\frac{\text{dpm incorporated into fatty acids/10 min/g or per whole tissue}}{\text{dpm in 1 µg of } ^3H \text{ atom of plasma water at 10 min}}$$

The relative contribution to the synthesis of body fatty acids of each tissue or organ, expressed in percentage, was computed as follows:

$$\frac{\text{µg of } ^3H \text{ atom incorporated into fatty acids/10 min/tissue}}{\text{µg of } ^3H \text{ atom incorporated into fatty acids/10 min/whole body}} \times 100$$

The rate of fatty acid synthesis in the whole body was obtained by calculating the sum of the synthesis rates measured in all the tissues and organs, expressed in µg of ^3H atom incorporated into the fatty acids/10 min/tissue or organ.

RESULTS AND DISCUSSION

Liver (Table 1)

In the 7-week old Wistar male rat, the liver was the main site of fatty acid synthesis; it alone synthesized 42% of all the fatty acids in the body. In rodents, the relative contribution of the liver to body fatty acid synthesis varies (10-50%) with the authors (3-9). This variability in results seems to be related to nutritional (3,14,18,19) and genetic (4,6,8) factors which considerably affect the rate of fatty acid synthesis in the liver. The dietary fat content (19), the type of carbohydrate supply (glucose-fructose) (3) and the rhythm of food intake (15, 18), in particular, modify the intensity of hepatic fatty acid synthesis. By feeding the animals a low-fat diet (0.3%, w/w) rich in carbohydrates (75%, w/w, 1/3 of which was sucrose), we created optimal conditions for observing de novo fatty acid synthesis in the liver.

Organs Besides the Liver (Tables 1 and 2)

The contribution of the 10 organs studied did not exceed 6% of the total fatty acid synthesis (Table 1). The kidneys, lungs, heart, stomach, thymus, spleen, brain and testes synthesized little lipids and thus contributed little to body synthesis of fatty acids (Table 3). These results agree with data on the mouse (9). The intestines synthesized 2.3% of the newly formed fatty acids in the body; this contribution was much less (3-10 times) than that found in the mouse (8,9). However, Hollands and Cawthorne (9), who reported that the contribution of the intestines to fatty acid synthesis equalled that of the liver in the mouse, did not separate those organs from the mesenteric adipose tissue. This tissue produces much fat, and its presence in the intestines thus leads to a considerable overestimate of their role in fatty acid synthesis. For the same reason, the contribution of the pancreas (1.8%) to body fatty

TABLE 1

Relative Contribution of the Liver, Organs, White Adipose Tissues, Skin and the Rest of the Carcass to Body Fatty Acid Synthesis in the Rat[a]

Tissues and organs	Weight (g)	Fatty acid synthesis (μg ^3H incorporated/10 min/tissue)	Contribution to body synthesis (%)
Liver	11.2 ± 0.8[e]	728 ± 146	42 ± 4
Σ Organs[b]	20.1 ± 1.3	107	6
White adipose tissues[c]	16.1 ± 3.1	464 ± 122	27 ± 3
Skin	43 ± 3	124 ± 37	7 ± 2
Rest of carcass[d]	116 ± 4	319 ± 53	18 ± 2
Whole animal	228 ± 8	1742 ± 304	100

[a]Each rat received 2.5 mCi of ^3H_2O by intraperitoneal injection 10 min before decapitation.

[b]All the organs except the liver (see list in Methods).

[c]All the dissectable white adipose tissues.

[d]Mostly the musculature and skeleton.

[e]Mean ± SD of 10 assays.

acid synthesis is probably also greatly overestimated in the present paper (Table 3); in fact, rat pancreas being diffuse, it is quite difficult to separate it completely from the mesenteric adipose tissue.

White Adipose Tissues (Tables 1 and 3)

In our nutritional conditions, the dissectable white adipose tissues represented 7% of the live weight in the 7-week old Wistar male rat. Their contribution to body synthesis of fatty acids was 27%, or ca. 50% of the fat synthesized in the extrahepatic tissues (Table 1). At equal weight, the rate of fatty acid synthesis was, on average, 2.5 times lower in adipose tissues than in the liver. However, this mean value masked a considerable heterogeneity in adipose tissue lipogenic activity which varied according to the anatomical site (Table 3). Thus, the mesenteric adipose tissue alone synthesized 40% of the labeled fatty acids found in the 6 white adipose tissues studied. At equal weight, the rate of fatty acid synthesis in this tissue was higher than in the other adipose tissue sites studied; it was 2.5-3 times higher than in other inner adipose tissues (epididymal and perirenal) and 6-9 times higher than in the outer adipose tissues (dorsal subcutaneous and inguinal). Most authors recognize that fatty acid synthesis is more intense in the inner than in the outer adipose tissues, whether the rate of synthesis is estimated in vivo with ^3H_2O (11,15) and ^{14}C-glucose (20) or in vitro with ^{14}C-acetate (21). Only Kannan and Baker (22) conclude that their experiments "provide no support for the hypothesis that there is a fundamental difference in the lipogenic capacities of various adipose tissue sites." An examination of the data of these authors (22) (Table 1, p. 48) effectively shows no significant difference in lipogenic capacity between inguinal and popliteal adipose tissues. On the other hand, there are highly significant differences (a) between epididymal and popliteal tissues ($P<0.001$), and (b) between epididymal and inguinal tissues ($P<0.01$). Consequently, when studied closely, Kannan and Baker's data agree with with those of other authors.

In any case, and whatever the experimental conditions, it appears to be impossible to obtain an accurate estimate of the contribution of the adipose tissues to whole body fatty acid synthesis by extrapolating the rate of fatty acid synthesis measured in a particular adipose tissue to the whole of the adipose mass of the animal. However, up to now, all estimates of the contribution of white adipose tissues to overall fatty acid synthesis in the rat have been obtained by this method of calculation (4,22), and therefore the validity of the results proposed should be accepted with great caution. In fact, the exact assessment of the contribution of white adipose tissues to fatty acid synthesis depends on a complete dissection of the main adipose sites of the animal. Using this methodology, frequently applied in the mouse, the contribution of white adipose tissues has been evaluated as 5-30% of the fatty acid synthesis in the body of the nonobese animal, depending on operative conditions (8,9). To our knowledge, only the present study shows an accurate contribution of the adipose tissues (27% of body synthesis) to the total fatty acid synthesis in the rat. This result is similar to the highest values observed in mouse.

TABLE 2

Relative Contribution of Different Organs to Body Fatty Acid Synthesis in the Rat[a]

Organs	Weight (g)	Fatty acid synthesis (μg ^{3}H incorporated/10 min/tissue)	Contribution to body synthesis (%)
Intestines	7.6 ± 0.4[b]	41[c]	2.3
Kidneys	1.8 ± 0.1	9	0.5
Lungs	1.5 ± 0.1	8	0.5
Heart	1.0 ± 0.1	2	0.1
Stomach	1.3 ± 0.1	4	0.2
Thymus	0.6 ± 0.1	4	0.2
Spleen	0.7 ± 0.1	1	0.1
Brain	1.9 ± 0.1	4	0.2
Testes	2.4 ± 0.2	2	0.1
Pancreas	1.3 ± 0.1	32	1.8
Total	20.1 ± 1.3	107	6.0

[a]Each rat received 2.5 mCi of ^3H_2O by intraperitoneal injection 10 min before decapitation.
[b]Mean ± SD of 10 assays.
[c]Owing to the low fat content of the organs, the rate of fatty acid synthesis was measured on pools of the equal amount of dry matter from the same organ of each rat.

TABLE 3

Relative Contribution of White Adipose Tissues to Body Fatty Acid Synthesis in Relation to Their Anatomical Site in the Rat[a]

Adipose tissues	Weight (g)	Fatty acid synthesis (μg ^{3}H incorporated/10 min/g or tissue) /g fresh tissue	whole tissue	Contribution to body synthesis (%)
Mesenteric	2.2 ± 0.5[c]	83 ± 23	183 ± 76	10.5 ± 3.0
Epididymal	2.9 ± 1.0	32 ± 10	91 ± 35	5.2 ± 2.0
Perirenal	2.1 ± 0.6	28 ± 5	57 ± 13	3.3 ± 0.4
Dorsal subcutaneous	4.6 ± 0.8	15 ± 5	70 ± 19	4.0 ± 0.9
Inguinal	1.8 ± 0.6	9 ± 4	16 ± 7	0.9 ± 0.5
Other adipose tissues[b]	2.5 ± 0.9	19 ± 9	47 ± 25	2.7 ± 1.3
Total	16.1 ± 3.1		464 ± 122	26.6 ± 2.8

[a]Each rat received 2.5 mCi of ^3H_2O by intraperitoneal injection 10 min before decapitation.
[b]Other adipose tissues = all the cover fat remaining after the inguinal and dorsal subcutaneous tissues were sampled.
[c]Mean ± SD of 10 assays.

Skin (Table 1)

The skin contributed 7% of the body fatty acid synthesis. In the mouse, the skin has a much more important role in this synthesis than in the rat since it contributes 15-30% of the fatty acids synthesized in the body (8,9).

Rest of Carcass (Table 1)

The rest of the carcass mostly included the musculature and the skeleton. This whole synthesizes 18% of the body fatty acids. If the bones are considered as only slightly synthesizing fatty acids (23), the muscles would synthesize, at most, 18% of the fatty acids in the body; therefore, contrary to data on the mouse (8,9), they are not the main site of fatty acid synthesis in the rat. There is a lively controversy among authors regarding the exact site of muscle lipogenesis, some authors attributing it to the muscle fibers (9) and others to intermuscular adipose tissue (22,24). Although this paper does not supply a direct answer to the question, we think it is more plausible to attribute fatty acid synthesis to the muscle

fibers themselves than to intermuscular adipose tissues. This hypothesis is based on two observations. (a) The fat content of the rest of the carcass is very low (54 mg/g) and half constituted of phospholipids, and therefore the intermuscular adipose tissue is probably very little developed. (b) If this adipose tissue was the lipogenic site of the rest of the carcass, its lipogenic capacity (at equal weight) would then be higher than that of all the other tissues and organs (liver or mesenteric adipose tissue included). This does not seem very likely.

To conclude, in 7-week old Wistar male rats fed a low-fat diet ad libitum, the relative contribution of the main tissues and organs to whole body fatty acid synthesis is: liver, 42%; dissectable white adipose tissues, 27%; muscles, 18%; skin, 7%; and organs besides the liver, 6%.

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Cooccurrence of C-24 Alkylated Δ^7- and Δ^5-Sterols in the Leaves of *Beta vulgaris*

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ABSTRACT

The 4-desmethylsterols from the leaves of *Beta vulgaris* are a mixture of Δ^7-sterols (71%) and Δ^5-sterols (29%). The Δ^7-sterols isolated are spinasterol (24α-ethylcholesta-7,22-dien-3β-ol; 45%), 22-dihydrospinasterol (24α-ethylcholest-7-en-3β-ol; 24%), and avenasterol (24-ethylcholesta-7,24(28)-dien-3β-ol; 1.5%). The Δ^5-sterols isolated are sitosterol (24α-ethylcholest-5-en-3β-ol; 15%), 24ξ-ethylcholesta-5,22-dien-3β-ol (7.5%), and 24ξ-methylcholest-5-en-3β-ol (7%).

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INTRODUCTION

Small amounts of 24-alkyl-Δ^5-sterols (<3%) were putatively identified in the seed oils of plants which produce predominately 24-alkyl-Δ^7-sterols (1). Since these assignments were originally based on gas liquid chromatography (GLC) and "a weak molecular ion" (1), the occurrence of Δ^5-sterols in plants producing predominately Δ^7-sterols was assumed to be "rare, if existent at all," and a genetic block for the conversion of $\Delta^7 \rightarrow \Delta^5$ sterols was postulated (2,3). Conversely, the presence of Δ^7-sterols in plants producing predominately Δ^5-sterols is, as expected, well documented (2,4). The majority of higher plant sterols are 24-alkyl-Δ^5-sterols (2,5,6) and the predominance of 24-alkyl-Δ^7-sterols appears to be restricted to a few plant families (2,7,8). However, the seed oil from the Δ^7-sterol producing plant *Trichosanthes kirilowii* was recently demonstrated to possess higher levels (25%) of Δ^5-sterols (9). This compositional data may reflect a developmental requirement of the seed and not be representative of the photosynthetic tissue. Since the leaves of *Spinacea oleracea* in the Chenopodiaceae biosynthesize Δ^7-sterols and are not reported to contain Δ^5-sterols (10) but the seed oil is reported to possess < 3% Δ^5-sterols (1), we investigated a related Chenopod *Beta vulgaris*. The beet leaf sterols provide unequivocal data for the occurrence of 24-alkyl-Δ^5-sterols in the photosynthetic tissue of a plant which by virtue of its dominant sterols is a 24-alkyl-Δ^7-sterol plant.

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MATERIALS AND METHODS

Beta vulgaris L. (table beet) was purchased in Silver Spring, MD in March, and Philadelphia, PA in July 1982. The leaf sterols from these 2 acquisitions were analyzed and comprise, respectively, samples 1 and 2 which were performed in duplicate. The leaves were washed, ground in a polytron and extracted in a Soxhlet with acetone for 48 hr. The extracted material was saponified in 5% (w/v) KOH in 70% ethanol/water. The neutral lipids after saponification (NLAS) were extracted with ether and were further fractionated by alumina chromatography using 2 column volumes of: hexane, hexane/benzene (1:1, v/v), benzene, ether, ether/methanol (1:1, v/v), and methanol. The 4-desmethylsterols eluted with the ether and were then recrystallized from methanol. Anasil B chromatography was performed with hexane/ether (8:2, v/v) (11). Argentation chromatography of sterol acetates was performed on Unisil impregnated with 5% $AgNO_3$ and eluted with 5% step increments of benzene graded into hexane.

GLC examination of the sterols was performed 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 in a Perkin-Elmer Sigma 3B with He at 35 ml/min. All RRT are to cholesterol and agree with previously published values (12,13).

Authentic standards were obtained and purified from the following sources: spinasterol and campesterol, Applied Science; stigmasterol, Sigma; sitosterol from cabbage; chondrillasterol and 22-dihydrochondrillasterol from *Chlorella*

ellipsoidea. Preparative reverse-phase liquid chromatography (RPLC) was performed on a Whatman M-20 column (25 cm x 2.5 cm) packed with Whatman ODS-3 chromatographed at 28 C with methanol/acetonitrile (1:9, v/v) 7.5 ml/min. Analytical RPLC was performed on a Zorbax ODS column (30 cm x 3 mm) with isopropanol/acetonitrile (2:8, v/v) at 45 C 1.5 ml/min on a Perkin-Elmer 3 liquid chromatograph with an 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) were calculated as previously described (14). Electron impact mass spectoscopy was performed at 70eV on a Finnigan model 4000 with a series 6000 data system. ^{1}H NMR was performed at 360 MHz at ambient temperature on a Bruker model WH 360, in $CDCl_3$ with TMS as an internal standard.

RESULTS AND DISCUSSION

Beta vulgaris leaves possess both Δ^7- and Δ^5-4-desmethylsterols with a predominance of the Δ^7-sterols (Table 1). Preliminary GLC data of the recrystallized sterols (0.02% of the wet weight) indicated the presence of only 3 components due to the chromatographic properties of the mixture on both GLC systems. Preparative RPLC of this sample also produced a chromatogram with a similar profile; however, GLC analysis of these RPLC fractions indicated the presence of 6 components (Fig. 1).

GLC and analytical RPLC of peak 1 indicated that only one sterol was present with a RRT on XE-60 of 1.88, on SE-30 of 1.87 and an α_c of 0.93. This sterol was putatively identified as avenasterol. Mass spectra further supported this identification with ions at m/e: 412 (M^+, 26%), 397 ($M\text{-}CH_3^+$, 24%), 381 ($M\text{-}CH_3\text{-}H_2O^+$, 25%), 314 ($M\text{-}C_7H_{14}^+$, 92%), 299 ($M\text{-}C_7H_{14}\text{-}CH_3^+$, 49%), 271 (M-side chain-2H$^+$, 100%), 255 (M-side chain-H_2O^+, 82%). The ion at m/e 314 is indicative of a 24(28) double bond (15). ^{1}H NMR analysis (Table 2) produced a spectrum consistent with that expected for avenasterol and agrees with the previously reported spectrum (16). The signals produced by the side chain protons are also similar to those reported for isofucosterol (17,18). The sterol in peak 1 is, therefore, determined to be avenasterol (24-ethylcholesta-7,24(28)-dien-3β-ol).

GLC of the fractions from the RPLC of peak 2 indicated that 3 sterols were present (Fig. 1). These were putatively identified as spinasterol, 24-methylcholest-5-en-3β-ol and 24-ethylcholesta-5,22-dien-3β-ol by GLC and analytical RPLC (Table 1). Separation of the spinasterol from the Δ^5-sterols was achieved by Anasil chromatography. The elution profile was Δ^5-, $\Delta^{5,22}$-, and $\Delta^{7,22}$-sterol. The $\Delta^{7,22}$-sterol was isolated in pure form. The mass spectrum of the $\Delta^{7,22}$-sterol produced the following 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^+,

TABLE 1

Composition[a] and Chromatographic Characteristics of 4-Desmethylsterols from *Beta vulgaris*

Sterol	% of total 4-desmethylsterols		GLC[b] RRT	GLC[b] RRT	RPLC[c]
	Sample 1[d]	Sample 2[d]	SE-30	XE-60	α_c
Δ^7 Sterols					
Spinasterol	43	47	1.57 ± 0.01	1.52 ± 0.03	1.10 ± 0.01
22-Dihydrospinasterol	23	25	1.81 ± 0.02	1.71 ± 0.06	1.26 ± 0.02
Avenasterol	1.6	1.4	1.87 ± 0.01	1.88 ± 0.02	0.93 ± 0.01
Δ^5 Sterols					
Sitosterol	16	13	1.62 ± 0.01	1.55 ± 0.02	1.23 ± 0.01
24ξ-Ethyl-cholesta-5,22-dien-3β-ol	8.5	7.1	1.42 ± 0.02	1.32 ± 0.02	1.07 ± 0.01
24ξ-Methyl-cholest-5-en-3β-ol	7.7	6.5	1.30 ± 0.01	1.30 ± 0.02	1.10 ± 0.01
Total					
Δ^7 Sterols	67.6	73.4			
Δ^5 Sterols	32.2	26.6			

[a]From GLC following preparative RPLC.

[b]RRT to cholesterol.

[c]α_c to cholesterol.

[d]Separate acquisitions and isolations performed in duplicate, see Methods.

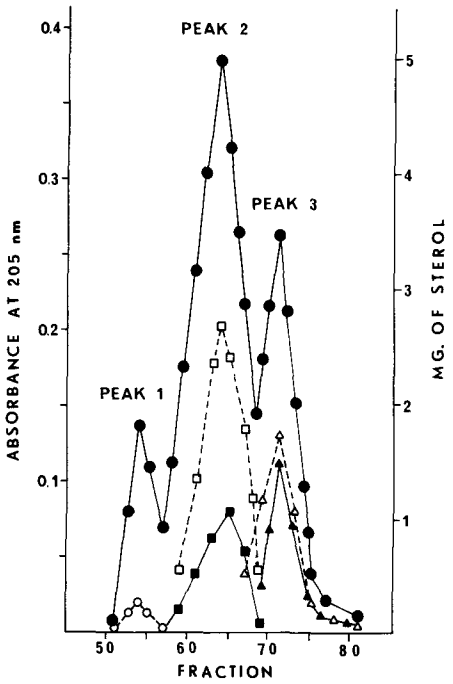

FIG. 1. Comparison of RPLC and GLC profiles of the sterols from *Beta vulgaris*. Elution of sterols from preparative RPLC, A at 205 nm (•). GLC analysis in mg/RPLC fraction (20 ml) on XE-60. Avenasterol (○), spinasterol (□), 22-dihydrospinasterol (△), Δ^5-24-methyl- and $\Delta^{5,22}$-24-ethylcholesterol (■), sitosterol (▲).

100%). The ^{1}H NMR analysis (Table 2) produced a spectrum consistant with that of authentic spinasterol run at 360 MHz and different from that of authentic chondrillasterol (Table 2). The chemical shifts of the isomeric standards are distinctly different as previously reported (13,19,20). The chemical shifts observed in this component indicate that it is the 24α-ethylcholesta-7,22-dien-3β-ol and not an isomeric mixture as observed in *Cucurbita pepo* seeds (20) or roots of *Trichosanthes japonica* (21). The two Δ^5-sterols eluted as an incompletely separated mixture from the Anasil column and, due to the limited amount of material (4 mg), further separation was not attempted. The mixed mass spectrum of the two sterols provided the following ions at m/e for the $\Delta^{5,22}$-ethylsterol: 412 (M^+, 57%), 397 ($M-CH_3^+$, 17%), 369 ($M-C_3H_7^+$, 22%), 271 (M-side chain-$2H^+$, 75%, 255 (M-side chain-H_2O^+, 100%); for the Δ^5-methylsterol: 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 mixed ^{1}H NMR analysis indicated chemical shifts for protons at C-18, C-19 and C-21 (Table 2). The nature of the mixture made assignments of additional chemical shifts difficult. The signal for the protons at C-18 for the methylsterol component was sufficiently broad to indicate the existence of an epimeric mixture at C-24 for the methylsterol component.

TABLE 2

^{1}H NMR Spectra of 4-Desmethylsterols from *Beta vulgaris* and Authentic Sterols

Sterol	Proton position[a]				
	3H, H-18 (s)	3H, H-19 (s)	3H, H-21 (d)J=6 Hz	6H, H-26, H-27 (d,d)J=6 Hz	3H, H-29 (t)J=7 Hz
Beta vulgaris					
Δ^7 Sterols					
Spinasterol	0.56	0.80	1.03	0.80, 0.86	0.81
22-Dihydrospinasterol	0.54	0.80	0.94	0.82, 0.84	0.85
Avenasterol	0.54	0.80	0.95	0.98, 0.98	1.58
Δ^5 Sterols					
Sitosterol	0.68	1.01	0.92	0.81, 0.83	0.85
24ξ-Ethyl-cholesta-5,22-dien-3β-ol	0.70	1.01	1.03	nd nd	nd
24ξ-Methyl-cholest-5-en-3β-ol	0.68	1.01	nd	nd nd	–
Authentic standards					
Spinasterol	0.56	0.80	1.03	0.80, 0.86	0.81
Chondrillasterol	0.55	0.80	1.03	0.83, 0.85	0.80
22-Dihydrochondrillasterol	0.55	0.80	0.94	0.82, 0.84	0.86
Sitosterol	0.68	1.01	0.92	0.81, 0.83	0.85

[a]Chemical shift in ppm from TMS at 360 MHz.
nd = Not determined due to mixture of these two compounds.

The GLC of the fractions from the RPLC of peak 3 indicated that 2 sterols were present (Fig. 1). These were putatively identified as 22-dihydrospinasterol and sitosterol by GLC and analytical RPLC (Table 1). The mixture was converted to the sterol acetates and then separated by argentation chromatography. Both compounds were isolated in pure form and then converted to the free alcohol. Mass spectrum of the Δ^7-ethylsterol produced the following 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 analysis (Table 2) produced a spectrum which was in accord with a 24α-ethylcholest-7-en-3β-ol. The spectra of the 24α- and 24β-isomers are very similar (13,20). Since our sample has a different chemical shift for the protons of C-29 from that of authentic 22-dihydrochondrillasterol, we assume it to be the 24α-ethyl isomer. The mass spectrum of the 24-ethylcholest-5-en-3β-ol produced the following ions at m/e: 414 (M^+, 92%), 399 ($M\text{-}CH_3^+$, 61%), 396 ($M\text{-}H_2O^+$, 43%), 381 ($M\text{-}CH_3\text{-}H_2O^+$, 16%), 303 ($M\text{-}C_7H_9\text{-}H_2O^+$, 61%), 273 (M-side chain$^+$, 61%), 255 (M-side chain-H_2O^+, 100%). The ^{1}H NMR analysis (Table 2) produced a spectrum identical with that of authentic sitosterol run at 360 MHz.

The reproducibility of the relative ratios of Δ^5-alkylsterols to Δ^7-alkylsterols from *Beta vulgaris* leaves obtained from 2 different sources indicates that the composition of this mature tissue maintains a fixed ratio of these products. UV scans of the RPLC peaks from the mixture and isolated components indicated the absence of any detectable quantities of $\Delta^{5,7}$-alkylsterols. This suggests either that the "normally assumed" biosynthetic route to Δ^5-sterols is operating and that the rate of introduction of the 5(6) double bond is limited while the reduction of the Δ^7 proceeds normally, or that 2 alternative pathways to each end product exist. A systematic examination of the sterol metabolism in beets and related species in the Chenopodiaceae, e.g. spinach, may yield insight into the regulatory mechanisms in these organisms.

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Absorption and Metabolic Fate of Dietary ^{3}H-Squalene in the Rat

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ABSTRACT

The absorption and metabolic fate of dietary squalene were investigated on the rat by administering a single oral dose of ^{3}H-squalene and ^{14}C-cholesterol. Experiments on rats with cannulated thoracic duct revealed that ^{3}H-squalene was, like ^{14}C-cholesterol, absorbed through the lymphatic vessels and that ca. 20% of absorbed ^{3}H-squalene was cyclized to sterols during the transit through the intestinal wall. Feces contained ^{3}H-sterols, indicating that newly synthesized mucosal sterols had been secreted into the gut lumen. In intact animals, ^{3}H-squalene appeared in the circulation more rapidly than ^{14}C-cholesterol and did not persist to any significant extent in the squalene-rich adipose and muscle tissues. The increase in dietary squalene load (8-48 mg) decreased the absorption percentage of ^{3}H-squalene (45-26%) but did not affect the absorption of ^{14}C-cholesterol (47%). Determination of fecal steroids revealed that during the first days absorbed ^{3}H-squalene was eliminated to a significantly higher extent than ^{14}C-cholesterol as fecal bile acids (34% vs 11%). The experiments indicate that the rat intestine has a marked capacity for absorbing dietary squalene and that the absorbed squalene is preferentially converted to bile acids in the liver.
Lipids 18:233-238, 1983.

Squalene is a minor constituent of serum lipoproteins (1-5) and is present in fairly high concentrations in triglyceride-rich mammalian tissues (4,6). Variable amounts of squalene have been detected in dietary ingredients (4,7). Certain fish and plant oils, olive oil, in particular, contain substantial amounts of squalene (7-10).

Intake of diets rich in squalene has clearly augmented the squalene concentrations in human serum (4). Furthermore, feeding squalene has increased squalene and sterol concentrations in serum and liver of the rat and enhanced fecal excretion of bile acids (11), suggesting that squalene absorbed from the diet participates in the overall cholesterol synthesis. However, the rate of absorption of dietary squalene and its metabolic fate have not been investigated in detail.

In this investigation, the rats were given a single oral dose of ^{3}H-squalene and the appearance of different ^{3}H-labeled lipids in serum, tissues and feces was followed. For comparison, the animals were simultaneously given ^{14}C-cholesterol.

MATERIALS AND METHODS

Isotopes

[4-^{14}C]-cholesterol (58 mCi/mol) was obtained from New England Nuclear Corp., Boston, MA. ^{3}H-squalene was prepared biochemically (12) from DL-(2-^{3}H)-mevalonic acid lactone (500 Ci/mol, Radiochemical Centre, Amersham, England). The specific activity of [1,1,5,5,9,9,16,16,20,20,23,23-^{3}H]-squalene was 969 Ci/mol. The final specific activities of the labeled lipids are given separately. Unlabeled squalene and the standard substrates, 5α-cholestane, lanosterol and α-tocopherol were purchased from Sigma Ltd. (St. Louis, MO).

Animals and Diets

Male Sprague-Dawley rats (Anima Ltd., Finland) weighing 260-280 g were used for the experiments. The rats, through an indwelling duodenal catheter, were given 2 ml of aqueous suspension containing 80 mg milk powder (Valio Ltd., Finland), 0.24 ml of olive oil (Fischer Scientific Co., NJ), 15.4 mg chromic oxide and ^{14}C-cholesterol (final specific activity 0.23 mCi/g), and various amounts of ^{3}H-squalene. Radioactive substrates were dissolved in acetone and mixed with milk powder-olive oil suspension. Subsequently, the solvent acetone was evaporated under nitrogen.

Cannulation of the Rats

For studies on the absorption route of ^{3}H-squalene, the thoracic duct of the rats was cannulated as described by Bollman et al. (13). The animals were placed in a restraining cage and given a 2 ml bolus of suspension containing ^{3}H-squalene and ^{14}C-cholesterol. Thereafter, Intralipid® (A.B. Vitrum, Sweden) was given as a constant duodenal infusion and the lymph was collected. Feces of the rats were gathered on the blotting paper placed under the restraining cage, dried in vacuo at 50 C and weighed.

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Analyses

The rats were exsanguinated under ether anesthesia and the organs were removed and washed with saline at room temperature. The samples were homogenized with Ultra-Turrax® and extracted with 20 vol of chloroform/methanol (2:1) in the usual manner (14). Squalene, different methyl sterols and cholesterol were separated on Silica Gel G (E. Merck, Darmstadt) and quantitated by gas chromatography (6).

Fecal neutral sterols were extracted as described by Miettinen et al. (15). After extraction of neutral sterols, the fecal bile acids were determined by the method of Grundy et al. (16). Chromic oxide was used as an unabsorbable marker and internal standard (17).

The radioactivity of the double labeled samples was counted in a Packard 3003 Tricarb scintillation counter at 4 C with 18 ml of 0.5% diphenyloxazole (PPO) in toluene. The counting efficiency and the shift of counts from the carbon to the tritium channel were determined for each sample. The absolute level of radioactivity (dpm) was determined by means of an external standard.

Calculations

Because four ^{3}H from the twelve ^{3}H of ^{3}H-squalene are lost during demethylation of ^{3}H-lanosterol to ^{3}H-cholesterol (18), the radioactivity (dpm) of ^{3}H-cholesterol was multiplied by 1.5. Because three further ^{3}H were lost during 7α-hydroxylation of cholesterol and the loss of terminal propionic acid, the radioactivity of ^{3}H-bile acids was multiplied by 1.6.

The results are given as a mean and a standard error of mean (SEM). The statistical significance of the difference was estimated by the Student's two-tailed t-test.

RESULTS

Absorption of ^{3}H-Squalene and ^{14}C-Cholesterol in Rats with Cannulated Thoracic Duct

After administration of a milk powder-olive oil suspension containing ^{3}H-squalene and ^{14}C-cholesterol through an indwelling duodenal catheter, the radioactivities appeared in the thoracic duct within 2 hr (Fig. 1). The absorption of ^{3}H-squalene appeared to be completed in 12 hr when ca. 15% of the dose appeared in chyle. ^{14}C-Cholesterol absorption continued up to 24 hr at least when ca. 10% of the dose was recorded. No radioactivity was found in serum of rats with cannulated thoracic duct.

Analyses of ^{3}H-lipids of chyle at 4 hr revealed that 8% and 13% was found in the methyl sterols and cholesterol fractions, respectively. In feces of the rats, up to 20% of ^{3}H-radioactivity was found in sterols but fecal bile acids were not labeled. Thus, a portion of sterols synthesized by the rat intestine from ^{3}H-squalene was excreted with feces. About one-third of the fecal ^{3}H-sterol counts were in the cholesterol fraction and two-thirds in the coprostanol fraction.

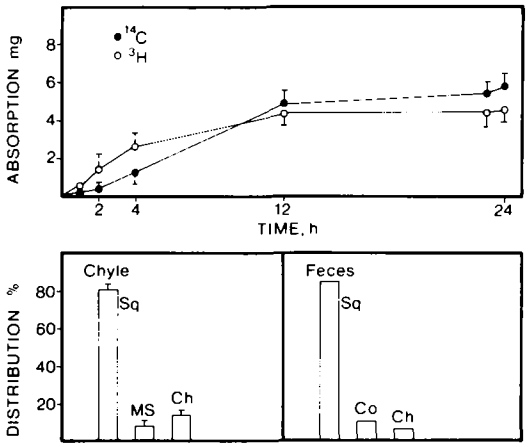

FIG. 1. Cumulative absorption of ^{3}H-squalene and ^{14}C-cholesterol into the thoracic duct and percentage distribution of ^{3}H-radioactivity between squalene (Sq), methyl sterols (MS), cholesterol (Ch) and coprostanol (Co) in the chyle and feces. Thoracic duct cannulated rats were given 2 ml of milk powder-olive oil suspension containing 33 mg of ^{3}H-squalene (77 μCi/g) and 65 mg of ^{14}C-cholesterol (17.5 μCi/g) via an indwelling duodenal catheter. The content of the thoracic duct was collected and the absorption rate of the compounds was calculated by dividing the total radioactivities in the chyle by the specific activities of the test lipids. Each point is a mean ± SEM for 3 animals. The distribution of ^{3}H-radioactivity of the chyle was determined at 4 hr.

Absorption of ^{3}H-Squalene in Intact Animals

When the rats were given a single dose of a mixture of ^{3}H-squalene (8 mg) and ^{14}C-cholesterol (15.4 mg) through the duodenal cannula, the absorption percentages were 42% and 48%, respectively (Table 1). A gradual increase of unlabeled squalene in the test mixture from 8 to 48 mg decreased the absorption percentage of squalene (r-value between dietary squalene load and the absorption percentage was -0.801, $p < 0.05$) but increased the absolute absorption of squalene from 3 mg to 12 mg. Variations in the dietary squalene did not affect the absorption rate of ^{14}C-cholesterol.

Kinetics of Absorbed ^{3}H-Squalene in Plasma

Kinetics of absorbed ^{3}H-squalene was studied by measuring the specific activities of plasma

TABLE 1

Absorption of Labeled Squalene and Cholesterol Administered as a Single Oral Dose

Rat	^{3}H-Squalene						^{14}C-Cholesterol (μCi)				
	mg			μCi			Diet	Feces			
	Diet	Feces	Abs%	Diet	Feces	Abs%		Cholesterol	Coprostanol	Total	Abs%
1	8	4.3	46	14.63	8.0	45	3.55	0.94	0.83	1.77	50
2	8	5.5	31	14.63	8.9	39	3.55	1.05	0.87	1.92	46
3	28	17.2	38	14.63	9.7	24	3.55	0.84	1.60	2.44	32
4	28	18.8	33	14.63	10.3	30	3.55	0.74	1.26	2.00	44
5	48	38.3	20	14.63	10.5	28	3.55	0.75	1.08	1.83	49
6	48	37.6	22	14.63	10.8	26	3.55	1.17	0.54	1.71	52
Mean ± SEM			32 ± 4.4			32 ± 3.7					46.5 ± 3.2

The rats were given 2 ml of aqueous mixture containing 80 mg of milk-powder, 0.24 ml olive oil, carmine red, 15.4 mg of chromic oxide and ^{14}C-cholesterol and various amounts of ^{3}H-squalene through an indwelling duodenal catheter. The radioactivities of feces collected during two days were measured and corrected against the recovery of chromic oxide.

squalene, methyl sterols and cholesterol serially after a single duodenal administration of ^{3}H-squalene and ^{14}C-cholesterol. Plasma ^{3}H-squalene was rapidly labeled, reaching the maximum specific activity at 6 hr (Fig. 2). At 24 hr, virtually no ^{3}H-squalene was found in serum, while the specific activity of ^{14}C-cholesterol increased up to 24 hr. The absorption of ^{3}H-squalene was followed by appearance of ^{3}H-methyl sterols and ^{3}H-cholesterol in plasma. The specific activity of ^{3}H-methyl sterols at 6 hr was clearly below that of ^{3}H-squalene, but markedly higher than that of ^{3}H-cholesterol at 24 hr.

Distribution of Absorbed ^{3}H-Squalene in Tissues

The rapid disappearance of absorbed ^{3}H-squalene from plasma could have been due to its effective conversion to cholesterol or transfer to tissues. The latter alternative was studied by tissue analysis after a single duodenal dose of the ^{3}H-squalene/^{14}C-cholesterol mixture. The results in Table 2 show that neither ^{3}H-squalene nor ^{3}H-methyl sterols endured in tissues. Up to 98% of ^{3}H-radioactivity discovered in the rat tissues after 2 days was found in the cholesterol fraction. Even in adipose tissue and skin the ^{3}H-radioactivity, retained as cholesterol precursors, was less than 0.1% of the absorbed dose of ^{3}H-squalene.

The ^{3}H/^{14}C ratio in cholesterol of different organs was quite similar, suggesting that the

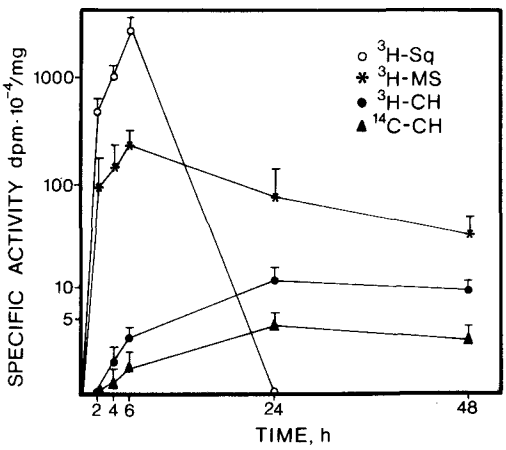

FIG. 2. Labeling of plasma squalene (Sq), methyl sterols (MS) and cholesterol (Ch) after a single oral dose of a mixture containing ^{3}H-squalene and ^{14}C-cholesterol. Six rats were given through a duodenal catheter 2 ml of milk powder-olive oil suspension containing 28 mg ^{3}H-squalene (0.52 mCi/g) and 15.4 mg ^{14}C-cholesterol (0.23 mCi/g) and blood samples were taken from the tail vein after different time intervals.

TABLE 2

Radioactivities of Organs Two Days After a Single Oral Dose of ^{3}H-Squalene and ^{14}C-Cholesterol

Organ	^{3}H-Radioactivity						^{14}C-Cholesterol		^{3}H-Ch[a] / ^{14}C-Ch
	Squalene		Methyl sterols		Cholesterol[a]				
	dpm mg	dpm×10^{-3} organ	dpm mg	dpm×10^{-3} organ	dpm mg	dpm×10^{-3} organ	dpm mg	dpm×10^{-3} organ	
Serum	–	–	550	0.03	103	337 (3.7)	49	149 (4.1)	2.33 ± 0.18
Liver	10	1.0	93	4.7	28	774 (8.6)	12	373 (10.4)	2.12 ± 0.19
Kidney	13	0.1	16	0.1	19	37 (0.4)	8	16 (0.5)	2.28 ± 0.23
Muscle	4	3.7	17	3.5 (0.04)	11	312 (3.4)	4	127 (3.5)	2.43 ± 0.18
Heart	15	0.2	71	0.1	22	35 (0.4)	10	15 (0.4)	2.49 ± 0.20
Lung	105	0.3	368	0.4	97	147 (1.6)	43	66 (1.8)	2.24 ± 0.19
Skin	19	9.6 (0.09)	10	1.5	22	353 (3.9)	9	120 (3.4)	2.39 ± 0.36
Adipose tissue	20	7.7 (0.08)	47	9.4 (0.1)	5	164 (1.8)	2	74 (2.1)	2.27 ± 0.25
Total		22.6 (0.20)		19.7 (0.2)		2159 (21.0)		940 (26.0)	

n = 5. Percent of absorbed dose in parenthesis.

[a]The loss of ^{3}H-radioactivity in Ch synthesis is corrected.

dietary ^{14}C-cholesterol and the newly formed ^{3}H-cholesterol had been similarly distributed in tissue pools.

Fecal Elimination of Absorbed ^{3}H-Squalene and ^{14}C-Cholesterol

Within 2 days after intraduodenal administration of the labels, 34% and 31% of the ^{3}H-squalene absorbed were excreted as fecal ^{3}H-bile acids and ^{3}H-neutral sterols, respectively, whereas ca. 11% of absorbed ^{14}C-cholesterol was eliminated as ^{14}C-bile acids (Table 3). Because a portion, about one-third, of ^{3}H-sterols originated from intestinal mucosa (Fig. 1), the liver eliminated newly synthesized ^{3}H-cholesterol into bile preferentially as bile acids and less so as cholesterol.

DISCUSSION

Virtually no studies are available in the literature on absorption and subsequent metabolism of dietary squalene in man or experimental animals. The experiments of the present study indicated that ^{3}H-squalene is effectively absorbed and transported, like sterols, through the thoracic duct. The more rapid appearance of dietary ^{3}H-squalene than ^{14}C-cholesterol in chyle and serum can be partly explained by the fairly high difference of squalene concentration between the test mixture and intestinal mucosa cells. Isotopic exchange could have also contributed to the different velocity of absorption. The dietary ^{14}C-cholesterol mixes in the gut lumen and mucosal cells with endogenous cholesterol, resulting in a decrease in the specific activity (19-21). Because of the low squalene concentration of intestinal mucosa (6), this dilution effect might be negligible for ^{3}H-squalene.

Labeling of methyl sterols and cholesterol of chyle and feces after duodenal administration of ^{3}H-squalene to thoracic duct cannulated rats indicates that a portion of the absorbed ^{3}H-squalene is cyclized to sterols during the first pass through the intestinal wall. A portion of these labeled sterols are excreted in feces either via sloughed mucosal cells, isotopic exchange or direct luminal secretion. The bulk apparently reaches the chyle. The intestinal mucosa releases also newly synthesized cholesterol precursors into the thoracic lymph. Thus, intestine contributes also to serum methyl sterols though bulk of them may originate from the liver (2).

The experiments on intact animals showed that the rat intestine has a remarkable capacity to absorb dietary squalene and that squalene transferred to the circulation is rapidly taken up by the liver, where it is cyclized to sterols and bile acids. Only a negligible portion of ^{3}H-

TABLE 3

Fecal Elimination of Absorbed ^{3}H-Squalene and ^{14}C-Cholesterol in the First 2 Days (n = 6)

	Fecal lipid (% of absorption)			
	Neutral sterols			
Absorbed lipid	Cholesterol	Coprostanol + methyl sterols	Total	Bile acids
^{3}H-Squalene	5.6 ± 0.2	25.5 ± 3.8	31.1 ± 3.8	34.0 ± 3.8
^{14}C-Cholesterol	nd	nd	nd	10.8 ± 0.6

$p < 0.001$; nd = not determined. The rats were given 2 ml of milk powder-olive oil suspension containing 15.4 mg of ^{14}C-cholesterol (sa = 0.23 mCi/g) and 28 mg of ^{3}H-squalene (sa = 0.52 mCi/g) through a duodenum catheter. Stool was collected during the 2 days after oral labeling of rats. The absorption rates were calculated from the fecal elimination of the labeled test compounds. The radioactivities of fecal bile acids were compared with the radioactivities of absorbed squalene and cholesterol. The corresponding calculation of the rate of the reelimination of ^{14}C-cholesterol was not possible.

squalene absorbed from a single oral dose was detected in squalene-rich adipose and muscle tissues. Recent human and experimental studies have indicated that the half-life of serum squalene is shorter than that of other lipoprotein constituents and that serum squalene is not taken up by adipose tissue to any significant extent (4,5,22).

The efficient uptake of ^{3}H-squalene absorbed from the diet might explain the rapid excretion of newly synthesized ^{3}H-sterols and ^{3}H-bile acids with feces. In fact, 21% of ^{3}H absorbed was found as ^{3}H-sterols in tissues, whereas 65% was eliminated with feces in 2 days. The remaining 14% might be distributed among tissues not checked by this study. Because the specific activities of ^{14}C-cholesterol of serum and liver had not equilibrated within 2 days, the exact calculation of the rate of reelimination of absorbed ^{14}C-cholesterol was not possible. Taking into consideration the retention of ^{14}C-cholesterol in tissues (26%), the rate of its elimination as ^{14}C-bile acids (11%), and the percentage recovery of ^{3}H-squalene (86%), it could be estimated that ca. 49% of adsorbed and absorbed ^{14}C-cholesterol was reeliminated as fecal neutral sterols within the first 2 days.

Thus, the dietary ^{3}H-squalene was eliminated more efficiently than ^{14}C-cholesterol as fecal bile acids. The observation supports earlier results which have pointed out to the role of newly synthesized hepatic cholesterol as a preferential source of bile acids (23-25). It is also in good agreement with experiments on the rat which have shown that serum ^{3}H-squalene is eliminated mainly as bile acids (22) and that feeding squalene significantly increases fecal bile acids (11).

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ERRATUM

In the article "Stimulation of Hepatic Squalene and Triglyceride Synthesis by Dimethylsulfoxide, in vitro" by F.P. Bell and E.V. Hubert (*Lipids* 17:900-904, 1982), three lines of text were misplaced. Instead of being placed after line 28 of the Discussion (p. 903), the lines "sisted under conditions of an oxygen-rich atmosphere (Table 3, exp. 1) as well as under an N_2 atmosphere (Table 3, exp. 2)." should follow on from the end of the Results. The last sentence of the Results (p. 902) should therefore read: "The results show that an increased incorporation of [^{14}C] mevalonate into squalene in the presence of DMSO persisted under conditions of an oxygen-rich atmosphere (Table 3, exp. 1) as well as under an N_2 atmosphere (Table 3, exp. 2)."

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METHODS

Determination of Lipase Specificity[1]

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ABSTRACT

Specificity of lipases is controlled by the molecular properties of the enzyme, structure of the substrate and factors affecting binding of the enzyme to the substrate. Types of specificity are as follows. I. Substrate: (a) different rates of lipolysis of TG, DG, and MG by the same enzyme; (b) separate enzymes from the same source for TG, DG and MG. II. Positional: (a) primary esters; (b) secondary esters; and (c) all three esters or nonspecific hydrolysis. III. Fatty acid, preference for similar fatty acids. IV. Stereospecificity: faster hydrolysis of one primary *sn* ester as compared to the other. V. Combinations of I-IV. Lipases with these specificities are: Ia, pancreatic; Ib, postheparin plasma. IIa, pancreatic; IIb, *Geotrichum candidum*, for fatty acids with *cis*-9-unsaturation, and IIc, *Candida cylindracea*. III, *G. candidum* for unsaturates. IV. *sn*-1, postheparin plasma and *sn*-3 human and rat lingual lipases. V. Rat lingual lipase. Methods for determination involve digestion of natural fats of known structure and synthetic acylglycerols followed by analysis of the lipolysis products. All of the types of specificity have been detected with use of synthetic acylglycerols. Detection of stereospecificity requires enantiomeric acylglycerols which are difficult to synthesize, so other methods have been developed. These involve the generation of 1,2-(2,3) DG and resolution of the enantiomers. Trioleoylglycerol or racemic TG can be used as substrates. If the lipase is stereospecific, then either the *sn*-1,2- or 2,3-enantiomer will predominate. The relative amounts of the enantiomers can be determined by measurement of specific rotation, and nuclear magnetic resonance spectra. The DG can also be separated by conversion to phospholipids and hydrolysis with phospholipases A-2 or C. Applications of these procedures are discussed and data on the specificity of various lipases presented.
Lipids 18:239-252, 1983.

Lipases or acylglycerol hydrolases are enzymes which catalyze the hydrolysis of long-chain aliphatic acids from acylglycerols at an oil/water interface. The systematic name is acylglycerol acylhydrolase. The interface is usually provided by emulsion globules or lipoprotein particles, the latter primarily chylomicrons and very low density lipoproteins. The element providing the interface has been termed the supersubstrate (1).

Enzymes are classified by the types of reaction catalyzed or specificity (2). Specificity is a comparative difference in rates of catalysis of certain reactions. After an enzyme has been identified as a lipase, several specificities within the class have been identified or can be expected to occur. We will discuss the types, control, examples and determination of lipase specificities in this review. A preliminary report on this information has been presented (3).

CONTROL OF SPECIFICITY

Specificity of lipases is controlled by: (a) the molecular properties of the enzyme, (b) the structure of the substrate, and (c) factors affecting binding of the enzyme to the substrate or otherwise influencing activity.

In (a), properties are usually beyond our control, except that the enzyme being investigated should meet the definition of a lipase. It is likely that the enzyme preparation under study may contain lipases with more than one type of specificity and purification or other strategies will be required.

For (b), the structure of the acylglycerol selected as a substrate obviously determines the identity of the digestion products used to identify the type of specificity. Consequently, the acylglycerol must be structured so that specificities are not confused. Examples will be given later. Presumably the digestion mixture used to assay for activity of the lipase can be employed

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Abbreviations: TG, triacylglycerols; DG, diacylglycerols; MG, monoacylglycerols; FFA, free fatty acids. Trioleoylglycerol is 18:1-18:1-18:1, etc. 1,2-dioleoyl-3-palmitoyl-*sn*-glycerol is *sn*-18:1-18:1-16:0, with the *sn*-1 ester to the left. If the TG is racemic, *rac* is omitted.

to determine specificity with suitable selections of substrate. Digestion conditions must provide the products of lipolysis needed to determine specificity. These are DG, MG, and FFA. The extent of digestion must be regulated so as to produce the amounts of DG, MG and FFA required, yet avoid or reduce acyl migration of the partial acylglycerols (4). With DG, the equilibrium mixture is 60:40 1,3: 1,2 (2,3) and MG, 90:10 1(3):2. Extremes of heat and pH and exposure to silicic acid hasten acyl migration. Fielding (5) stabilized 2-MG in a digestion mixture to acyl migration with 2mM 5,5′ dithiobis (2-nitrobenzoic acid). The effect of the compound on acyl migration in DG has not been reported. Detection and assay of lipases have been reviewed (4). Methods for recovery and identification of DG, MG and FFA are described in (1) and (6). High performance liquid chromatography (HPLC) has been employed to resolve these lipids (7). The method has the advantage of speed as compared to the separations obtained by thin layer chromatography (TLC). High temperature gas liquid chromatography (GLC) is also effective, equaling TLC and HPLC in resolution, and is better than both in quantitation of the lipolysis products. Examples of this procedure will be presented later.

Implicit in (c) are the needs for stable and comparable emulsions or lipoprotein particles which provide maximum surface areas and for rapid mixing of the digestion mixtures. Dervichian and Barque (8) have found that the quantity of lipase absorbed onto the substrate is one of the controlling factors in hydrolysis and that mixing hastens the diffusion of enzyme to the oil/water interface. Mixing will also help stabilize the emulsion by preventing the coalescence of globules that would occur during quiescence. Finally, mixing will assist in removal of FFA from the lipase-substrate domain and this is important because FFA inhibits many lipases (4).

TYPES OF SPECIFICITY

The known and expected types are as follows: I. Substrate; (a) different rates of lipolysis of TG, DG and MG by the same enzyme, (b) separate enzymes from the same source for TG, DG and MG. II. Positional; (a) primary esters, (b) secondary esters and (c) all three esters, nonspecific or random hydrolysis. III. Fatty acid, preference for types of fatty acids, e.g., short-chain, etc. IV. Stereospecificity; faster hydrolysis of one primary *sn* ester as compared to the other. V. Combinations of I-IV. Examples are given in Table 1 and discussed below.

TABLE 1

Types of Lipase (Acylglycerol Hydrolase) Specificity

Type	Source of lipase	Reaction	References
I. Substrate			
a. Different acylglycerols: Same lipase	Pancreas	TG>DG>MG 16:0-16:0-4:0>16:0-4:0-4:0	9,10,11
b. Different lipases: for TG, DG and MG	Postheparin plasma (lipoprotein lipase)	DG→MG→glycerol	5,18-27
II. Positional			
a. Primary esters	Pancreas	TG→1,2(2,3)DG→2-MG	9,10,11
b. Secondary esters	*Geotrichum candidum*	Acid must contain *cis*-9-double	33
c. All esters	*Candida cylindracea*	TG→1,2+2,3+1,3 DG→1-+2-MG	34
III. Fatty acid			
a. 4:0-10:0	Pregastric esterase	4:0-10:0>12:0-18:0, 18:1	40
b. *cis*-9-unsaturation	*G. candidum*	Acid must contain *cis*-9 double	33
c. 8:0-12:0	Rat and human lingual preparations	8:0-12:0>16:0, 18:1	41-44
IV. Stereospecificity			
a. *sn*-1-ester	Postheparin plasma, bovine and human milks, adipose tissue, liver, (lipoprotein lipase)	*sn* 1:*sn*-3,2:1	45-48
b. *sn*-3-ester	Rat and human lingual preparations; termite	*sn* 3:*sn*:1,4:1	43,46,49,50
V. Combinations			
a. Fatty acid and stereospecificities	Rat lingual preparations. Human milk lipoprotein lipase	*sn*-R-R[a]-12:0>*sn*-R-R-16:0 8:0, 10:0, 12:0 and 18:0 TG>16:0-16:0-16:0. *sn*-1:*sn*-3,2:1	43,44,45

[a]R-long-chain fatty acids in human milk.

EXAMPLES OF SPECIFICITY

I. Substrate Specificity

(a) Different acylglycerols, same lipase. Ia. Acylglycerols are hydrolyzed by pancreatic lipase at the following relative rates (9,10): TG > 1,2 (2,3)-DG > 1,3-DG > 1(3)-MG > 2-MG. The 1,3-DG and 1(3)-MG are the result of spontaneous acyl migration, not enzymatic action, as pancreatic lipase is almost completely specific for primary esters (10,11). These data were obtained by measuring the acylglycerols at intervals during a digestion except that the isomers of DG and MG were not separated. The experiment should be repeated at time intervals with quantitation of the isomers. Another difficulty was the presence of a nonspecific esterase or carboxyl ester hydrolase, at least in crude preparations of pancreatic lipase, dependent on bile salts, which digested primary and secondary esters of alcohols (12,13). The activity was eliminated by storage of pancreatic juice solution at pH 9 and 40 C for 2 hr, absence of bile salts during digestion or addition of the esterase inhibitor, diethyl-*p*-nitrophenyl phosphate. Nonspecific esterase was also eliminated by treatment with chymotrypsin or purification (14). The specificity has also been investigated by separately digesting the acylglycerols, with the exception of 1,2 (2,3) DG in the reaction sequence above and the same rate order obtained (15,16).

Triacylglycerols of divergent molecular weights were lipolyzed at different rates by pancreatic lipase (17). Several acylglycerols containing 4:0 and 16:0 and dissolved in 18:1-18:1-18:1 were digesteed in the following rate order: 16:0-4:0-4:0 > 16:0-16:0-4:0 > 16:0-4:0-16:0 > 16:0-OH-4:0. Pancreatic lipase always released primary 16:0 and 4:0 equimolarily, but did differentiate among the acylglycerols tested. We designated this as intermolecular specificity. Intramolecular specificity would be fatty acid or stereospecificities.

(b) Different lipases. Several lipase systems contain enzymes which hydrolyze DG or MG more rapidly than TG. Monoacylglycerol hydrolase activities have been found for example, in human (18,19) and rat postheparin plasma (5), rat liver (20,21), rat preadipocytes (22), human (23-25) and rat platelets (5) and bovine milk lipoprotein lipase (21). Diacylglycerol hydrolases were detected in rat brain (26) and human platelet intracellular membranes (27). Specificities were established by control of digestion conditions, use of specific antibodies (20), employment of purified enzymes (in some cases) and selection of suitable substrates. Fielding (5) observed both 1(3)-and 2-MG acylhydrolase activities in human plasma and platelets. These were apparently separate entities. Lipolysis of 2-MG has usually been attributed to acyl migration and subsequent digestion of the resultant 1(3)-MG. Fielding inhibited acyl migration of 2- to 1(3)-MG with 5,5′ dithiobis (2-nitrobenzoic acid). Therefore, a 2-MG lipase was certainly present. It is likely that many natural systems, in which TG are hydrolyzed by a lipase with specificity for primary esters, will also contain hydrolases for DG and MG. Nevertheless, there are very few references to hydrolysis of 2-MG because they are not easily synthesized and because of the confounding effect of acyl migration.

The occurrence of more than one lipase from the same source can yield confusing results because the origin of the final products may be obscured. The best method for identification of different lipases is the use of specific antibodies.

II. Positional

(a) Primary esters. Pancreatic lipase is the best example. Mattson and Beck (28,29), with various oleoyl TG as substrates, observed in vitro and in vivo that the sequence of digestion was: TG > 1,2(2,3) DG > 2-MG, and consequently pancreatic lipase was specific for primary esters. This has subsequently been confirmed many times with a large variety of TG (10,11). Pancreatic lipolysis is accepted as a method for identification of fatty acids in the 1 +3-(FFA) and 2-(MG) positions of TG. The specificity enables us to test the structure of synthetic TG (10) and to produce DG for the structural analysis of natural TG (30). It is the best method for preparing small quantities of 1,2-(2,3)-DG and 2-MG (10).

Some other reported examples of specificity for primary esters are: serum and bovine milk lipoprotein lipases (31) and the lipase from *Rhizopus arrhizus* (32), all tested with synthetic TG. Specificity for primary esters can be readily determined by digestions of natural and synthetic acylglycerols in which the primary and secondary positions differ.

(b) Secondary esters. There has been one lipase discovered, that from the microorganism *G. candidum*, which lipolyzes secondary esters from TG (33). However, this represents a preference for fatty acids with *cis*-9 unsaturation and will be discussed in the section on fatty acid specificity. Another lipase, the MG hydrolase in rat serum and platelets, digests 2-MG apparently without prior conversion to 1-MG(5).

(c) Nonspecific or random hydrolysis. This type of specificity implies lipolysis of all esters from all positions of the acylglycerols in the digestion mixture at equimolar rates, a very

unlikely occurrence. There are several lipases which have no marked positional specificity, but which are probably not totally nonspecific. The presence of hydrolases for DG and MG may account for the apparent nonspecificity. Further, prolonged digestion of a TG will finally produce FFA and glycerol or complete hydrolysis. This is not random hydrolysis since it is a function of acyl migration because, with the exception noted above, lipolysis of 2-position esters has not been observed to occur.

Lipases from *Candida cylindracea* (34), human breast milk, bile salt stimulated (35) and *Chromobacterium viscosum* (36) have no marked positional specificity. All hydrolyzed the primary and secondary esters of natural or synthetic TG. Further, *C. cylindracea* cleared the ester from 1,3-hexadecyl-2-oleoyl glycerol. A lipase which randomly digests TG must hydrolyze the 2-position ester. A test for nonspecificity would employ the dialkylacyl glycerol above or preferably, since it is much easier to synthesize, 2,3-dioleoyl butanediol. If a search is being made for nonspecific lipases and the preparation does not hydrolyze the butanediol derivative, then the enzyme is incapable of hydrolyzing all of the esters of TG. Lipases which rapidly hydrolyze TG alone or in combination with various esterases are key reagents for determination of TG manually or in continuous flow systems (37). Glycerol is actually assayed, usually enzymatically, in these analyses. Another potential industrial application is the complete hydrolysis, in 12-16 hr, of fats by *C. cylindracea* lipase (38). Since the conditions are very mild as compared to alkaline saponification, this lipolysis could release polyunsaturated fatty acids from natural fats with minimal oxidation.

Ory and St. Angelo (39) have introduced another complication into the determination of positional specificity. They investigated castor bean lipase, which was believed to be somewhat nonspecific. They found that the *sn*-2 acid of TG was cleared and transacylated to *sn*-1 or 3 by active acyl transferase and not spontaneous acyl migration. Reesterification has been noted by others (31). It is possible that the acyl transferase activity could be more readily differentiated by inhibition of acyl migration with 5,5′-dithiobis (2-nitrobenzoic acid) (5).

III. Fatty Acid

Very few lipases have been found that are specific for groups of similar fatty acids, none for individual fatty acids. An enzyme secreted from the pharyngeal regions of suckling ruminant milk fats and from synthetic TG (40). The enzyme preparations are used in the manufacture of Italian type cheeses, in which free 4:0-10:0 contribute to the characteristic flavors.

A unique type of fatty acid specificity is a characteristic of an extracellular lipase produced by the mold *G. candidum* (33). The enzyme is partially specific for fatty acids with *cis*-9 configuration, releasing 89.7 M% 18:1 from, for example, 18:1-16:0-16:0.

A partial specificity for 8:0-12:0 has been observed in rat (41,43) and human lingual (44) lipases. Wang et al. (45) obtained similar results with purified human lipoprotein lipase (the serum, not the bile salt stimulated enzyme). Wang prepared equimolar mixtures of several monoacid TG and found that 8:0-12:0 TG and 18:1-18:1-18:1 were digested more rapidly than 16:0-16:0-16:0 and 18:0-18:0-18:0. By using a mixture of the TG, they avoided the mistake of digesting, for example, equal amounts of 4:0-4:0-4:0 and 18:0-18:0-18:0 separately and then comparing the results from two completely different molar amounts and emulsions.

Miller et al. (21) demonstrated specificity by both hepatic and lipoprotein lipases toward unsaturated as compared to saturated 1-MG. They assayed both alone and in mixtures, thus eliminating to some extent the problems of preparing comparable emulsions. They attributed the much lower activity toward 18:0 MG to the rigid structure this compound forms as compared to 18:1 MG. They made this interpretation from fluorescence depolarization data.

Chau and Tai (23) observed preferential release of 20:4 from *sn*-1,2,-DG by a human platelet DG lipase. The *sn*-1 ester was deacylated first. This could be a stereospecific hydrolysis, although *sn*-2,3-DG were not tested.

IV. Stereospecificity

Stereospecificity is a novel characteristic of lipases. The property has been observed in lipoprotein lipases found in postheparin plasma in vitro (46,47) and in vivo (48) adipose tissue (46) and in bovine (47) and human milks (45) and liver, heparin released (49). The specificity of these lipases is ca. 2:1 for the *sn*-1-ester of TG. Lipases which exhibit partial stereospecificity for the *sn*-3 ester are: rat (43,46) and human lingual lipases (50), the enzyme found in the termite, *Reticulitermes flavipes* (51), and in the fat body and hemolymph of the American cockroach (52).

Akesson et al. (53) noted that the lipase from *Pseudomonas fluorescens* did not differentiate between *sn*-1 and *sn*-3 esters of alkyldiacylglycerols, but did hydrolyze the 2-acyl-3-alkyl-*sn* glycerol more rapidly than the enantiomer. We have mentioned another lipase apparently stereospecific for *sn*-1,2-DG, however, with the *sn*-1 ester deacylated first (23).

Determinations were made in these studies by digestion of enantiomeric acylglycerols or alkyldiacylglycerols and identification of the products. In another procedure, racemic acylglycerols can be utilized and the 1,2-(2,3) DG separated by various means.

V. Combinations of Types

Examples are bovine milk lipoprotein and human lingual lipases which are partially stereospecific for *sn*-1 and *sn*-3 esters (46,44), respectively, and therefore must be and are specific for primary esters.

In some instances, positional and stereospecificity may create an apparent fatty acid preference. In rat milk, most of the 8:0-12:0 is esterified to the *sn*-3 position and the enzyme is partially specific for primary and *sn*-3 esters (43,44). Obviously, relatively large amounts of 8:0-12:0 in the FFA from a digestion of these products could be due solely to positional and stereo- and not fatty acid specificities. Here we see the influence of structure. If a fatty acid is located mostly in a position of the TG for which the lipase has specificity, then the acid will appear, often predominantly in the FFA. However, it is possible to have all of these types. Staggers et al. (43) found that more 8:0-12:0 were released by rat lingual lipase than were present in the *sn*-3 positions of the milk TG. Wang et al. (45) found that human milk lipoprotein lipase preferentially hydrolyzed 8:0, 10:0 and 12:0 and further was stereospecific for the *sn*-1 position.

DETERMINATION

After the enzyme has been identified as a lipase, determination of specificity can proceed as described below. First, however, we must caution the investigator to be certain that conditions for digestion of substrates are the same for each trial. The primary problem is preparation of comparable emulsions with substrates of widely differing melting points. The same precaution applies when lipoproteins are employed as substrates. Lipoprotein particles can be sized by ultracentrifugation (5). Whatever the method used to prepare the emulsions (it has usually been sonication), the substrate must be liquid when emulsified and digested. Cosolvents can be used for high melting substrates. Otherwise, the differing physical states of the substrate may cause a spurious specificity.

Many of the method we will describe utilize synthetic acylglycerols or alkyldiacylglycerols of known structure as substrates. Methods for synthesis of these compounds are described by Jensen and Pitas (54), Buchnea (55) and in the relevent references. We have found that synthesis, insofar as following a recipe is concerned, is not arduous. The "rate-limiting step" is purification, which can be extremely difficult. Some of the synthetic acylglycerols can be purchased, but availability is limited. Reliable suppliers, with whom we have had experience, are Supelco, Inc. (Bellefonte, PA) and Serdary Research Labs, Inc. (London, Ontario, Canada). Other suppliers may be equally satisfactory, but we have not dealt with them. Many of the procedures used have been discussed by Litchfield (56) and Myher (57). The methods they describe were developed to determine the structure of TG, but are applicable to the study of lipase specificity.

Positional

Positional specificity should be ascertained first. If the enzyme is nonspecific, then positional, fatty acid and stereospecificities cannot be properties of the enzyme. This can be done by lipolyses of (a) olive oil or 18:1-18:1-18:1, (b) 2,3 dioleoylbutanediol, (c) cocoa butter and unrearranged lard and (d) synthetic TG, e.g. 16:0-18:1-16:0 and 18:1-16:0-18:1.

The least expensive method for determination of specificity for primary esters is (a) digestion of olive oil or 18:1-18:1-18:1 purified with a column of alumina (54). A digestion of 5 min or less should yield DG and MG not isomerized by acyl migration. Separation of the products by boric acid-TLC (6) will reveal if 1,3-DG and 1(3) MG are present. Since a lipase with specificity for primary esters would produce primarily 1,2(2,3)-DG and 2-MG, the presence 1,3-DG and 1(3)-MG in amounts approaching 33M% each of the total digestate would indicate departure from absolute primary position specificity. Hydrolysis of 2,3-dioleoyl butanediol or 1,3-hexadecyl-2-oleoyl glycerol (b) differentiated between pancreatic lipase and nonspecific esterase (12,13). Lipases with marked specificity for primary esters would hydrolyze very little 18:1 from 2,3-dioleoylbutanediol. If substantial hydrolysis occurs, then the enzyme is probably nonspecific. Acyl migration cannot happen with this compound. The compound is not commercially available, but is easy to prepare (54).

Another inexpensive test is (c), the digestion of purified cocoa butter and unrearranged lard TG and analysis of the products. Unrearranged lard can be obtained from most meat packing houses and must be used because the commercial product has been randomized. Cocoa butter TG are primarily 16:0-18:1-18:0 (58,59) and the 2-position ester of unrearranged lard is mostly 16:0 (60). Incidentally, the authors of

this paper describe one of the most widely quoted procedures for structural analysis of TG with pancreatic lipase. The results which have been obtained with both primary positional pancreatic lipase and nonspecific *C. cylindracea* lipase from these and synthetic substrates (d) are presented in Table 2. Note that the specificity of pancreatic lipase is clearly evident, particularly with the synthetic TG where the amounts of fatty acids in the 2-MG and FFA (1+3) positions are very close to theoretical values. The amount of 16:0 in the 2-MG from 16:0-18:1-16:0 should theoretically be 0% and was 0.9%. If a lipase is truly nonspecific, then the amounts of each fatty acid in the TG, MG and FFA should be equal. With the *C. cylindracea* lipase, the FFA conform, but the MG do not. The 18:0 MG from cocoa butter and the 16:0 MG from 16:0-18:1-16:0 are apparently resistant to digestion. An added advantage of these substrates is that the specificity cannot be due to saturated or unsaturated acids in the primary positions and this type of fatty acid specificity is eliminated. In fact, a better test would be digestion of an equimolar mixture of the synthetic TG because differences caused by digestion of separate emulsions could not occur.

Wang et al. (45) separated the DG and MG from the hydrolysis of 16:0-18:1-16:0 by human milk lipoprotein lipase according to molecular weight by GLC, to show preferential attack on the primary positions. They also tested acetylated 1(3)-16:0 and 2-18:1 finding that the primary ester was digested 4.3 times faster than the secondary ester. The MG were acetylated to prevent acyl migration of 16:0 and 18:1.

Substrate

If positional specificity has been found, some information on substrate specificity is already available. A nonspecific lipase will digest TG, DG and MG at the same rates, although DG or MG lipases may be in the enzyme preparation. Purification may be needed to find if more than one type of lipase is present (5). A lipase that is positionally specific and highly purified, e.g., pancreatic lipase (16), produces DG, MG, and glycerol so it is likely that only one lipase is hydrolyzing the acylglycerols, but at different rates. Other sources of lipase in which one enzyme molecule apparently has several activities are: rat liver which hydrolyzes TG, MG and phosphatidylcholines (20); and the hormone

TABLE 2

Determination of Primary Positional Specificity of Pancreatic Lipase and Nonspecificity of *C. cylindracea* lipase[a] by Digestion of Natural and Synthetic Triacylglycerols

Substrate and digestion products	Fatty acid (M%)		
	16:0	18:0	18:1
Cocoa butter[b]			
TG[c]	25.2[d] (27.8)[e]	35.5 (33.0)	35.2 (35.6)
2-MG[f]	2.4 (27.3)	1.6 (42.6)	89.0 (27.4)
FFA[f]	36.6 (34.5)	52.5 (18.8)	8.3 (39.3)
Lard[g]			
TG	27.8[c]	12.7	42.6
2-MG	68.9	3.8	13.5
FFA	6.3	18.6	58.4
16:0-18:1-16:0[h]			
TG	66.6	–	33.4
2-MG	0.9	–	99.1
FFA	99.1	–	0.9
18:1-16:0-18:1[h]			
TG	33.3 (33.3)[e]	–	66.7 (66.7)
2-MG	99.0 (55.5)	–	1.0 (44.5)
FFA	1.0 (32.4)	–	99.0 (67.6)

[a]Figures in parentheses are for *C. cylindracea* lipase. All others for pancreatic lipase.

[b]Reference 58.

[c]TG is triacylglycerol.

[d]Amounts do not total 100%. Minor acids omitted.

[e]Reference 34.

[f]2-MG, the fatty acids in the 2-position. FFA, the fatty acids in the 1+3 positions.

[g]Reference 58. 16:0-18:1-16:0 is 1,3-dipalmitoyl-2-oleoyl-*rac*-glycerol.

[h]Reference 52.

sensitive lipase from rat adipose tissue lipolyzing TG, DG, MG and cholesteryl oleate (61).

Specificity for different acylglycerols by the same enzyme has been studied by separate digestion of 12:0 acylglycerols, except for 1,2 (2,3)-DG, with bovine milk lipoprotein and porcine pancreatic lipases, and the rate order TG > 1,3-DG > 1(3) MG > 2-MG observed (15). Spontaneous acyl migration occurred as seen in the 1-MG content of the 15 min 2-MG controls (see Table 3). The 1,2(2,3) DG were not tested because, at the time, they were extremely difficult to prepare (54). Another approach would be to employ alkyldiacylglycerols as substrates (46). The alkyl group cannot migrate, but these compounds are not easy to prepare. Oleoyl compounds should be synthesized for these studies because they will be liquid at 37-42; the usual temperatures of emulsification and comparable emulsions can be prepared and digested. Unfortunately, these compounds are poor substrates for some lipases (46).

Coleman (62) realized that rate constants obtained from separate digestions of TG, DG and MG would probably not be the same as the rates from a mixed digestion. He hydrolyzed illipe butter and lard with pancreatic lipase and found that the constants for lipolysis of TG and 1,2 (2,3)-DG in the digestion mixture were similar and concluded that TG and DG were hydrolyzed at the same rates.

The intramolecular specificity for TG of widely different molecular weights documented by Sampugna et al. (17) can be detected best by digestion of selected synthetic TG. Data from these experiments are depicted in Table 4. Specificity for primary esters was maintained, but 16:0-4:0-4:0 was lipolyzed more rapidly than 16:0-16:0-4:0, probably because of the difference in molecular weight and configuration.

Reaction conditions can be manipulated to stimulate the desired lipase and inhibit others. Use of the desired substrate is obvious. Triacylglycerol and MG lipase activities in postheparin plasma (19) were identified because the latter was not affected by NaCl, protamine and pyrophosphate and was normal in type I hyperlipoproteinemia. This familial type of hyperlipoproteinemia is characterized by absence of TG lipase. The response of the TG enzyme to apo-C-II was of greater magnitude than that of the MG lipase (5). The adipocyte enzyme is inhibited by 0.4% Triton X-100 (22). Another example of control by reaction conditions is the influence of bile salts on human milk lipase. Activity is nil unless the salts are present; the enzyme is defined as bile salt stimulated lipase (35). Serum stimulated lipoprotein lipase is also present.

More recently, immunological techniques have been utilized to measure amounts of enzyme protein. Nanogram quantities of lipoprotein lipase protein activity have been determined by radioimmunoassay (63) of liver TG lipases (20,64). If an immunoassay can be developed, it provides the most sensitive and specific method for identifying a lipase; one of few methods for direct determination of the enzyme protein.

TABLE 3

Lipolysis of Laurate Acylglycerols by Bovine Milk Lipoprotein and Porcine Pancreatic Lipases[a]

Micorequiv FFA released/meq available ester

Substrate[b]	Milk lipase		Pancreatic lipase	
	5 min	15 min	5 min	15 min
12:0-12:0-12:0	203	410	216	273
12:0-OH-12:0	44	66	103	140
1-(3)-12:0	8	28	55	129
2-12:0	0	6	58[c]	135[c]

[a]Reference 15. The lipase preparations did not have the same specific activity.

[b]12:0-12:0-12:0, trilauroylglycerol; 12:0-OH-12:0 1,3 dilauroylglycerol; 1-(3)-12:0, 1(3) monolauroylglycerol and 2-12:0, 2-monoacylglycerol.

[c]1-MG contents of 15 min controls were 71.4 and 85.8%.

TABLE 4

Triacylglycerol Specificity of Pancreatic Lipase: Products From the Lipolysis of Butyrate Triacylglycerols[a]

Substrate[b]	M%	
	4:0	16:0
16:0-4:0-4:0		
MG	100	0
FFA (63.4)[c]	41.7	58.3
16:0-16:0-4:0		
MG	0	100
FFA (52.7)[c]	50	50

[a]Reference 17.

[b]Digestions were 2.5 min and substrates were mixed with equimolar quantities of 18:1-18:1-18:1.16:0-4:0-4:0, 1-palmitoyl-2,3-dibutyryl-*rac*-glycerol.

[c]μeq of fatty acid released/min.

Fatty Acid

The methods are: (a) release of a relatively large amount of a particular group of acids from natural fats, (b) hydrolysis of relevant,

mixed acid TG and (c) lipolysis of equimolar mixtures of monoacid TG. We have used both (a) and (b) to study the specificity of *G. candidum* lipase (33). From our summarized data in Table 5, it is clear that the enzyme is highly specific for fatty acids containing *cis*-9-unsaturation. With (a), the investigator must be certain that an apparent fatty acid specificity is not due to the structure of the natural TG and positional specificity of the enzyme. We discussed this problem in Types of Specificity: V. Combinations.

Staggers et al. (43) took a much more difficult approach with (a): rat milk TG and lingual lipase. They performed a stereospecific analysis of the TG, identifying the fatty acids in the *sn*-1, 2 and 3 positions. They then determined the FFA released from the TG by lingual lipase in the stomach of the nursing rat. Some of the data they obtained were: (M%) milk TG, *sn*-3 and FFA: 8:0; 11.9, 10.9, 10:0; 28.7, 40.1 and 12:0; 15.3, 28.3. Thus, the enzyme preferentially released 10:0 and 12:0, but we know that rat lingual lipase has partial stereospecificity for for *sn*-3 esters; 2:1 (46). Nevertheless, the amounts of these acids in the FFA are greater than can be attributed to stereospecificity, so the enzyme does have partial specificity for 10:0 and 12:0.

Wang et al. (45) lipolyzed equimolar mixtures of monoacid TG, shown in Table 6, with human milk (serum stimulated) lipase, finding marked specificities for 8:0, 10:0 and 12:0. Also tested was an equimolar mixture of 14:0-14:0-14:0, 16:0-16:0-16:0, 18:0-18:0-18:0, 18:1-18:1-18:1, 18:2-18:2-18:2 and 18:3:18:3:18:3. We have recalculated these data as % digestion at 60 min relative to 18:1-18:1-18:1 which was the same as for 8:0-8:0-8:0. Setting 18:1-18:1-18:1 at 100%, the rate order was 18:3, 83; 18:2, 67; 14:0, 50; 16:0, 38 and 18:0, 17. The authors avoided the problem of separate digestions ofmarkedly different emulsions by using equimolar mixtures; an absolute necessity.

We present in Table 7 data from which we can discern the problems inherent in separate digestion of monoacid TG with a wide range of melting points (65). Note the extreme difference in melting points. Observe that equal weights of substrate were available, but that the difference in the amount of material exposed to the lipase was large. If the emulsion globules were the same diameter, there would have been three times more 4:0-4:0-4:0 available to the lipase than 18:1-18:1-18:1.

TABLE 5

Specificity of the Lipase from *G. candidum*[a]

Compounds[b] hydrolyzed	Compounds hydrolyzed slowly[c]
cis-9-18:1[d]	4:0-18:0
cis-9-16:1	*trans*-9-18:1
cis-9-14:1	*trans, trans*-9,12-18:2
cis, cis-9,12-18:2	Positional isomers of *cis*-18:1 other than Δ 9
cis, trans-9,12-18:2	*cis*-5-14:1
	Arachidonic acid
trans, cis-9,12-18:2	Octadecynoic acid
Palmityloleate	Erucic acid
Cholesteryl oleate	Oleylpalmitate
Linolenic acid	Dilinoleoyl-*sn*-glycerophosphoryl choline[e]

[a]Reference 33.

[b]Substrates were triglycerides with obvious exceptions.

[c]Relative to *cis*-9-18:1 or *cis, cis*-9,12-18:2.

[d]Hydrolyzed, regardless of location at positions *sn*-1,2, or 3, no positional or stereospecificity.

[e]Not hydrolyzed.

TABLE 6

Hydrolysis of an Equimolar Mixture of Triacylglycerols by Human Milk Lipoprotein Lipase[a]

Substrate	Length of hydrolysis (min)		
	20	40	60
6:0-6:0-6:0[b]	0	25	68
8:0-8:0-8:0	50	90	100
10:0-10:0-10:0	30	65	88
12:0-12:0-12:0	20	45	75
14:0-14:0-14:0	0	14	23
16:0-16:0-16:0	0	16	14
18:0-18:0-18:0	0	4	8

[a]Reference 45. The enzyme is serum, not bile salt stimulated.

[b]6:0-6:0-6:0, trihexanoylglycerol.

Stereospecificity

Some lipases have the property of preferentially deacylating one primary *sn*-ester from TG as compared to the other. Several of the procedures for determination of this specificity have been borrowed from methods for the stereospecific analyses of TG (56,57,66,67). Those which have been or can be applied to the characterization of stereospecificity are: (a) stereospecific analysis of a natural TG and identification of the digestion products, as was previously discussed (43); (b) lipolysis of enantiomeric alkyldiacylglycerols or TG and (c) hydrolysis of 18:1-18:1-18:1 or racemic TG and resolution of the 1,2 and 2,3-DG.

For (b), Paultauf et al. (46) digested racemic

mixtures containing equimolar amounts of 1-octadecyl-2,3-dioctadecenoyl-*sn*-glycerol, labeled with ^{3}H in the alkyl moiety or with ^{14}C in both acyl groups and 3-octadecyl-1,2-dioctadecenoyl-*sn*-glycerol labeled with ^{14}C in the alkyl moiety or ^{3}H in the acyl groups. Analysis of the isotope ratios in the hydrolysis products from incubation with various lipases indicates the presence or absence of stereospecificity and these data are in Table 8. The stereospecificities of rat lingual lipase for *sn*-3-esters are revealed in the faster disappearance of the *sn*-3 rather than the *sn*-1 ester. Accompanying these is the accumulation of the "DG" with the *sn*-3-OH. Opposite or *sn*-1 specificities are seen with postheparin serum, bovine milk and adipose tissues lipases. Pancreatic lipase exhibited little or no stereospecificity. These substrates are not easy to synthesize, but partially eliminate the complication of acyl migration and only the

TABLE 7

Hydrolysis of Triacylglycerols by Bovine Milk Lipase[a]

Substrate	MP (C)	Relative hydrolysis (% FFA)	Substrate available (g	mmol)
Milk fat	36	100	1	1.3[b]
Olive oil		60	1	1.1[c]
4:0-4:0-4:0[d]	-75.0	128	1	3.3
6:0-6:0-6:0	-25.0	94	1	2.6
8:0-8:0-8:0	8.3	67	1	2.1
12:0-12:0-12:0	46.4	55	1	1.6
14:0-14:0-14:0	57.0	50	1	1.4
16:0-16:0-16:0	63.5	22	1	1.2
18:0-18:0-18:0	73.1	22	1	1.1
18:1-18:1-18:1	5.5	69	1	1.1

[a]Reference 65.

[b]Calculation based on an average molecular weight of 750.

[c]Calculated as 18:1-18:1-18:1.

[d]4:0-4:0-4:0, tributyrylglycerol.

TABLE 8

Lipolysis of Enantiomeric 1(3)-Alkyl-2,3 (1,2) Diacyl-*sn*-Glycerols by Several Lipases[a]

	Source of enzyme							
	Pancreas[b]		Lingual[b]		Postheparin serum[b]		Bovine milk	Adipose tissue[b]
Substrates and products	0[c]	10[c]	0	20	0[d]	40[d]	40[d]	40[d]
1-(^{3}H)-alkyl-2,3-diacyl-[e] *sn*-glycerol	1600	838	2000	1468	900	873	869	–
3-(^{14}C)-alkyl-1,2[f]-diacyl-*sn*-glycerol	1600	880	2000	1638	900	798	764	–
1-alkyl-2-acyl-*sn*-glycerol		710		436		17.1	17.1	10.8
3-alkyl-2-acyl-*sn*-glycerol		580		254		45.8	72.5	40.0
1-alkyl-3-acyl-*sn*-glycerol		38		64		4.8	6.0	1.8
3-alkyl-1-acyl-*sn*-glycerol		30		60		14.4	26.6	4.5
1-alkyl-*sn*-glycerol		14		36		4.2	7.2	5.0
3-alkyl-*sn*-glycerol		110		46		41.4	36.6	20.0

[a]Adapted from Reference 46.

[b]Source was the rat.

[c]Min of digestion. Some data from other lenghs of digestion are omitted.

[d]Zero time lingual was control for postheparin serum, milk and adipose tissue.

[e]1-(9′,10′-$^{3}H_{2}$)octadecyl-2,3-dioctadecenoyl-*sn*-glycerol.

[f]3-(1′-^{14}C) dioctadecyl-1,2-dioctadecenoyl-*sn*-glycerol.

chirality of the compounds, not fatty acid differences, can explain preferential lipolysis of one enantiomer. Digestion of both enantiomers as a racemic mixture alleviates differences in preparing emulsions and other experimental errors. With dialkylacylglycerols, absence of a carbonyl oxygen at *sn*-2 causes a loss of stereospecificity (68).

With enantiomeric TG as substrates, (b) identification of stereospecificity is relatively simple, unfortunately synthesis is not (52,53). Nevertheless, the compounds have been tested with several lipases. The data obtained by Morley and Kuksis for bovine milk lipoprotein lipase (69) and Jensen et al. (50) for human lingual lipase are presented in Table 9. Morley and Kuksis detected stereospecificity by GLC and argentation-TLC of the 1,2 and 2,3-*sn* DG (see also ref. 45). The ratio of 1,2:2,3-*sn*-DG of 1:3-4 indicates an accumulation of the 2,3-*sn* isomer and more rapid hydrolysis of the *sn*-1 ester. Hydrolysis of the asymmetric TG selected for substrates produced DG separable by argentation-TLC; that is, containing either 1 or 2 double bonds. We (50) identified the fatty acids in the FFA and MG from lipolysis by human lingual lipase. We chose this method because the relatively low optimum pH of the enzyme 5.2 and long periods of digestion, 30 or 60 min, catalyze acyl migration of the *sn* 1,2 (2,3)-DG to the 1,3 isomer. The preponderance of *sn*-3 16:0 or 18:1 in the FFA is evidence for *sn*-3 stereospecificity.

For (c), 18:1-18:1-18:1 or racemic TG are lipolyzed and the 1,2-(2,3) DG separated by several approaches. These DG must first be separated from 1,3-DG by boric acid-TLC or HPLC. The methods for separation and identification are: (i) separation by argentation-TLC or GLC, (ii) determination of optical rotation, (iii) use of NMR with a shift reagent and (iv) conversion to phospholipids and lipolysis with phospholipases A-2 or C.

(i) the *sn*-1,2 and 2,3-DG must have different numbers of double bonds as previously mentioned for TLC (66), but only different acids for GLC. With GLC, the carbon numbers (acyl groups of the DGs) can be obtained (45) or the fatty acids identified.

(ii) the *sn*-1,2 and 2,3-DG have specific rotations of -2.8° or +2.8° (55). These will vanish as the ratio of the DG approaches 1:1 or a racemic mixture. Nevertheless, the polarimeter can provide a rapid answer to the search for stereospecificity. If there is no rotation of the 1,2 (2,3) DG when 18:1-18:1-18:1 is hydrolyzed, then the lipase is not stereospecific. The method lacks sensitivity, but would be helpful in screening. We obtained a specific rotation of -2.8° when we analyzed the *sn*-1,2(2,3) DG from a digestion of 18:1-18:1-18:1 with human infant lingual lipase (50), supporting our findings from lipolyses of enantiomeric TG. The *sn*-1,2 DG accumulated, thus the *sn*-3 ester was hydrolyzed more rapidly than *sn*-1. Specific rotation can be greatly increased by derivatization (56). Lok (70) tritylated the DG, finding +11.30° for the *sn*-1,2 and -12.2° for *sn*-2,3 enantiomers.

(iii) Lok (70) used ^{1}H NMR with a shift reagent to differentiate between the two tritylated DG enantiomers. The signals of the enantiomers were clearly discernible in the spectra. Lok was determining the identity of the *sn* 1,2-DG from fresh bovine milk fat and found only 7% of its enantiomer; a relatively sensitive method which has apparently not been applied to lipase specificities.

(iv) In this separation, advantage is taken of the stereospecificity of phospholipases; A-2 for

TABLE 9

Composition of Products from the Lipolyses of Enantiomeric Triacylglycerols by Bovine Milk Lipoprotein[a] and Human Lingual Lipases[b]

	Lipoprotein lipase[a]		Human lingual lipase[b]			
	DG[c] isomer (%)		MG[c] M%		FFA[c]	
Substrates	*sn* 1,2	*sn* 2,3	16:0	18:1	16:0	18:1
sn-16:0-18:1-18:2[d]	20.3	79.7	–	–	–	–
sn-18:2-18:1-16:0	17.5	82.5	–	–	–	–
sn-18:1-16:0-16:0	24.1	75.9	84.8	15.2	20.6	79.4
sn-16:0-16:0-18:1	34.6	65.4	77.8	22.2	85.2	14.8
sn-16:0-18:1-18:1	–	–	27.5	72.5	12.5	87.5

[a]Reference 69. Determined by GLC of the DG.

[b]Reference 50. Determined by GLC of the fatty acid methyl esters.

[c]DG, diacylglycerol; MG, monoacylglycerol and FFA, free fatty acids.

[d]*sn*-16:0-18:1-18:2,1-palmitoyl-2-oleoyl-3-linoleoyl-*sn*-glycerol.

sn-2 esters of *sn*-3 phospholipids and C for the phosphoryl moiety. The schemes are shown in Figure 1. In both procedures, the 1,2(2,3)-DG are separated from a digestion mixture, which can be from the lipolysis of 18:1-18:1-18:1 or a racemic TG and converted to phosphatidylphenols (71) or cholines (72). Phospholipase A-2 cleaves the *sn*-2 ester only in *sn*-3 phospholipids. The reaction products are the unhydrolyzed *sn*-1 phosphatidylphenol, an *sn*-3 lysophosphatidylphenol and FFA (30). All are separable by TLC. A preponderance of either phosphatidylphenol enantiomer indicates stereospecificity.

Phospholipase C hydrolyzes *sn*-3 phosphatidylcholine producing, in 2 min, an *sn* 1,2-DG and the phosphorylcholine moiety. The *sn*-1 enantiomer is digested in 2 hr, thus providing a means for resolution of the two enantiomers by TLC and identification by GLC or GLC-MS. (72).

Staggers et al. (43) provided data supporting the partial *sn*-3-stereospecificity of rat lingual lipase with a Brockerhoff analysis (30,68) of rat milk TG and identification of the FFA in the suckling rat's stomach. Digestion of dietary TG by lingual lipase occurs in the stomach. Hydrolysis of 10:0 and 12:0 was preferential, but the amounts of these acids at *sn*-3 are much greater than at *sn*-1 and there appears to be a specificity for both. Therefore, we cannot conclude with certainty, based on these data, that the lipase is stereospecific. Employment of TG such as *sn*-10:0-18:1-18:1 and *sn*-18:1-18:1-18:1-10:0 or perhaps an equimolar mixture of 12:0-12:0-12:0 and 18:1-18:1-18:1 would profide the required data after appropriate analysis. Except for the work of Staggers et al. (43), the highly specific enzymatic methods have apparently not been applied to the determination of specificity. These procedures have the advantages of assuring excellent resolution of the DG enantiomers and use of 18:1-18:1-18:1 and racemic TG. The only disadvantage is that preparation of the phospholipids can be troublesome. Moisture must be excluded.

Combinations

The examples given in V: Combinations of Types were determined by the methods discussed above for fatty acid and stereospecificities.

A flow sheet for determination of lipase specificity is given in Table 10.

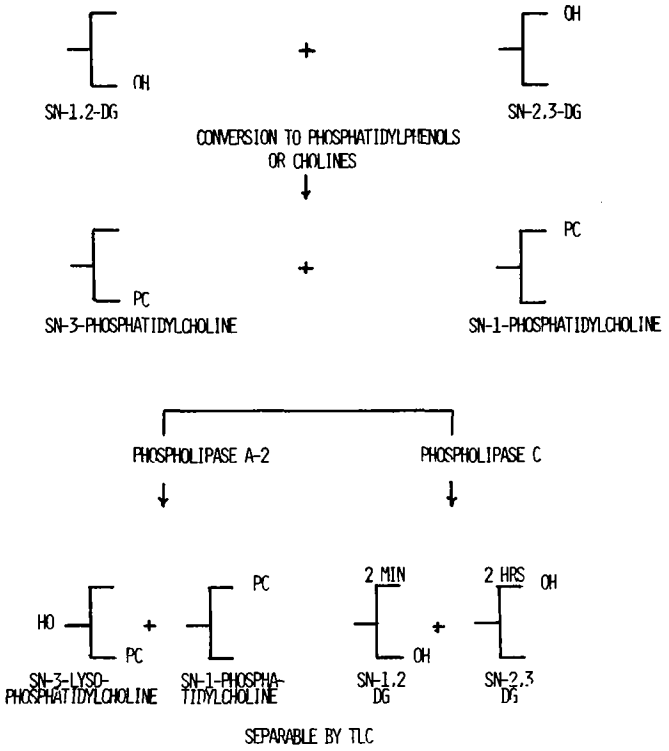

FIG. 1. Enzymatic resolution of 1,2 (2,3) diacylglycerols.

TABLE 10

Determination of Lipase Specificities

Enzyme identified as a lipase.

1. Digestion of:
   a. 18:1-18:1-18:1 or olive oil,
      (i) No 1,3-DG or 1,(3)MG after short digestion. Positional specificity, go to 2. For substrate specificity, to 3
      (ii) 1,3-DG and 1(3)MG found in relatively large amounts after short digestion. Possibly nonspecific. Proceed to 2b.
2. Positional specificity.
   (a) Primary:
      (i) Little or no digestion of 2,3-oleoylbutanediol, go to (ii). If digested, to b.
      (ii) Hydrolysis of cocoa butter and unrearranged lard followed by 18:1-16:0-18:1 and 16:0-18:1-16:0 for confirmation. If the fatty acids differ from the amounts which should be in the FFA, (1+3 positions) and MG (2-positions), go to 4 and 5. For substrate specificity, to 3.
   b. Nonspecific
      (i) The fatty acid composition of the substrate TG; and DG, MG and FFA from 2a (ii) should be very similar after a short (incomplete) digestion. Lipase is nonspecific.
3. Substrate:
   a. Different acylglycerols. Separate lipolysis of TG, DG and MG by same enzyme, or determination of rate constants for TG→DG→MG→glycerol in digestion mixture. Preferential release of fatty acids, go to 4.
   b. Different lipases. Arrangement of conditions so that the desired lipase is stimulated, others inhibited; use of desired acylglycerols. Employment of antisera. Preferential hydrolysis of esters, to 4.
4. Fatty acid:
   (a) Liberation of relatively large amounts of closely related groups of fatty acids from cocoa butter, unrearranged lard, bovine milk fat, rat milk fat, etc. Do not confuse with positional or stereospecificities. Proceed to 5 and 6 if amounts are very large.
   (b) Lipolysis of equimolar mixtures of suitable monoacid TG.
   (c) Lipolysis of synthetic TG tailored for the fatty acids in question; e.g., 12:0-18:1-18:1, etc.
5. Stereospecificity: primary position specificity required.
   (a) Digest 18:1-18:1-18:1, recover 1,2 (2,3) DG and:
      (i) Determine specific rotation. If O, lipase is not stereospecific. If rotation is small derivatize and repeat. Proceed to (ii) and c or d.
      (ii) Obtain NMR spectra of derivatized DG. If difference in amounts of enantiomers is greater than experimental error, lipase is stereospecific.
   (b) Stereospecifically analyze a natural fat and identify the products of hydrolysis. Identification is not positive unless the acids released are found in relatively large quantities in both *sn*-1 and 3-positions.
   (c) Prepare and hydrolyze suitable enantiomeric TG or alkyldiacylglycerols.
   (d) Recover 1,2(2,3)-DG, convert to phosphatidylcholines, and resolve enantiomers by digestion with phospholipase C. Separate DG by GLC if possible.
6. Combinations:
   (a) Should be apparent from 4 and 5.

Significance of Lipase Specificity

Why is it important to determine lipase specificity? Some of the reasons are: (a) to prepare TG for transfer through membranes by conversion to the more polar FFA and 2-MG, (b) fatty acids must be free to be activated prior to oxidation, biosynthesis of prostaglandins and reesterification, (c) fatty acid specificity releases shorter acids which are absorbed directly and travel to the liver via the portal vein where they are oxidized, (d) *sn*-1 specificity diverts *sn*-2,3-DG from biosynthesis of phospholipids and toward TG; *sn*-3 specificity directs *sn*-1,2 DG toward biosynthesis of phospholipids and (e) the enzymes have many uses; determination of natural TG structure, structural analysis of synthetic acylglycerols, biosynthesis of 1,2(2,3)-DG and 2-MG, clinical determination of plasma TG and mild hydrolysis of natural fats. Other applications will undoubtedly be developed.

SUMMARY

The types of lipase specificity have been identified as: substrate, positional, fatty acids, stereospecificity and combinations of these. Examples of lipases with each type are given and methods for their determination described. Application of the methods should reveal interesting and useful specificities in lipases.

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Determination of Lycopene, α- and β-Carotene and Retinyl Esters in Human Serum by Reversed-Phase High Performance Liquid Chromatography

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ABSTRACT

A rapid specific, microdetermination of the major human blood carotenoids by high performance liquid chromatography (HPLC) separation and quantitation at 466 nm is detailed in this paper. Serum retinyl esters can also be quantified utilizing the same separation procedure but detected at 325 nm. One hundred microliters of deproteinated serum were extracted with chloroform and injected on a reverse-phase column. Separation occurred within 16 min for all compounds of interest employing a mobile solvent of MeOH/AcN/$CHCl_3$ (47:47:6). All compounds were quantified at the wavelengths cited by integrated peak areas using retinyl acetate as a daily standard. Analysis of serum from a hypercarotenemic anorexia nervosa patient and a person suffering from hypervitaminosis A are presented as examples of the clincial application of this procedure.
Lipids 18:253-258, 1983.

The development of high performance liquid chromatography (HPLC) has made possible the efficient separation and detection of diverse classes of compounds. Among those of nutritional interest are the fat soluble vitamins (1-4).

Clinical carotenoid analysis has been classically accomplished by measurement of total carotenoids via absorbance at a fixed wavelength (5). This method lacks specificity and yields no information about individual carotenoid levels. Separation of carotenoids into the various compounds has been accomplished by gravity flow liquid chromatography (6), but this is extremely time-consuming and impractical. The use of HPLC for resolution of plant carotenoids has recently been published (4). The current paper employs a similar procedure in order to determine the concentrations of lycopene, α- and β-carotene and retinyl esters in human blood sera.

Application of these techniques to clinical diagnosis may prove particularly expedient in cases of suspected hypervitaminosis A or hypercarotenemia. General clinical assessment and nutritional surveys may also benefit from this rapid method with respect to correlating dietary consumption of vitamin A and carotenoids with resulting blood concentrations.

MATERIALS AND METHODS

The HPLC equipment was composed of a Tracor 950 chromatographic pump and 970 A variable wavelength detector (Tracor Instruments, Austin, TX), Rheodyne 7125 sample injection port (Cotati, CA) and a Supelcosil LC-18 (Supelco Inc., Bellefonte, PA) stainless steel column (4.6 × 25 cm) packed with ODS C18 of 5 μ particle size. A precolumn of similar packing material was also used. A Hewlett Packard (Avondale, PA) reporting integrator (Model 3390A) was used for recording the chromatographs. Spectrophotometric determinations were made with a Gilford Model 250 Spectrophotometer (Gilford Instrument Laboratories Inc.).

Development of the chromatograph was accomplished with a solvent system composed of methanol/acetonitrile/chloroform (47:47:6). All solvents used were of spectrograde quality. Prior to use, solvents were filtered through Whatman No. 50 filter paper and degassed. Finally, all solvent was drawn through a 7 μm inlet filter prior to entry into the system. Samples were injected into a 100 μl loop (Rheodyne, Cotati, CA) with a 100 μl gas-tight syringe (Rheodyne, Cotati, CA).

Standards

Crystalline lycopene, α-carotene, β-carotene, retinyl acetate and retinyl palmitate (oil solution) were gifts from Hoffmann-LaRoche Inc. (Nutley, NJ) or purchased from Sigma Chemical Co. (St. Louis, MO). β-Carotene was recrystallized using a modification of the procedure of Britton and Goodwin (7). For HPLC analysis, all standards were dissolved in chloroform and dilutions were made to allow for the injection of standards in a range similar to the

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amounts of compound present in the injected test samples. The typical final concentrations of the carotenoids, retinyl acetate, and retinyl palmitate standard solutions were: 100 ng/100 μl, 50 ng/100 μl, and 200 ng/100 μl, respectively. The purity of all standards were determined spectrophotometrically. Extinction coefficients used were (E 1%cm): β-carotene, 2396 at 465 nm in $CHCl_3$; α-carotene, 2800 at 444 nm in petroleum ether; lycopene, 3450 at 472 nm in petroleum ether; retinyl acetate, 1550 at 326 nm in EtOH; retinyl palmitate, 975 at 325 nm in EtOH (8).

Only retinyl acetate was used as a standard on a daily basis because it was observed that the carotenoids were not reliably stable. Immediately after opening a 1-g vial of crystalline retinyl acetate, the contents were weighed into separate vials in ca. 20 mg quantities. These smaller vials were sealed under nitrogen, covered with foil, and stored in a desiccator under nitrogen at -10 C. This method protects the stock retinyl acetate standard from repeated exposure to oxygen and/or light.

Standard Curve and Recovery of Standards from Blood

Initially standard curves were generated comparing quantity of compound vs integrated peak areas for the carotenoids, retinyl acetate, and retinyl palmitate.

In order to determine the recovery efficiency of carotenoids and retinyl palmitate, these compounds were added to rat serum in chloroform at 3 concentration levels. Human serum usually contains significant levels of various carotenoids. Rat serum was chosen for the recovery studies because rats fed typical stock laboratory diets do not have detectable blood carotenoid levels (unpublished observations), and have less than 5% of total vitamin A in the ester form (9).

Method of Extraction and Analysis of Carotenoids and Retinyl Esters

One hundred μl of serum was pipetted into a polyethylene microcentrifuge tube (Brinkman Instruments, Westburg, NY). One hundred μl of methanol was added, and the mixture was vortexed for 15 sec. Carotenoids and retinyl esters were extracted by adding 200 μl of chloroform, vortexing for 60 sec, and centrifuging at 5000 rpm in a table-top laboratory centrifuge for 10 min. Fifty μl of extract from the bottom (chloroform) layer was then injected on the column and developed with MeOH/AcN/$CHCl_3$ (47:47:6) with a solvent flow rate of 2 ml/min. Samples were injected twice and monitored at 2 different wavelengths. This was necessary because retinyl palmitate has an absorption maxima at 325 nm, but does not absorb at 466 nm, while the converse is true for β-carotene. Simultaneous determination could easily be accomplished with a spectrophotometer capable of monitoring at 2 wavelengths or 2 detectors connected in series. Unfortunately, β-carotene and retinyl palmitate have approximately the same retention time, making measurement of both in the same injection impossible with a single detector.

Because of the similar retention time of the 2 compounds, a standard containing both β-carotene and retinyl acetate in predetermined quantities was measured at 325 nm and at 466 nm. The absorbance of either compound was not affected by the presence of the other. Therefore, the quantitation of retinyl palmitate or β-carotene is not influenced in samples containing both compounds.

Identification of the compounds of interest in serum was verified by comparison of retention time with standards and cochromatography with added standards.

RESULTS AND DISCUSSION

Zakaria et al. (4) have compared the use of nonpolar reverse-phase packing vs straight phase columns for carotenoid separation. They concluded that the long retention time and lack of resolution exhibited by the use of the straight phase columns contraindicated their use. The resulting retention times for the Supelcosil LC-18 used in our work compares favorably in resolution and retention times to the Partisil PXS-5/ODS column used by Zakaria et al. (4). The retention times for the previous and present work are shown in Table 1. The resolution between α-carotene and β-carotene was > 1.5 for Zakaria et al. and 1.2 ± .2 for the present study. Resolution (RS) is defined (4) as:

$$RS = \frac{2\,(V_{\beta\ \text{carotene}} - V_{\alpha\ \text{carotene}})}{(W_{\beta\ \text{carotene}} + W_{\alpha\ \text{carotene}})}$$

where V is the retention time and w is the width of the curve. Although the resolution was less than that achieved by Zakaria et al., it was more than adequate for quantitation of both compounds from blood.

The acetonitrile/chloroform (92:8) system chosen by Zakaria et al. (4) is quite capable of good resolution and quick separations when used in conjunction with our column. The high cost associated with using acetonitrile as 92% of the mobile phase was substantially reduced by replacing some of the acetonitrile with methanol. At best, methanol/chloroform mixtures were capable of resolving α- and β-caro-

TABLE 1

Retention Time (min) of Retinyl Acetate, Retinyl Palmitate and Carotenoids

Compound	Zakaria et al.[a] (4) 92:8 AcN/$CHCl_3$	Present work[b] 92:8 AcN/$CHCl_3$	Present work[b] 47:47:6 AcN/MeOH/$CHCl_3$
Retinyl acetate	–	–	10.62
Retinyl palmitate	–	–	15.59
Lycopene	7.82	10.4	9.26
α-Carotene	12.4	18.3	13.99
β-Carotene	13.2	20.0	15.01

[a]Column, Partisil PXS-5-ODS 5 μm. λ=410 nm, 2.0 ml/min flow-rate.

[b]Column, Supelcosil LC-18 5 μm. Retinyl acetate, λ=325 nm, 0.5 ml/min. Retinyl palmitate, λ=325 nm, 2.0 ml/min. Lycopene, α-carotene, β-carotene, λ=466 nm, 2.0 ml/min.

tene quite well, but no combination of these 2 solvents alone could resolve lycopene and α-carotene at a level comparable to that provided by acetonitrile/chloroform (92:8). Consequently, it was determined that a methanol/acetonitrile/chloroform mixture (47:47:6) best resolved the compounds of interest (Table 1, Fig. 1).

Standards

A major problem in the development of this assay occurs in the use of carotenoids as standards. The 3 compounds of interest, lycopene, α-carotene, and β-carotene, are highly light-sensitive and are subject to isomerization, oxidation, and subsequent loss of optical activity. It was found that storage of these compounds in chloroform was possible for only a few days before deterioration made them unreliable for standards. Other storage solvents were tried and found to be less reliable.

In addition to the above problem, the purity of commercially available standards was unpredictable and varied with the lot purchased. The high cost of these standards also discouraged their use. One solution was to recrystallize the carotenoids. This method produces pure standards. However, the process is time consuming and was not always practical for daily operation. Therefore, we chose to employ a more stable, inexpensive standard for routine calibration of the instrument. Carotenoid and retinyl palmitate standards were used periodically to insure accurate quantification.

All-*trans*, synthetic crystalline retinyl acetate was chosen for a day-to-day standard. Dilutions were made in chloroform to a final concentration of 50 ng/100 μl. Purity was checked spectrophotometrically.

Retinyl acetate has an absorption maxima at 325 nm and does not absorb above 380 nm. Therefore, it was necessary to measure the response of retinyl acetate at 325 nm while utilizing 466 nm for the measurement of the carotenoid compounds. Retinyl esters were also monitored at 325 nm.

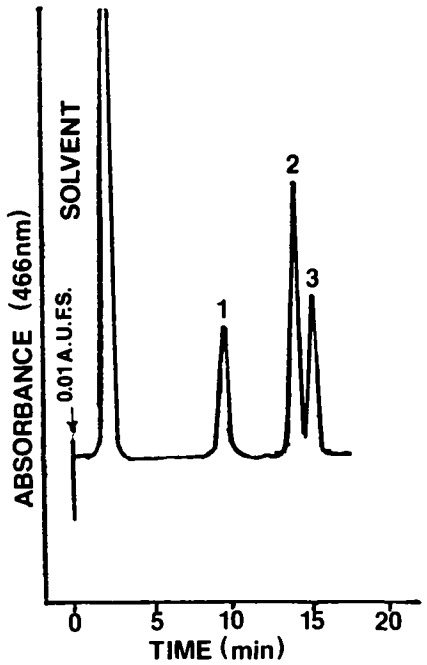

FIG. 1. HPLC chromatogram of carotenoid standards: 1=lycopene; 2=α-carotene; 3=β-carotene. Chromatographic conditions: column, Supelcosil LC-18, 5 μm; eluent, 47:47:6 AcN/MeOH/$CHCl_3$; flow-rate, 2.0 ml/min; detection, 466 nm; sensitivity, 0.01 AUFS.

Recoveries of Compounds

Recovery of standards added to rat serum in chloroform, vortexed and taken through the extraction procedure, and injected into the HPLC is shown in Table 2. For the additional

TABLE 2

Percentage Recovery of Retinyl Palmitate and Carotenoids Added to Carotenoid and Retinyl Palmitate Free Rat Serum[a]

Compound	% Recovery
Lycopene	98.2 ± 7.7
α-Carotene	94.3 ± 10.8
β-Carotene	93.6 ± 16.8
Retinyl palmitate	87.8 ± 6.6

[a]Mean ± SD of % recovery of 10, 20, 30 ng of compound added in duplicate to 100 μl of rat serum.

range of 10-30 ng, 93.6-98.2% of the carotenoids were recovered, while retinyl palmitate was recovered at 87.8%. The poorer recovery of retinyl palmitate may be due to the difficulty in adding this nonpolar compound to serum. The retinyl palmitate has a tendency to cling to the side of the tube and is not easily mixed into the serum.

Reproducibility Study

To demonstrate the reproducibility of this method, 10 aliquots from a sample of hypercarotenemic serum were taken through the extraction and separation procedure. The mean ± SD concentrations of the 3 carotenoids, lycopene, α- and β-carotene, in the serum were 69.2 ± 9.35, 39.0 ± 4.62, and 86.0 ± 5.5 μg/100 ml, respectively. These results are within the degree of variability expected for this type of analysis.

Standard Curves

Standard curves of retinyl acetate, retinyl palmitate, α-carotene, β-carotene, and lycopene were all generated on the same day (see Table 3). A high degree of linearity of integrated peak areas over a given concentration of compound was achieved in all cases.

Response ratios of retinyl acetate to lycopene, α- and β-carotene, and retinyl palmitate were determined from the standard curve data. The calculated ratios for retinyl acetate (in area units/ng) to each of the following compounds (in area units/ng) are: lycopene, 10.9; α-carotene, 5.35; β-carotene, 5.60; and retinyl palmitate, 2.31.

Calculation of Serum Carotenoid and Retinyl Ester Concentrations

Daily variations in the performance of the instrument can be corrected by the use of the response ratios of retinyl acetate to the compounds of interest. To calculate sample concentrations of the carotenoids or retinyl palmitate, only the integrated peak area of a given amount of retinyl acetate standard is used. The formula for calculation of the amount of β-carotene in a serum is:

$$\text{Sample ng } \beta\text{-carotene} = \frac{\text{Response ratio of retinyl acetate to } \beta\text{-carotene (5.6)} \cdot \beta\text{-carotene peak area} \cdot \text{ng retinyl acetate used}}{\text{area retinyl acetate}}$$

The term, ng retinyl acetate, is the amount of retinyl acetate injected as the daily standard. The area generated by that amount is the term, area retinyl acetate. The sample ng of β-carotene is multiplied by any dilution factors used and is divided by the predetermined percent recovery of that compound from serum. The above calculations will give the concentration of β-carotene in the serum sample. The concentrations of the other carotenoids and retinyl esters are determined similarly.

Freezing Study

Often, serum saples are thawed and refrozen several times during the course of a study. We were concerned that this refreezing of samples may affect the carotenoid and/or retinyl palmitate concentrations determined from frozen and thawed serum.

Blood was drawn from a hypercarotenemic patient and the serum was separated into 2 fractions. One fraction was stored in a large vial at -10 C, while the other fraction was separated

TABLE 3

Standard Curve Data for Retinyl Acetate, Retinyl Palmitate and Carotenoids

	r	Slope (area counts × 10^4/ng)	y-Intercept (area counts × 10^4)	Linear region[b] (ng)
Retinyl acetate	.976	54.2	8.44	15-25
Retinyl palmitate	.983	8.01	89.10	37-92
Lycopene	.994	7.73	-13.00	9-45
α-Carotene	.999	12.29	-9.14	9-42
β-Carotene	.999	13.23	-60.80	18-46

[a]One area count = .125 μV - sec.

[b]Quantity injected to obtain standard curve.

into 100 μl aliquots and stored at the same temperature directly in the polyethylene tubes used for the analysis. For 8 of the next 10 days, 2 aliquots of serum were removed from the large vial for analysis and the large vial was refrozen. Two small vials were thawed and analyzed at the same time. Thus, the large vial was thawed and refrozen 8 times, while the serum stored separately was never exposed to thawing and refreezing. Fresh serum which had never been frozen was also analyzed.

The results of this study showed that there were no significant differences in the levels of the carotenoids or retinyl palmitate over the duration of the study due to thawing and freezing. Also, there was no significant difference between fresh serum and the frozen serum.

Clinical Applications

One recent clinical use in our laboratory for this procedure has been for the screening of patients with anorexia nervosa for hypercarotenemia (10). Anorexia nervosa-induced hypercarotenemia is currently assessed by spectrophotometric analysis for total serum carotenoid levels (11,12). Analysis for specific serum carotenoids has proved useful in elucidation of the etiology of the hypercarotenemia (10). Figure 2 depicts a typical chromatograph of an individual with this disease. Some patients with anorexia nervosa also demonstrate elevated retinyl esters.

Hypervitaminosis A results in very high serum levels of retinyl esters. Normally, very low concentrations are found in serum. Figure 3 depicts the chromatogram associated with the analysis of serum from an individual diagnosed (13) for vitamin A intoxication. It should be noted that the present separation technique will not differentiate between various esters of retinol. A procedure has been developed by Ross (14) further to quantify the type of fatty acid esterified to retinol. Although other methods are certainly successful in their separation of retinyl esters (15,16), the current method is useful because it is rapid and can be accomplished routinely with the same solvents and column used to separate the carotenoids.

Simpson and Chichester have pointed out a need for the correlation of plasma provitamin A carotenoids with their dietary intake (17). The current procedure may help to accomplish this goal and to understand better the metabolism of carotenoids in man.

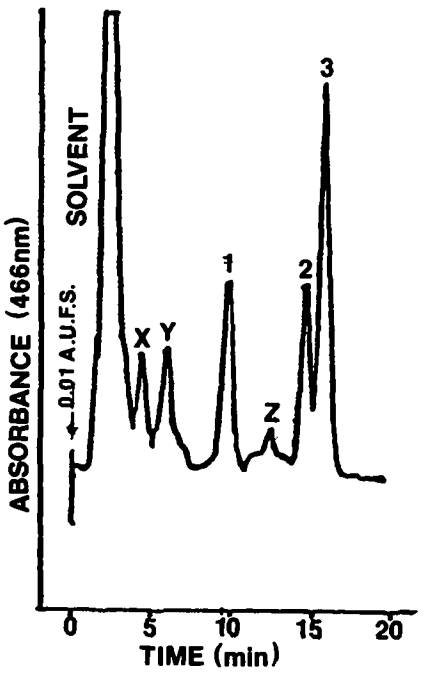

FIG. 2. HPLC chromatogram of serum carotenoids from a patient with anorexia nervosa-induced hypercarotenemia. Peaks 1, 2, and 3 are lycopene, α-carotene, and β-carotene, respectively. Peaks X and Y are probably xanthophylls, while peak Z might be γ-carotene. Chromatographic conditions as in Figure 1.

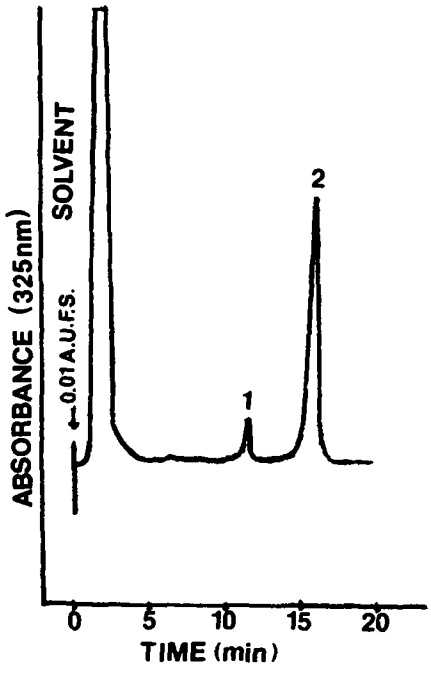

FIG. 3. HPLC chromatogram of serum retinyl esters from a patient with hypervitaminosis A. Peak 1 is unidentified. Peak 2=retinyl esters. Chromatographic conditions as in Figure 1 with the exception of detection at 325 nm.

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COMMUNICATIONS

Effect of Antioxidants on the Photooxidation of Fatty Acids

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ABSTRACT

The effect of butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tocopherol acetate on photooxidation of the fatty acids was studied. A marked increase in the photooxidation was observed in the presence of BHT, and this effect was further potentiated by hexabromobiphenyls. Conversely, BHA and tocopherol acetate as such did not show any significant effect, but greatly enhanced the photooxidation when hexabromobiphenyls were also present. Hexabromobiphenyls by themselves did not show any notable effect on the photooxidation.
Lipids 18:259-263, 1983.

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are antioxidants commonly used as food preservatives. These are hindered phenolic compounds and prevent autoxidation by donating a hydrogen atom to free radicals. It is estimated that Americans daily consume ca. 0.1 mg/kg body wt of these antioxidants. In recent years, there has been accumulating evidence that antioxidants reduce the incidence of chemically induced carcinogenesis in experimental animals (1-6). It has also been reported that an antioxidant-supplemented diet provides considerable protection against photocarcinogenesis (7-9). The mechanisms by which antioxidants provide such protection are, however, poorly understood at this time. In chemical carcinogenesis, induction of P-450 microsomal system (10), epoxide hydrase (11,12) and glutathione S-transferase activities (13,14) by antioxidants have been implicated in their anticarcinogenic action. These enzymes are generally known to have detoxification functions, even though epoxide hydrase is also involved in the activation of certain carcinogens (15,16). In photocarcinogenesis, the inhibitory action of antioxidants is believed to act through free radical quenching, thus preventing lipid peroxidation of cellular membranes (17). In support of this, it has been observed (9) that feeding a diet containing a mixture of added antioxidants delayed the appearance of lipid oxidation products. Most normal skin lipids are unlikely to function as chromophores for carcinogenic ultraviolet light (280-320 nm). Lipid peroxides were reported, however, by several workers following irradiation of skin (18,19); thus, the likelihood of endogenously photosensitized reactions in skin does exist.

Although photosensitized oxidation of lipids, in general, involves singlet oxygen, the participation of triplet state oxygen has also been reported. Using two different sensitizers, erythrosine and riboflavine, for oxidation of methyl oleate and methyl linoleate, Chan showed that erythrosine sensitization involves singlet oxygen while riboflavine-sensitized oxidations involve triplet oxygen (20). Although riboflavine-sensitized oxidations primarily involve radical formation like 'dark' oxidation, photooxidation involving triplet oxygen does not involve chain reactions. Besides, no induction period was observed in the latter case, while the former reactions involve long induction periods. This was supported by the relatively small inhibitory action of the antioxidant BHT in the riboflavine reactions (20). The observation implies that prevention of photosensitized oxidation involving either singlet or triplet oxygen should not be possible through the antioxidants commonly used to inhibit 'dark' oxidation. The inhibitory effect of antioxidants on photocarcinogenesis may thus be operating through some other mechanism than by simple free radical quenching. In order to determine whether or not the antioxidants can prevent the photooxidation of lipids, we have studied the effect of some commonly used antioxidants on photooxidation of unsaturated fatty acids. The present study shows that BHT enhances photooxidation of fatty acids rather than reduces it.

MATERIALS AND METHODS

BHA, BHT, dl-α-tocopherol, tocopherol

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acetate, methyl linoleate, methyl linolenate, and methyl arachidonate (99.9% pure) were purchased from Sigma Chemical Company (St. Louis, MO). The purity of the fatty acid esters was checked by thin layer chromatography (silica gel, hexane/ether 95:5 or 50:50). Hexabromobiphenyls were obtained from Ultra Scientific (Hope, RI). 1,4-Diazabicyclo(2.2.2) octane (DABCO) was purchased from Aldrich Chemical Company. Reference samples of methyl esters of 9-hydroxy-10,12-, 10-hydroxy-12,13-, 12-hydroxy-9,13- and 13-hydroxy-9,11-octadecadienoate were prepared as described by Thomas and Pryor (21), except that methylene blue was replaced by sensitox (rose bengal deposited on a polymeric surface, 0.08%), and characterized by ultraviolet, infrared and nuclear magnetic resonance (NMR) spectroscopy.

Chromatography

F & M model 402 equipped with hydrogen flame ionization detector was used for gas liquid chromatography (GLC). The analyses were made on 6 ft × ¼ in. od glass column packed with 4% OV-1 on 100-120 mesh Gas-Chrom Q, Applied Science Laboratories (State College, PA). The carrier gas was nitrogen, and the analyses were run at 200 C using methyl palmitate as internal standard. The photolyzed samples of tocopherol acetate were analyzed on 1% OV-1, 4 ft glass column, at 250 C using squalene as internal standard.

Spectra Physics model 3500 equipped with a variable wavelength detector was used for high performance liquid chromatography (HPLC). Isomers of methyl linoleate alcohol were separated on a 30 cm × 3.19 mm μ-porasil column using 0.3% 2-propanol in hexane as eluent (21). Conjugated and nonconjugated isomers were detected at 254 nm and 210 nm, respectively.

Irradiation

For studying the photooxidation of fatty acid methyl esters, a solution of these compounds in hexane (18 mg/ml), in the absence or presence of the antioxidants (10 mg/ml), was irradiated in a pyrex tube with unfiltered black light using F40 BLB fluorescent lamps (20 W × 10) which emit a continuous spectrum between 300-400 nm with ca. 1% of the fluence below 320 nm. The lamps were arranged in a concave well covered with a top. The test tubes to be irradiated were hung from the top in the middle of the well so all the samples received irradiation. The control and test samples were always put next to each other. In this arrangement, several samples could be irradiated simultaneously. The samples were cooled through a constant circulation of air in the well by a fan. Following irradiation, any loss in the amount of solvent was compensated and the samples were analyzed by GLC (4% OV-1 on Gas Chrom Q, 200 C) using methyl palmitate as internal standard.

Spectroscopic Measurements

Infrared spectra were determined with a Perkin-Elmer 237-B spectrophotometer as KBr pellets or as solid or liquid film on AgCl plates. Ultraviolet spectra were recorded on a Cary Model 118. Proton magnetic resonance spectra were recorded at 360 MHz on a Varian HR-360 NMR spectrometer using tetramethylsilane as internal standard.

Singlet Oxygen Quenching Studies

For quenching studies with DABCO, a solution of fatty acid methyl esters (18 mg/ml) in hexane was treated with DABCO (5 mg/ml, 44 mmol/l), in the presence or absence of BHT; the resulting solutions were irradiated as described above.

RESULTS AND DISCUSSION

Photooxidation of methyl linoleate, linolenate and arachidonate in the presence of BHT is shown in Table 1. Irradiation of methyl linoleate for 4 hr with black light showed less than 1% consumption. When this irradiation was done in the presence of BHT, a marked increase in the consumption (18%) of methyl linoleate was observed. Similarly, consumption of methyl linolenate and arachidonate was much lower with black light alone (15.5 and 19.8%, respectively) in comparison to when BHT was present (57.9% and 67.6%, respectively). The concentration of BHT used routinely in our studies was 10 mg/ml. However, a much lower concentration (0.2 mg/ml) was found to be equally effective in enhancing the photooxidation. That this effect was photochemical in nature was apparent because no significant consumption of these fatty acids was observed in the dark in the presence or absence of BHT. The enhancing effect of BHT is possible due to a direct or indirect (through photoproducts of BHT) photosensitization by this compound. This was supported by the fact that BHT itself underwent photooxidation under our experimental conditions.

Irradiation for 4 hr consumed BHT almost completely and the solution turned yellow, possibly due to the formation of quinones.

TABLE 1

The Effect of BHT on Photooxidation of Unsaturated Fatty Acids in the Presence or Absence of Hexabromobiphenyls[a]

Fatty acid	Percent loss of fatty acid under conditions indicated						
	Experimental conditions						
	hv	hv + BHT	hv + BHT + HBB	hv + HBB	BHT	BHT + HBB	HBB
Methyl linoleate[b]	<1	18.0 ± 3.2	36.5 ± 4.5	7.5 ± 1.5	<1	<1	<1
Methyl linolenate[b]	15.5 ± 2.5	57.9 ± 5.5	62.2 ± 5.2	19.5 ± 3.5	<1	<1	<1
Methyl arachidonate[b]	19.8 ± 3.2	67.6 ± 6.8	81.3 ± 7.4	27.4 ± 3.8	<1	<1	<1

[a]Irradiation was done for 4 hr using unfiltered black light (10 fluorescent tubes, 20 W each). The basic spectral output of this source was in the range of 320-400 nm with less than 1% below 320 nm. Each value represents the mean ± SE of 5 separate experiments.

[b]% Consumption.

When methyl linolenate was irradiated in the presence of the photooxidized mixture of BHT, a similar enhancing effect (as with BHT alone) in its consumption was observed, indicating that the enhanced photooxidation of unsaturated fatty acids was possible due to photosensitized reactions by quinones resulting from the oxidation of BHT. This was further supported by the fact that the time courses of the photooxidation of methyl linolenate and of BHT ran parallel to each other (Fig. 1). A marked increase in the consumption of methyl linolenate was observed after 3 hr when sufficient accumulation of the oxidized products of BHT occurred. Photooxidation in the absence of BHT, on the other hand, did not show any significant increase within this time period. As most photosensitized reactions involve singlet oxygen, one would expect the participation of this reactive oxygen species in BHT-induced photooxidation of fatty acids. This was indeed found to be the case, as evident by the analysis of fatty acid hydroperoxides. It has been shown by several workers that oxidation of fatty acids by free radicals can be distinguished from that by singlet oxygen by the nature of the hydroperoxides formed (20). For example, in free radical oxidation, methyl linoleate gives two conjugated hydroperoxides (9-hydroperoxy-10,12- and 13-hydroperoxy-9,11-octadecadienoate) in equal proportions. Oxidation by $^1O_2$, on the other hand, gives a mixture of the two conjugated (9- and 13-isomers) and two nonconjugated diene hydroperoxides (10-hydroperoxy-8,12- and 12-hydroperoxy-9,13-octadecadienoate). HPLC of the methyl linoleate hydroperoxides obtained from the photooxidation in presence of BHT showed the formation of conjugated as well as nonconjugated hydroperoxides, indicating the participation of $^1O_2$. This was further supported by the fact that, when BHT-induced photooxidation of methyl linoleate was carried out in the presence of DABCO (5 mg/ml), a well known singlet oxygen quencher (22-24), a significant decline (47%) in the consumption of this fatty acid was observed (Fig. 2).

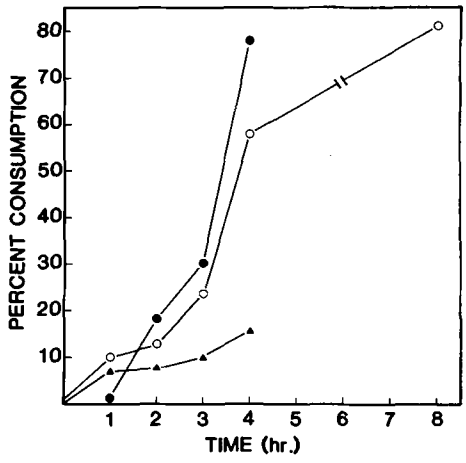

FIG. 1. Photooxidation of methyl linolenate in the presence (○) or absence (▲) or BHT. Irradiation was done as described in Methods. BHT alone (●) was photooxidized under the same conditions.

In order to see whether the enhancement of photooxidation was specific to BHT or was a general effect of phenolic antioxidants, we studied the effect of some other commonly used antioxidants, BHA, dl-α-tocopherol, and tocopherol acetate. As shown in Table 2, no enhancing effect by these antioxidants was observed. This could be due to the relatively slow rate of photooxidation of these compounds, thus preventing the accumulation of quinones from these compounds. α-Tocopherol acetate showed less than 10% consumption in 4 hr, while BHA exhibited ca. 30% consumption in the same time period. Conversely, 75-

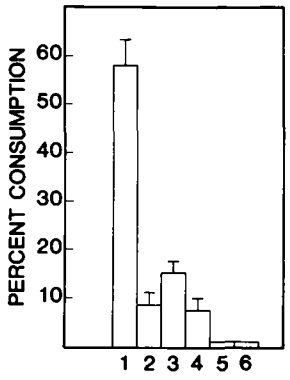

FIG. 2. Effect of DABCO on BHT-induced photooxidation of methyl linolenate (ML). (1) ML + hv + BHT; (2) ML + hv + BHT + DABCO; (3) ML + hv; (4) ML + hv + DABCO; (5) ML + BHT + DABCO; (6) ML + DABCO. Irradiation was done for 4 hr as described in Methods. Each straight bar is the mean ± SE of 5 separate experiments.

80% BHT was oxidized under these conditions.

In connection with some other studies going on in our laboratory concerning the interactions of ultraviolet light with polybromobiphenyls, we studied the effect of UVA (320-400 nm) on fatty acid peroxidation in the presence of hexabromobiphenyls (HBB). As expected, no effect of these chemicals was observed (Table 1) since HBB do not show any significant absorption beyond 320 nm. However, it was interesting to observe that presence of HBB potentiated BHT-induced enhancement of photooxidation (Table 1). For example, consumption of methyl linoleate was increased almost by 100% when both BHT and hexabromobiphenyls were present in the mixture as compared to the presence of BHT alone. The enhancing effect was even greater (ca. 300%) in the case of BHA and tocopherol acetate (Table 2). As described above, these antioxidants by themselves did not show any enhancing effect on the photooxidation. Since polybromobiphenyls are present widely as environmental pollutants (25) and tend to accumulate in subcutaneous fat (26), our observation that these compounds can greatly enhance the photooxidation of fatty acids in the presence of antioxidants could have significant biological implications.

ACKNOWLEDGMENTS

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TABLE 2

The Effect of BHA and Tocopherol Acetate on Photooxidation of Methyl Linolenate in Presence or Absence of Hexabromobiphenyls

Experimental conditions[a]	Percentage consumption[b]
hv	15.5 ± 2.8
hv + BHA	8.3 ± 3.5
hv + BHA + HBB	27.6 ± 4.2
BHA + HBB	<1
BHA	<1
hv + TA	8.9 ± 1.8
hv + TA + HBB	30.0 ± 4.5
TA + HBB	<1
TA	<1
hv + HBB	19.5 ± 3.3
HBB	<1
hv + Tocopherol	8.5 ± 3.5

[a]Irradiation conditions were the same as described in Table 1.

[b]Each value represents the mean ± SE of 5 separate experiments.

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Desmosterol in Human Milk[1]

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ABSTRACT

Milk samples were collected from mothers at 2, 6, 12 and 16 weeks postpartum. Desmosterol was found to be present in all the milk samples. Identification of desmosterol was based on retention times with two gas liquid chromatography (GLC) columns and verified by GC-mass spectrometry. The concentration of desmosterol in breast milk increased significantly ($P<.05$) from 0.6 mg/100 ml at 2 weeks to 1.3 mg/100 ml at 16 weeks postpartum. Desmosterol was not significantly correlated with total lipid, total cholesterol or free cholesterol in the milk.
Lipids 18:264-266, 1983.

Recently, we reported total cholesterol concentrations in breast milk that are less than those in many earlier reports (1). These earlier reports have been reviewed (2,3). The major difference between our work and the others was the method of cholesterol analysis. We used gas liquid chromatography (GLC), whereas most other workers used a less specific colorimetric assay. Although the results using the colorimetric assay were reported as cholesterol, they may have represented a wider range of sterols (4). Therefore, we decided a detailed sterol analysis of human milk was needed. During the analysis, we observed the presence of desmosterol, a previously unidentified sterol in human milk. In this paper, the basis for our identification of desmosterol and its concentration in breast milk collected from mothers between 2 and 16 weeks postpartum are reported.

METHODS

Milk samples were collected from 10 mothers at 2, 6, 12 and 16 weeks postpartum by the use of an electric breast pump. On the day of collection, total milk from one breast was taken in the morning (9:30-11:30 A.M.) and again in the afternoon (1:30-3:30 P.M.). After collection, the milk was immediately placed on dry ice and transported to the laboratory for storage at -70 C. For more information on the sampling see (1).

On the day of analysis milk was quickly thawed to 38 C and equal volumes of A.M. and P.M. milk pooled for analysis. A known amount of 5 α-cholestane (0.2 mg/ml milk) in chloroform/methanol (2:1, v/v) was added to the milk as an internal standard. The total lipids then were extracted by a modified Folch procedure as previously described (1). After the solvents were removed from the lipid extract, the total lipids were quantified by weighing. The sterols were isolated from other lipid classes on Silica Gel G thin layer chromatographic plates developed with petroleum ether/ethyl ether/glacial acetic acid (90:30:2, v/v/v). Further analysis of sterols was by GLC using stainless steel columns, 1.83 m x 2 mm, packed with 3% SE-30 on 100/120 Gas Chrom Q GA 2318 (Supelco Inc., Bellefonte, PA). The operating conditions for separation of sterols were injection port temperature 260 C, oven temperature 240 C, detector temperature 260 C and carrier gas flow 38 ml He/min. Peak areas were recorded and measured by the use of a Hewlett-Packard Model 3380-A recorder-integrator and quantified by calculations of relative response to 5 α-cholestane.

The identification of desmosterol was verified by GC-mass spectrometry. A 1.52 m x 2 mm glass column packed with 3% OV-17 on Chromasorb WHP 100/120 mesh (Supelco Inc., Bellefonte, PA) was used. The injector temperature was 300 C, the source temperature was 220 C and the jet-separator temperature was 300 C. The column temperature was programmed from 200 C to 290 C at 9 deg/min with a 15 min hold at 290 C. Helium was used as a carrier gas at a flow rate of 25 ml/min. The mass spectrometer (DuPont DP 102) was operated in the electron impact mode using 70 eV potential.

Statistical analysis of desmosterol concentrations in breast milk with time postpartum was based on a complete block design with repeated measurements (5). Once a significant change with time was detected, orthogonal contrasts between times were performed to determine if the change was linear or quadratic. Correlation analyses among total lipid, total cholesterol, free cholesterol and desmosterol in the breast milk were performed.

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RESULTS AND DISCUSSION

Desmosterol is found in mammalian tissues but to our knowledge has not been observed in human milk (6). In this study, the GLC peak we tentatively identified as desmosterol had identical retention times with a standard of desmosterol on both the SE-30 and OV-17 columns. The relative retention time for desmosterol to cholesterol was 1.08 for both GLC columns. Because of the close retention time with cholesterol, care must be taken to fully resolve desmosterol from cholesterol. This may explain why there are no reports of desmosterol in breast milk.

The mass spectrum of the peak tentatively identified as desmosterol in breast milk is compared to a standard mass spectrum of desmosterol analyzed under identical conditions (Fig. 1). The GLC peak of each of the compounds shown in Figure 1 had a relative retention time to cholesterol of 1.08. The mass spectrum of the compound in breast milk was similar to the mass spectrum of desmosterol standard and included the base peak at m/e 69, the major ion at m/e 271 and the molecular ion at m/e 384. Based on the mass spectrum data and the retention times observed with two GLC columns, we have concluded that desmosterol is present in human milk.

Desmosterol was present in all of the milk samples analyzed as determined by GC analysis on SE-30. The concentration ranged from 0.2 to 1.9 mg/100 ml. For comparison with desmosterol, the average concentrations of total lipid, total cholesterol, and free cholesterol in these milk samples from 2 to 16 weeks postpartum are given in Table 1.

The average concentration of desmosterol increased significantly ($P<.05$) from 0.6 mg/100 ml at 2 weeks to 1.3 mg/100 ml at 16 weeks postpartum. The increase had significant ($P<.05$) linear and quadratic components. The quadratic component was due to the small increase observed between 12 and 16 weeks. Desmosterol was not significantly correlated with total lipid, free cholesterol or total cholesterol in the milk.

The role of desmosterol in the mammary cell and the effect of its presence in breast milk on infant nutrition are not known. However, the desmosterol concentration was equal to ca. 10%

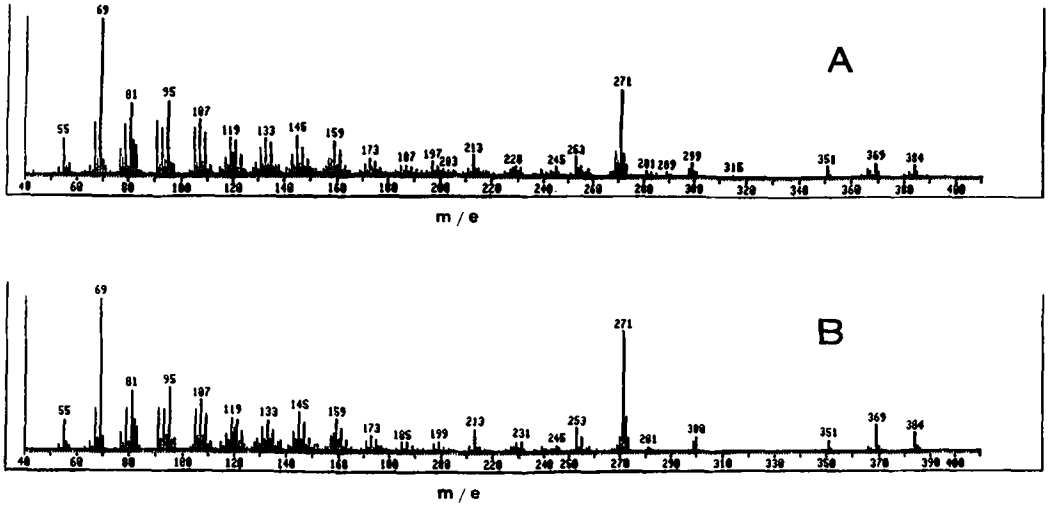

FIG. 1. Mass spectrum of a standard of desmosterol (A) and a compound tentatively identified as desmosterol in human milk (B).

TABLE 1

Lipids in Human Milk[a]

Parameters	2	6	12	16	CV[b]
Total lipid (g/100 ml)[c]	3.9	4.1	4.6	5.2	14.5
Total cholesterol (mg/100 ml)[c]	11.0	9.7	10.3	10.4	16.3
Free cholesterol (mg/100 ml)[c]	8.7	8.1	8.5	7.9	16.7
Desmosterol (mg/100 ml)	0.6	0.9	1.2	1.3	40.6

[a]Mean of 10 observations.

[b]Coefficient of variation (error mean square $0.5/\bar{x}$) X 100.

[c]Data previously reported (1).

of the total cholesterol concentration, making it a major sterol in human milk.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Ann M. Ferris for her help in the collection of milk samples. The research reported in this publication was supported in part by Federal funds made available through the provision of the Hatch Act and by NIH contract N01-HD-2817.

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Modulation of Platelet Thromboxane and Malonaldehyde by Dietary Vitamin E and Linoleate[1]

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ABSTRACT

Thromboxane (TXB_2) and malonaldehyde (MDA) production by thrombin-stimulated washed platelet were evaluated in rats fed 6 combinations of dietary vitamin E (0, 100, 1000 ppm) and linoleate (6.5 and 17.0 en%) for 23 weeks. The molar ratio of MDA:TXB_2 was consistently near 3 in all groups studied. In animals receiving the lower linoleate diets, TXB_2 and MDA synthesis were inversely related to the dietary vitamin E concentrations and the levels of MDA and TXB_2 were positively correlated (r = 0.99) with decreasing vitamin E in the diet. High dietary linoleate (17.0 en%), independent of vitamin E status, reduces TXB_2 and MDA synthesis. The importance of dietary antioxidant on platelet prostanoid synthesis is discussed.
Lipids 18:267-269, 1983.

INTRODUCTION

Platelets selectively incorporate arachidonic acid (AA) into their phospholipids and, upon stimulation, AA is released and can be metabolized via the lipoxygenase and the cyclooxygenase pathways (1,2). The predominant products from the cyclooxygenase pathway are thromboxane A_2 (TXA_2), 12-hydroxyheptadecatrienoic acid (HHT) and malonaldehyde (MDA) with minor formation of prostaglandin (PG) E_2, PGD_2 and $PGF_{2\alpha}$. The direct measurement of TXB_2, the aqueous decay product of TXA_2 as well as the indirect measurement of MDA, have been used to reflect the cyclooxygenase activity in stimulated platelets. Originally shown to be a potent vasoconstrictor and platelet aggregator (3,4), the induction role of TXA_2 in platelet aggregation has been challenged by the fact that aggregation and the release reaction can occur without detectable synthesis of TXA_2 (5).

Certain dietary factors such as vitamin E (6-9) and the types of fat (10-12) have been shown to influence platelet function and TXA_2 synthesis. The present study was designed to evaluate the quantitative difference between platelet TXA_2 and MDA production from rats fed six combinations of dietary vitamin E and linoleate for 23 weeks.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents were reagent grade. Dietary ingredients were purchased from ICN Biochemicals (Cleveland, OH) with the exception of "tocopherol stripped" corn oil which was from Eastman-Kodak (Rochester, NY). [^{3}H]Thromboxane B_2, sp act 125 Ci/mmol, was from New England Nuclear (Lachine, Quebec). The thromboxane standard and antiplasma to TXB_2 were gifts from Dr. J. Pike (Upjohn Co.) and Dr. L.K. Steel (NIH), respectively. Thrombin, of bovine source, was from Sigma Chemicals (St. Louis, MO). Dextran T70 was from Pharmacia Fine Chemicals (Uppsala, Sweden). All glassware was treated with Siliclad® (Clay-Adams Inc., New York) before use.

Animals and Diets

Young male Sprague-Dawley rats, with an average weight of 85 g were divided into 6 groups and fed purified diets containing a combination of 2 levels of linoleate (6.5 and 17.0 en%) and 3 levels of [dl]-α-tocopherol acetate (0, 100, 1000 ppm) for 23 weeks. Details of the dietary composition are described elsewhere (13). The spontaneous red blood cell hemolysis test and plasma pyruvate kinase activity were determined and used as indicators of vitamin E status. The results were reported in a separate communication (13).

Platelet Thromboxane (TXB_2) and Malonaldehyde (MDA) Production

After 23 weeks of feeding, blood was drawn via the abdominal aorta from pentobarbitol anaesthetized rat (50 mg/kg) using sodium citrate (3.8%) as anticoagulant (1:9 v/v). Platelets from 5 ml of blood were isolated by differential centrifugation and washed with calcium-free Tyrode buffer, pH 7.4. The washed platelets were resuspended in 1 ml of complete Tyrode and 200 μl were removed for protein determination. The remaining platelets were stimu-

[1]A portion of this work was presented at the conference on Vitamin E: Biochemical, Hematological and Clinical Aspects, New York Academy of Science, November 1981.

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lated with thrombin (5 U/ml) at 37 C for 5 min and the reaction was terminated with 1 N formate to pH 3.5. An aliquot of 200 μl was frozen at -25 C for TXB_2 determination and 500 μl were added to 3 ml of 0.5% 2-thiobarbituric acid. The mixture was capped and boiled for 10 min and MDA was detected from the chromogen formed (532 nm), using 1,1,3,3-tetraethoxypropane as standard. TXB_2 was determined by radioimmunoassay (RIA) as previously described for 6-keto-$PGF_{1\alpha}$ (14). TXB_2 production by stimulated platelet was linear from 0.5-2.0 mg of platelet protein (data not shown). RIA data were calculated using a logit transformation according to Rodbard et al. (15). Platelet protein was determined by the method of Lowry et al. (16) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

The platelet protein content per blood volume was significantly higher ($P<0.01$) in both groups of vitamin E-deficient rats (Table 1), representing a 140% and 172% increase (for 6.5 and 17.0 en% linoleate, respectively) of circulating platelets when compared with the vitamin E-supplemented rats. This observation is in agreement with that reported by Machlin and coworkers (17), who found an elevated platelet count in the vitamin E-deficient rats.

Synthesis of TXB_2 and MDA by thrombin-stimulated platelets is presented in Figure 1. The molar ratio of MDA:TXB_2 was consistently near 3 in all groups studied. In contrast, it has been reported that equal molar of MDA and TXB_2 was produced from the TXA_2 synthetic pathway (18). The higher molar ratio observed from this study suggests that MDA production by stimulated platelets was not solely derived from the TXA_2 synthetic pathway. Indeed, the lipoxygenase product HPETE (hydroperoxy eicosatetraenoic acid), which has been shown to react positively with thiobarbituric acid (19), is likely to contribute to the high MDA values observed herein.

At 6.5 en% of linoleate, both TXB_2 and MDA production were inversely related to the vitamin E concentrations in the diet; moreover, levels of MDA and TXB_2 were positively correlated ($r = 0.99$) with decreasing dietary vitamin E concentrations (Fig. 2). Such a relationship was not found to exist at a higher linoleate intake (17.0 en%). When comparing dietary linoleate levels, the MDA and TXB_2 production was relatively lower in rats receiving a higher linoleate intake. This can be explained by a reduced platelet arachidonate pool which has been shown to decrease and be replaced by linoleate when dietary linoleate was increased (20).

In vivo, vitamin E has been demonstrated to inhibit platelet aggregation in rats and humans (6-8) and to reduce TXB_2 synthesis from collagen-stimulated platelets (9). The effect of varying dietary vitamin E levels on platelet MDA and TXB_2 production observed in this study is in line with these observations only at the low

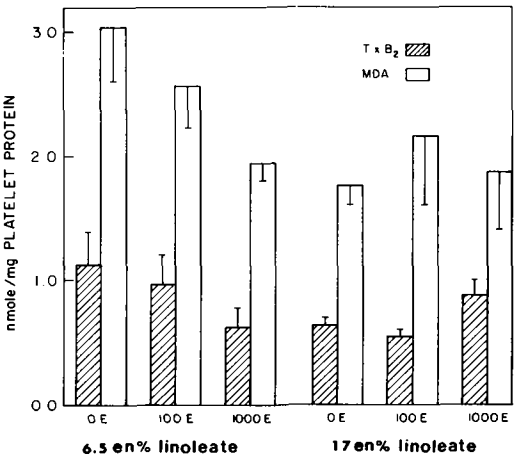

FIG. 1. Thromboxane (TXB_2) and malonaldehyde (MDA) production by thrombin-stimulated platelets from rats fed varying levels of vitamin E and linoleate for 23 weeks. Values are means ± SEM of 4-6 rats.

TABLE 1

Platelet Protein Concentration (mg/ml Blood) from Rats Fed Purified Diets Containing Different Combinations of Vitamin E and Linoleate for 23 Weeks

Dietary [dl]-α-tocopherol acetate (ppm)	Linoleate	
	6.5 en%	17.0 en%
0	0.91 ± 0.08[a]	1.12 ± 0.08[a]
100	0.65 ± 0.07	0.71 ± 0.06
1000	0.65 ± 0.03	0.65 ± 0.02

Values are mean ± SEM of 5 to 6 animals per group.

[a]Significantly different ($P<0.01$) from tocopherol-supplemented groups by analysis of variance.

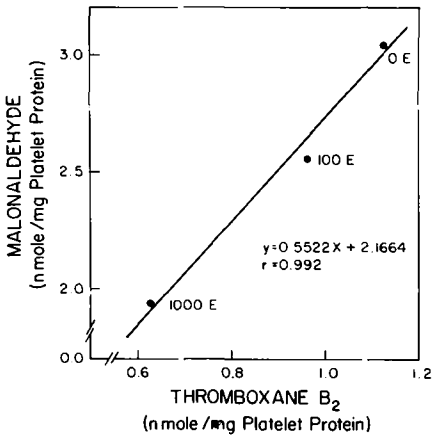

FIG. 2. Correlation between platelet malonaldehyde and thromboxane B_2 production from rats fed 6.5 en% linoleate and various concentrations (0, 100, 1000 ppm) of vitamin E. Values are means of 4-6 rats.

linoleate intake (6.5 en%). Using butter or corn oil as the sole dietary fat source, Agradi and coworkers (10) have reported a decrease in ^{14}C-TXB_2 incorporation from labeled arachidonate from the corn oil fed rabbit platelets. Judging by results from their study, this decrease in TXB_2 formation could in part be due to the higher dietary vitamin E content in the corn oil diet.

The mechanisms(s) by which vitamin E inhibits MDA and TXB_2 production is unclear at present. Preliminary evidence from this laboratory has demonstrated that platelet phospholipase A_2 can be inhibited by vitamin E in vitro and in vivo (Chan, unpublished observation). In conclusion, data from this experiment clearly demonstrated that dietary vitamin E and fatty acid can modulate the levels of TXB_2 and MDA production in thrombin-stimulated washed platelets. The results further suggest that the level of dietary antioxidant should be carefully considered when dietary fats, which contain varying amounts of antioxidant, were used to test for the thromboxane synthetic capacity of platelets.

ACKNOWLEDGMENTS

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The Effect of Vitamin C on in vivo Lipid Peroxidation in Guinea Pigs as Measured by Pentane and Ethane Production

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ABSTRACT

Measurements of pentane and ethane as indices of in vivo lipid peroxidation were made on samples of breath from vitamin C-sufficient and vitamin C-deficient guinea pigs injected with 23 μl carbon tetrachloride (CCl_4)/ 100 g body wt. Vitamin C-deficient animals produced significantly more pentane and ethane after CCl_4 treatment than did vitamin C-sufficient guinea pigs. Pretreatment of vitamin C-deficient animals with 75 mg ascorbic acid/ 100 g body wt significantly lowered both pentane and ethane evolution. Protection against in vivo lipid peroxidation similar to that provided by ascorbic acid was also found when vitamin C-deficient guinea pigs were pretreated with isoascorbic acid, reduced glutathione, α-tocopherol or β-carotene. When animals were pretreated with the radical scavenger mannitol, a protective effect was also observed as measured by pentane evolution.
Lipids, 18:271-274, 1983.

The deleterious effect of many xenobiotics, including carbon tetrachloride (CCl_4), is believed to be peroxidation of polyunsaturated fatty acids in biological membranes. Investigators who have undertaken studies of hydrocarbon gas evolution in the breath of animals as a measure of in vivo lipid peroxidation have been attracted to the use of CCl_4 as a powerful initiator of peroxidation (1,2). Protection of test animals against CCl_4-induced lipid peroxidation requires a suitable amount of the lipid-soluble antioxidant vitamin E (3-5). The involvement of other compounds, particularly of water-soluble ascorbic acid, in CCl_4 toxicity has also been reported (6,7). The oral administration of CCl_4 produced a marked decrease in the hepatic concentration of ascorbic acid (6). In rats, ascorbic acid has been shown to reduce mortality due to CCl_4 exposure (7).

Ascorbic acid, a vitamin in vivo, is a reducing agent that protects against lipid peroxidation in the guinea pig (8) and mouse (9). Tappel (10), Tappel et al. (11) and others (12,13) have proposed that ascorbic acid is an antioxidant-synergist with vitamin E. The antioxidant role can be explained by the rapid interaction between vitamin E and ascorbic acid. Vitamin E acts as a primary antioxidant, and ascorbic acid reductively regenerates oxidized vitamin E. Recently, the interaction between these vitamins has been observed by pulse radiolysis techniques (14). Several investigators have shown the antioxidative action of ascorbic acid in vitro (13,15,16). However, Chen (17) and Chen and Barnes (18) provided evidence that over a 1-2 month period, supplementation of rats with high levels of vitamin C in diets with vitamin E levels normally considered to be adequate leads to erythrocyte hemolysis. Because of these contradictory reports, there is further need to explore the antioxidative action of ascorbic acid in vivo.

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The present study demonstrated some new aspects of both protection from in vivo lipid peroxidation by ascorbic acid and the efficiency of the vitamin to decrease peroxidation as compared with other protective agents. The sensitive measurements of expired pentane and ethane, decomposition products of ω6- and ω3-unsaturated fatty acid hydroperoxides, respectively (19), were used to show antioxidant protection of guinea pigs against lipid peroxidation. Peroxidation was initiated by CCl_4 because the effects of CCl_4 develop in a relatively short time and the differences in hydrocarbon gas production between CCl_4-treated and untreated animals are highly significant.

METHODS

Male Hartley guinea pigs were housed two per cage in a room kept at 20-22C with a 14-hr light and 10-hr dark cycle. The animals were fed vitamin C-deficient and vitamin C-sufficient diets (20) beginning at 3 wk of age. At 5 wk of age, the guinea pigs fed the vitamin C-deficient diet had lost ca. 10-15% of their maximal body weight. Four hours prior to measurement of pentane and ethane production, these animals were injected intraperitoneally with either 75 mg of L-ascorbic acid, isoascorbic acid, reduced glutathione or mannitol in 0.9% saline/ 100 g body wt. When either 75 mg of dl-α-tocopherol or β-carotene in mineral oil/ 100 g body wt were injected, basal hydrocarbon gas evolution was measured after 48 hr.

Collection of expired air from guinea pigs fasted overnight and subsequent analysis of pentane and ethane were done essentially according to the procedure of Dillard et al. (21). The animals were placed into the chamber 15 min prior to collection of the sample, and the sample collection time was ca. 5 min. The calculation of ethane was based upon the gas chromatographic instrument response to pentane (22). After the basal levels of pentane and ethane were determined, each animal was ad-

ministered 23 μl of CCl_4 in mineral oil/100 g body wt by intraperitoneal injection.

The significance of differences in pentane and ethane production between vitamin C-sufficient and vitamin C-deficient animals was determined by the Student's two-tailed t-test. p Values ≤ 0.05 were considered significant. The significance of the influence of dietary vitamin C, vitamin C deficiency and of injected antioxidants on pentane and ethane production by guinea pigs was determined by one-way analysis of variance using weighted squares of the means.

RESULTS

The time course for expired pentane and ethane by vitamin C-sufficient and vitamin C-deficient guinea pigs following a single injection of 23 μl CCl_4 in mineral oil/100 g body wt is shown in Figure 1. The mean basal values were significantly greater for pentane ($p = 0.004$) and ethane ($p = 0.004$) in vitamin C-deficient guinea pigs than in vitamin C-sufficient animals. Injection of mineral oil alone did not increase hydrocarbon gas production in either group of animals. The concentration of pentane in the breath of guinea pigs reached a maximum mean value 60 min after CCl_4 injection, whereas the maximum concentration of ethane was reached 15 min after CCl_4 injection. After 120 min, vitamin C-deficient animals had 2.1-fold higher pentane production ($p = 0.0004$) than those fed the vitamin C-supplemented diet (Fig. 1A). Pentane production by the two groups was not significantly different ($p > 0.05$) 15, 30 and 60 min after CCl_4 injection. Maximal ethane concentration in the breath 15 min after CCl_4 injection was significantly greater ($p = 0.006$) in vitamin C-deficient than in vitamin C-sufficient guinea pigs (Fig. 1B).

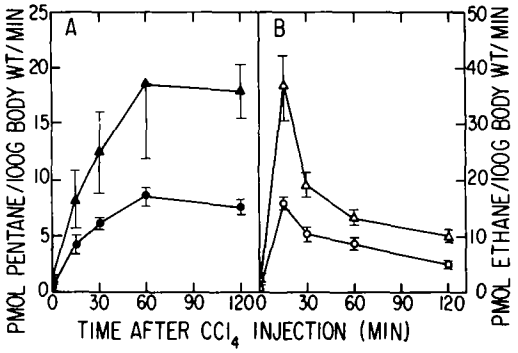

FIG. 1. Time-response relationship for total (A) pentane and (B) ethane produced after intraperitoneal administration of 23 μl CCl_4/100 g body wt. Guinea pigs were fed a vitamin C-supplemented diet (●,○) or a vitamin C-deficient diet (▲,△). Basal values at 0 time shown represent the mean ± SE of 13 vitamin C-sufficient and 30 vitamin C-deficient animals. Data shown at all other time points represent the mean ± SE of 6 animals in each group.

Neither administration of the water-soluble agents ascorbic acid, isoascorbic acid, glutathione or mannitol nor the administration of the lipid-soluble agents α-tocopherol or β-carotene over 4 hr or 48 hr, respectively, had a significant effect on either basal pentane or basal ethane evolution in vitamin C-deficient guinea pigs. Following an injection of a single dose of 23 μl CCl_4 in mineral oil/100 g body wt, vitamin C-deficient animals pretreated with either ascorbic acid or isoascorbic acid produced a significantly lower ($p = 0.01$) total amount of pentane over a 120-min time period than nonpretreated vitamin C-deficient guinea pigs (Fig. 2A). Total pentane evolution was also significantly lower ($p < 0.01$) by vitamin C-deficient guinea pigs pretreated with either glutathione, α-tocopherol, β-carotene or mannitol than by untreated animals.

A significantly lower ($p < 0.01$) total amount of ethane was produced over a 30-min time period by vitamin C-deficient guinea pigs pretreated with either ascorbic acid, isoascorbic acid, reduced glutathione, α-tocopherol or β-carotene than by untreated animals fed the vitamin C-deficient diet (Fig. 2B). The total amount of ethane evolved by mannitol-pretreated vitamin C-deficient guinea pigs was not significantly lower than that by

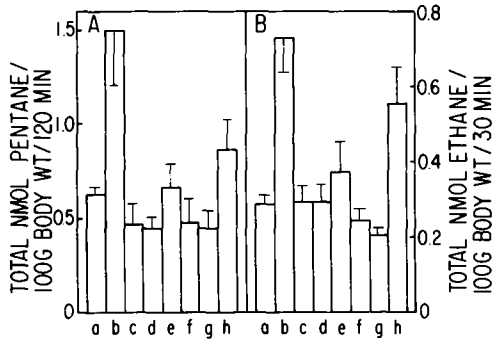

FIG. 2 Total net production of pentane (A) and ethane (B) above basal production over a 120-min and 30-min time period, respectively, after intraperitoneal administration of 23 μl CCl_4/100 g body wt. The net total amount of pentane and ethane above basal amounts was calculated by integration of values obtained at 15, 30 and 120 min for pentane and at 15 min and 30 min for ethane. The basal rate of ethane or pentane production was subtracted from the rate at each time point. Guinea pigs were fed a vitamin C-supplemented diet (a) or a vitamin C-deficient diet (b). Vitamin C-deficient animals were pretreated with 75 mg/100 g body wt of either ascorbic acid (c), isoascorbic acid (d), reduced glutathione (e) or mannitol (h) 4 hr prior to injection of CCl_4 or pretreated with α-tocopherol (f) or β-carotene (g) 48 hr prior to injection of CCl_4. Data shown represent the mean ± SE of 5 animals in each group.

vitamin C-deficient animals. Among the various pretreated vitamin C-deficient groups of animals, β-carotene- ($p<0.01$), α-tocopherol- ($p<0.01$), isoascorbic acid - ($p<0.02$) and ascorbic acid-pretreated guinea pigs had a significantly lower total amount of ethane than mannitol-pretreated vitamin C-deficient animals.

DISCUSSION

Many studies have shown expired hydrocarbons to be useful indices of in vivo lipid peroxidation (23). Several methods have been used to measure peroxidation, but most of the methods are time-consuming and are only applicable to postmortem tissue (24). Measurement of ethane and pentane seems to provide a sensitive indication of in vivo lipid peroxidation. The advantage of this noninvasive methodology is that a time course for peroxidative reactions can be followed in vivo after toxic substances such as CCl_4 are administered.

A large volume of evidence indicates that CCl_4 is a powerful initiator of in vivo lipid peroxidation (1,2) and administration of CCl_4 has been shown to elevate expired pentane or ethane dramatically (4,5). In our experiments, we demonstrated that CCl_4-treated guinea pigs also expired significantly increased amounts of hydrocarbons. More ethane than pentane was produced by CCl_4-injected guinea pigs in a short time period (Fig. 1) even though they had been fed a diet that contained 2.6% ω-unsaturated fatty acid for 2 wk. However, this phenomenon seems partly to be dependent on the differential solubility in fat of alkanes with different chain lengths (25). Therefore, ethane appears to be more readily released from the animal than does pentane, and more pentane could have been produced than was measured in the breath of the animals.

A variety of small molecules including vitamin E and ascorbic acid have been shown to decrease lipid peroxidation. Both basal and CCl_4-induced hydrocarbon gas production was greater in vitamin E-deficient animals (3-5,26) than in vitamin E-supplemented animals. In our studies, dietary vitamin C protected guinea pigs against lipid peroxidation. Consequently, both basal and CCl_4-induced production of pentane and ethane was lower in animals fed a vitamin C-supplemented diet than in those fed a vitamin C-deficient diet. However, CCl_4-induced lipid peroxidation in vitamin C-deficient guinea pigs as measured by pentane production was diminished by prior injection of ascorbic acid, isoascorbic acid, glutathione, mannitol, α-tocopherol or β-carotene. Guinea pigs were pretreated with relatively high amounts of these compounds to obtain a highly protective effect in short time experiments. Ascorbic acid exhibited an antioxidant capacity comparable to that of the other agents used. However, among the compounds tested, mannitol provided the least protection to vitamin C-deficient guinea pigs as measured by pentane production. Further, ethane production by mannitol-pretreated animals was not significantly lower than that by vitamin C-deficient guinea pigs. Recently, Bors et al. (27) determined the relative reaction rates of various radical scavengers with alkoxy radicals. Among the compounds tested, those compounds most reactive with alkoxy radicals were ascorbic acid and isoascorbic acid, and the least reactive were polyhydroxylated agents, including mannitol.

Overall, our experiments demonstrated that ascorbic acid enters into antioxygenic reactions in vivo. In addition to the known antioxidant protection against membrane damage provided to animals by vitamin E, protection in vivo is also provided by ascorbic acid. This interpretation of the results is strongly supported by the finding that both vitamin C-supplemented or ascorbic acid-pretreated guinea pigs were more protected against CCl_4-induced lipid peroxidation than were vitamin C-deficient animals not treated with ascorbic acid. However, the antioxidant function of ascorbic acid appears to be relatively unspecific when CCl_4 is used to induce peroxidation. Antioxidant protection similar to that of ascorbic acid was also provided by isoascorbic acid, glutathione, α-tocopherol and β-carotene. It should be of interest to examine in more detail the action of both water-soluble and lipid-soluble antioxidants in in vivo lipid peroxidation.

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Biosynthesis of Long-Chain Polyenoic Acids from Arachidonic Acid in Cultures of Enriched Spermatocytes and Spermatids from Mouse Testis

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ABSTRACT

Primary spermatocytes (PS), round spermatids (RS) and condensing spermatids (CS) from mouse testes were enriched on Sta-Put 1×g density gradients and cultured for 22 or 44 hr in the presence of [1-^{14}C]arachidonate. Mass and radioactivity were measured by gas radiochromatography of constituent fatty acids of the various complex lipid classes fractionated by thin layer chromatography. Patterns and levels of incorporation were compared with those of whole testis, both in vitro and in vivo. The 20:4, 22:4, 22:5, 24:4 and 24:5 of the germinal cells contained levels of radioactivity in each lipid class which were consistent with an important role for the germinal cells in long-chain polyenoic acid (LCPA) metabolism. Cells which represented later stages of spermatogenesis (RS, CS) incorporated much higher percentages and absolute amounts of radioactivity into the fatty acids derived from 20:4 by elongation-desaturation pathways than did PS or whole testis in vitro. These differences were most pronounced in triacylglycerol of CS. Distributions of mass and radioactivity among lipid classes suggest synthesis of triacylglycerol by CS with a high degree of specificity for 22 or 24 carbon LCPA at the *sn*-3 position.
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INTRODUCTION

Long-chain polyenoic acids (LCPA) derived from linoleic (n-6) or linolenic (n-3) acid are found in high concentrations in the testes of many species (1-3). These fatty acids are specifically associated with later stages of germinal cell differentiation and 22:5n-6, which is the predominant LCPA in testes of mice and rats, has been shown to occur in highest concentrations in the spermatids of those species (4-6). The pathways of biosynthesis of LCPA in the testis by a series of elongation-desaturation reactions have been demonstrated in the rat (3,7-9) but very little is known about the specific membrane-bound enzymes involved. Especially notable and unusual for this type of fatty acid is the accumulation of 22:5 in triacylglycerol of condensing spermatids in testes of mice and rats (4,5). Since these and other lines of indirect experimental evidence suggest that LCPA play an important role in spermatogenesis (1-3), the mechanisms involved in accumulation of these fatty acids and regulation of this process are of considerable interest.

The germinal cells differentiate (spermatogonia → primary spermatocytes (PS) → round spermatids (RS) → condensing spermatids (CS) → spermatozoa) in close communication with each other and sustentacular Sertoli cells through intercellular junctions (10). Evidence of mass movement of lipid among the associated cell types has been available for many years (11) and such movement may complicate interpretation of compositional analyses or metabolic studies in which intratesticular associations remain entirely or partially intact during experimentation. Beckman and Coniglio (8) have isolated rat Sertoli and mixed germinal cells *after* intratesticular injections of [^{14}C]arachidonate (20:4) and noted selective accumulation of radioactivity in 22:5 of Sertoli cells with a time course suggestive of a precursor-product relationship between 20:4 and 22:4 and 22:4 and 22:5. However, Grogan and Lam (12) isolated PS, RS and CS from mice *prior to* incubation with [^{14}C] acetate and obtained presumptive evidence for the presence of elongation-desaturation activities sufficient to account for the biosynthesis of LCPA from [^{14}C]acetate which was seen in whole testis in vivo or in vitro. In addition, the enriched germinal cells were able to incorporate acetate into the full range of fatty acids found in testicular lipids, to levels comparable with or higher than those seen in whole testis.

In the present work, we have studied the in vitro incorporation of [1-^{14}C]arachidonate into LCPA of various lipid classes by enriched PS, RS and CS in order to localize unambiguously the various biosynthetic capacities in the mouse testis. Metabolism of [^{14}C]arachidonate by germinal cells is contrasted with that of whole testis by in vitro

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Abbreviations: LCPA = long-chain polyenoic acids; 20:4(n-6) = all *cis*-5,8,11,14-eicosatetraenoic (arachidonic) acid; 22:4(n-6) = all *cis*-7,10,13,16-docosatetraenoic acid; 22:5(n-6) = all *cis*-4,7,10,13,16-docosapentaenoic acid; 22:6(n-3) = all *cis*-4,7,10,13,19-docosahexaenoic acid; 24:4(n-6) = all *cis*-9,12,15,18-tetracosatetraenoic acid; 24:5(n-6) = all *cis*-6,9,12,15,18-tetracosapentaenoic acid; PS = primary spermatocytes; RS = round spermatids; CS = condensing spermatids; RB = residual bodies; WT = whole testis; RA = radioactivity; SRA = specific radioactivity.

incubations of whole testis and by in vivo intratesticular injection of the labeled fatty acid.

METHODS AND MATERIALS

Germinal Cell Enrichment

Testes from 5 white Swiss mice (Microbiological Associates) were decapsulated and dispersed in Eagle's Minimum Essential Media (MEM) for suspension cultures (Flow Laboratories) with hyaluronidase (Sigma, Type V) and collagenase (Sigma, type II). Cells were sedimented at $1 \times g$ on an albumin density gradient using a Sta-Put apparatus as we have described in detail elsewhere (13). Fractions 12-17 (PS), 24-30 (RS) and 31-36 (CS) from the 40 fraction gradients were pooled and found to be enriched in primary spermatocytes, round spermatids and condensing spermatids, respectively, by histological evaluation as described previously (13). Cell compositions of the respective pooled fractions were 61, 30, 4 and 0%; 6, 63, 27 and 3%; and 3, 11, 62 and 24% PS, RS, CS and RB, respectively. All solutions were filtered through a 0.2-μ filter, and glassware and apparatus were autoclaved prior to use. Solutions were additionally supplemented with penicillin (25 units/ml), streptomycin (25 μg/ml) and Fungizone (2.5 μg/ml, Gibco). Concentrations of cells were determined by hemocytometer counts. Viability was checked by trypan blue exclusion, by O_2 uptake using a Clarke oxygen probe (Yellow Springs Instrument Co.), by phase contrast microscopy and by [^{14}C]arachidonate uptake.

[^{14}C]Arachidonate Incorporation

Dispersed cells (6×10^6 PS, 11×10^6 RS or 17×10^6 CS) or single whole decapsulated testes were incubated in 125 ml plastic culture flasks in an air/CO_2 (95:5) incubator at 37 C for 22 or 44 hr. Five ml of MEM contained 120 μmol glucose and 10 μCi[1-^{14}C]arachidonate (New England Nuclear, 56 mCi/mmol). The pH of incubations was monitored by phenol red indicator included in the commercial MEM. For whole testis in vivo studies, mice were ether anesthetized and injected intratesticularly with 10 μCi [^{14}C]arachidonate through small abdominal incisions which were then closed with surgical staples. Mice were sacrificed after 22 or 44 hr and testes were removed and decapsulated. Germinal cell suspensions were centrifuged at $60 \times$ g for 10 min and washed twice with phosphate buffered saline. All experiments were terminated by extraction of cells or tissue with $CHCl_3$/MeOH (1:1) as described below.

Gas Radiochromatographic Analyses

Total lipids were extracted from cells and tissues by the modified Folch procedure of Bridges and Coniglio (14). Total radioactivities of the extracts were measured by liquid scintillation counting of aliquots. The extract was dried under a stream of N_2 and methyl esters of the fatty acids were formed by transesterification with sodium methoxide as we described earlier (4). Methyl esters were analyzed for mass and radioactivity by a Packard 427 gas chromatograph equipped with a sample splitter (9:1) which divided the column effluent between a calibrated Packard 497 gas proportional radiation counter (83% efficiency) and a flame ionization mass detector. Gas chromatography was otherwise carried out as we have described previously (4). Radioactivity was assigned to specific fatty acids by corresponding retention times.

Radiochromatographic Analysis of Lipid Classes

Total lipids were extracted from tissues or pelleted cells by the modified Folch procedure of Bridges and Coniglio (14). Radioactivity was measured in aliquots of the lipid extract in a liquid scintillation counter. Extracts were dried under a stream of N_2, redissolved in $CHCl_3$ and separated on Whatman LHP-K silica gel thin layer plates developed with petroleum ether/ethyl ether/HOAc (80:20:1). Bands of phospholipid, triglyceride and cholesterol esters were visualized with Rhodamine 6G spray (Supelco) under ultraviolet light and scraped into screw-capped vials for methylation by transesterification with methanolic sodium methoxide (Supelco). Samples were sealed under N_2 and allowed to stand overnight at room temperature. Three vol of water were added and methyl esters were extracted at 4 C with petroleum ether for analysis by gas radiochromatography as already described. Radioactivity was measured in aliquots of the methyl ester extracts by liquid scintillation counting. All values were corrected to 100% of total radioactivity to adjust for extraction efficiency and pipetting or subsampling losses (dpm in a lipid class = % dpm in that class $\times$ total lipid-soluble RA; dpm in a specific fatty acid = % dpm in that fatty acid $\times$ RA in total fatty acid). Mass analyses were internally standardized by total radioactivity of fatty acid measured by both liquid scintillation counting and the calibrated gas proportional counter, using the assumption that recovery of mass = recovery of RA and the equality, total mass (mg) = total RA (dpm) $\div$ SRA (dpm/mg).

RESULTS

Fatty acid compositions of lipid classes from eriched germinal cells and whole testis (WT) (Table 1) were not significantly different after 22 or 44 hr of incubation from those which we have reported previously for fresh cells (4). Composition of

TABLE 1

Fatty Acid Distributions of Enriched Germinal Cell Suspensions and Whole Testes after Incubation with [^{14}C]Arachidonate[a]

Cell type (N)	Percent total fatty acid ± SEM									
	16:0	18:0	18:1	18:2	20:4	22:4	22:5	22:6	24:4	24:5
Total lipid										
PS (6)	34±0.9	10±0.5	15±1.1	4±0.6	14±0.8	2±0.3	11±0.9	7±0.6	1±0.2	1±0.2
RS (6)	36±1.8	7±0.4	10±0.8	4±0.2	13±0.3	2±0.3	16±0.8	10±0.5	1±0.2	1±0.0
CS (6)	37±2.7	7±0.3	10±1.0	4±0.3	11±0.7	3±0.2	17±1.1	9±0.7	2±0.3	1±0.2
WT (14)	32±0.4	9±0.4	16±1.4	4±0.8	12±0.8	3±0.2	13±0.8	8±0.5	2±0.2	2±0.2
Phospholipid										
PS (6)	41±0.7	11±0.4	13±0.8	4±0.2	14±0.4	1±0.0	10±1.0	6±0.4	1±0.1	1±0.1
RS (6)	45±1.4	9±0.3	10±0.9	3±0.3	11±0.7	1±0.2	12±1.3	7±0.5	1±0.2	1±0.0
CS (6)	44±1.9	10±0.4	9±0.4	3±0.3	10±0.5	2±0.2	13±0.8	8±0.9	1±0.0	1±0.0
WT (14)	37±0.9	11±0.1	12±0.3	2±0.2	12±0.7	2±0.0	13±0.4	8±0.2	1±0.1	1±0.2
Triacylglycerol										
PS (6)	25±0.7	9±0.8	21±0.5	6±0.8	11±1.1	5±0.5	12±0.2	7±0.5	2±0.2	2±0.2
RS (6)	26±0.7	6±0.8	16±1.3	4±0.3	10±0.8	6±0.5	20±2.2	6±0.5	2±0.5	2±0.2
CS (6)	30±0.8	4±0.6	15±0.4	3±0.3	6±0.5	6±0.6	27±1.6	5±0.6	2±0.7	2±0.3
WT (14)	28±0.8	6±0.5	25±2.4	9±1.4	10±1.1	3±0.4	11±1.1	5±0.7	1±0.0	2±0.2
Diacylglycerol										
PS (5)	39±3.6	11±1.2	14±0.7	6±2.1	11±1.2	2±1.2	10±2.6	7±1.2	1±0.0	1±0.3
RS (5)	45±3.3	10±1.4	11±1.6	6±1.6	9±0.5	2±0.2	11±1.1	6±0.6	1±0.1	1±0.3
CS (5)	50±2.0	10±0.5	11±0.5	3±0.2	7±0.6	2±0.0	10±0.8	6±0.8	1±0.2	1±0.2
WT (10)	36±1.3	12±0.8	14±0.3	4±0.9	12±0.8	2±0.2	11±0.6	6±0.5	1±0.0	2±0.2

[a]Cell suspensions or whole decapsulated testes were incubated for 22 or 44 hr, washed free of exogenous fatty acids and analyzed by gas chromatography. N = number of experiments.

diacylglycerol is reported for the first time. The 20:4 declined as a percentage of total fatty acid, whereas the percentage of 22:5 increased, with progressive stages of spermatogenesis (PS→RS→CS). The same trend was seen in phospholipid, but it was most pronounced in the fatty acid composition of triacylglycerol. The corresponding masses of n-6 LCPA shown in Table 2 have been weighted for number of cells and mean volumes of individual cells in the respective cell suspensions, as estimated from cell diameters and the positions of the cells in the Sta-Put gradient (15). This yields a true concentration parameter which is corrected for the large differences in cell volume among the various cell types. The concentrations of n-6 LCPA were higher in all lipid classes of later stages of spermatogenesis (RS, CS) than in PS. The concentrations of other fatty acids were also higher in the later stages (data not shown) but the differential was smaller, as may be deduced from the higher percentages of n-6 LCPA found in the later stages. Differential increases in n-6 LPCA of later stages can be largely accounted for by disproportionate and progressive increases in concentration of LCPA in triacylglycerol which are reflected in progressive increases of percentage composition from 32→40→43% of fatty acid in LCPA (see Table 1). Increases in the concentrations of 22:4, 22:5, 24:4 and 24:5 were proportionally larger than increases in 20:4 in all lipid classes. For example, 22:5 was 4-fold higher in triacylglycerol of CS than in PS, whereas 20:4 was only 1.7-fold higher.

Time Course of ^{14}C Incorporation

Incorporation of radioactivity from [^{14}C]arachidonate into the n-6 LCPA of testis cell suspensions was approximately linear for the first 22 hr (Fig. 1). After this time, discontinuities occurred, suggestive of altered metabolic function. Distribution of radioactivity among LCPA remained constant after sufficient radioactivity was present for radiochromatography, suggesting that equilibration of radioactivity among the various precursor pools was achieved early in the time course. Cell viability was 99% after 6 hr, 87% after 11 hr and remained at 70-80% for all time points thereafter. Cell composition did not change significantly. Suspensions enriched in PS, RS or CS by the Sta-Put gradient showed no net increase in specific radioactivity or total radioactivity (Tables 3 and 4) in any fatty acid after 22 hr. Distributions of radioactivity and fatty acid compositions of these suspensions also remained unchanged from 22-44 hr. This was sug-

TABLE 2

Concentration of n-6 Fatty Acids in Lipid Classes of Enriched Germinal Cell Types[a]

Lipid class	Fatty acid (μg/10^6 cells/100 μ^3 ± SEM)					
	20:4	22:4	22:5	24:4	24:5	Total n-6
	Primary spermatocytes					
Total lipid	174±31	26± 5	140± 23	9± 2	14±2	365± 62
Phospholipid	125±24	10± 2	91± 16	6± 7	7±3	238± 44
Triacylglycerol	26± 5	12± 2	29± 5	4± 1	4±1	75± 14
Diacylglycerol	6± 2	1± 0.3	4± 1	0.4± 0.1	1±0.3	13± 2
Methyl ester	4± 1	1± 0.5	3± 1	3± 1	4±2	16± 5
	Round spermatids					
Total lipid	374±44[b]	69± 9[b]	457± 37[b]	30± 9	27±5[c]	974± 95[b]
Phospholipid	252±36[c]	30± 4[c]	260± 28[b]	24± 6[c]	18±3[c]	583± 70[b]
Triacylglycerol	44± 9	27± 5[c]	89± 14[b]	10± 3	7±2	178± 26[b]
Diacylglycerol	14± 2[c]	2± 0.4	11± 3	1± 0.2[c]	2±0.6	33± 5[b]
Methyl ester	9± 2	3± 0.6[c]	9± 1[b]	5± 2	8±2	34± 8
	Condensing spermatids					
Total lipid	408±95[c]	95±16[b]	547±154[c]	60±13[b]	44±7[b]	1235±227[b]
Phospholipid	286±49[c]	47± 8[b]	268± 76	27± 8	20±6	743±109[b]
Triacylglycerol	40± 7	39±1[c]	174± 42[b]	21±11	18±6	291± 75[c]
Diacylglycerol	14± 5	4± 1[c]	20± 7	3± 2	2±0.7	43± 15
Methyl ester	9± 3	6± 2[c]	12± 5	5± 0.7	12±4	45± 14

[a]Lipids were extracted from suspensions of germinal cells after 22 or 44 hr incubations. Lipid classes were separated by thin layer chromatography and constituent fatty acids were analyzed by gas chromatography. Values are expressed as a ratio to cell volume to reflect accurately contribution to whole testis composition. N = 5 in each case. Statistical analysis is by Student's t-test.

[b]Different from value for primary spermatocytes, $p < .01$.

[c]Different from value for primary spermatocytes, $p < .05$.

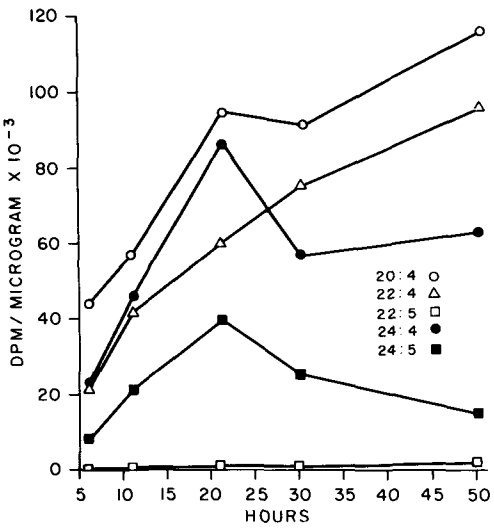

FIG. 1. Incorporation of [1-^{14}C]arachidonate into LCPA by germinal cell suspensions as a function of time. Dispersions of testis cells containing 95% germinal cells were incubated for the times specified. Lipids were extracted and constituent fatty acids were analyzed by gas radiochromatography. Value plotted on the ordinate is specific radioactivity of respective LCPA.

gestive of a metabolically quiescent state of these cells during this time period and allowed pooling of results from these time points. On the other hand, whole testis in vitro incorporated additional radioactivity and increased specific radioactivities of n-6 LCPA from 22 to 44 hr and this data is reported separately (Table 5).

Distribution of Radioactivity

Enriched germinal cell suspensions and whole testis incorporated ^{14}C from [1-^{14}C]arachidonate into all major lipid classes and their constituent n-6 LCPA (Tables 3-7). In every case, at least 99% of radioactivity was incorporated into complex lipid (Table 7). In addition, low levels (<1%) of radioactivity were occasionally found in 16:0, 18:0, 18:1 and fatty acids with chain lengths >24 carbons (data not shown). Oxidation is apparently not an important metabolic fate of n-6 LCPA in these cells.

All in vitro incubations, including both whole testis and germinal cell suspensions, incorporated 2% or less radioactivity into 22:5, the major n-6 LCPA of the mouse testis (Table 6). Whole testis in vivo incorporated radioactivity into 22:5 and 24:5 to a higher percentage and specific radioactivity

TABLE 3

Specific Radioactivities of n-6 Fatty Acids and Lipid Classes of Enriched Germinal Cells Incubated with [1-^{14}C]Arachidonate[a]

Lipid class	Specific radioactivity (dpm/μg fatty acid × 10^{-2} ± SEM				
	20:4	22:4	22:5	24:4	24:5
	Primary spermatocytes				
Total lipid	659 ± 123	466 ± 113	9 ± 2	336 ± 79	69 ± 25
Phospholipid	617 ± 125	513 ± 119	8 ± 2	463 ± 121	69 ± 27
Triacylglycerol	989 ± 160	587 ± 118	16 ± 3	676 ± 182	118 ± 28
Diacylglycerol	415 ± 107	416 ± 109	18 ± 5	323 ± 91	99 ± 10
Methyl ester	952 ± 308	898 ± 154	36 ± 8	625 ± 187	43 ± 17
	Round spermatids				
Total lipid	979 ± 60[b]	748 ± 46[b]	15 ± 4	1177 ± 247[b]	188 ± 28[b]
Phospholipid	1008 ± 67[b]	793 ± 173	11 ± 3	564 ± 144	148 ± 30
Triacylglycerol	1356 ± 120	941 ± 119	22 ± 7	1850 ± 505[b]	279 ± 69
Diacylglycerol	650 ± 80	836 ± 121[b]	17 ± 3	400 ± 125	207 ± 130
Methyl ester	1255 ± 185	1619 ± 232[b]	46 ± 11	1334 ± 426	70 ± 12
	Condensing spermatids				
Total lipid	1084 ± 68[b]	994 ± 80[c]	11 ± 4	987 ± 145[c]	200 ± 59
Phospholipid	1075 ± 125[b]	814 ± 135	15 ± 4	804 ± 176	162 ± 43
Triacylglycerol	1914 ± 121[c]	1302 ± 155[c]	19 ± 2	2290 ± 445[c]	324 ± 72[b]
Diacylglycerol	846 ± 81[b]	1155 ± 167[c]	22 ± 5	803 ± 159[b]	206 ± 33[b]
Methyl ester	1999 ± 50[b]	1474 ± 117[b]	46 ± 4	2348 ± 1245	95 ± 28

[a]Enriched germinal cells were incubated with 10 μCi [1-^{14}C]arachidonate for 22 or 44 hr. Lipids were extracted, fractionated into lipid classes by thin layer chromatography and analyzed by gas radiochromatography. N = 5 in each case. Statistical analyses by Student's t-test.

[b]Different from value for primary spermatocytes, $p < .05$.

[c]Different from value for primary spermatocytes, $p < .01$.

than did whole testis in vitro, but specific radioactivities for the other n-6 LCPA were quite comparable (Table 5). On the other hand, 22:5 and 24:5 of germinal cell suspensions (Table 3) had specific radioactivities comparable to those of whole testis in vivo, but the other n-6 LCPA of germinal cells had specific radioactivities much higher than those of whole testis in vivo. While it is difficult to interpret these findings without precise knowledge of the specific radioactivities of the respective LCPA precursor pools, specific radioactivities of the various fatty acids shown in Table 5 are comparable in whole testis in vivo and in vitro after 22 hr, suggesting that the precursor pools also have comparable specific radioactivities. The decrease in relative and absolute incorporation into the pentaenoic acids may be more a function of in vitro incubation than cell composition. Incubation of duplicate suspensions or whole testis in an atmosphere of O_2/CO_2 (95:5) did not affect the distribution of radioactivity (data not shown). Somewhat surprisingly, the distribution of radioactivity among lipid classes in germinal cell suspensions was practically identical to that of whole testis in vivo but very different from whole testis in vitro (Table 7).

The concentration of radioactivity with respect to cell volume (Table 4) was dramatically higher in RS and CS than in PS for each n-6 LCPA in each lipid class. This trend was progressive for the stages of differentiation, PS→RS→CS. In each lipid class, concentrations of radioactivity in 22:4, 22:5, 24:4 and 24:5 were disproportionately higher in RS and CS than were concentrations of radioactivity in 20:4. For example, concentrations in 20:4, 22:4, 22:5 and 24:4 were 2.7- , 7.3- , 7.3- , and 14-fold higher in triacylglycerol of CS than in PS.

The specific radioactivities of the n-6 LCPA were also higher in the later stages than in PS (Table 3) but the disproportionate progressive increases in concentration of radioactivity among the LCPA were masked somewhat by offsetting disproportionate increases in the concentrations of mass of some of the LCPA, with progressive stage of differentiation. This was especially true for 22:5 which was 8-fold higher in radioactivity of triacylglycerol in CS than PS, but also 6-fold higher in mass.

The distribution of ^{14}C in diacylglycerol of germinal cells was unlike that of either phospholipid or triacylglycerol (Table 6). The pattern of incorporation into diacylglycerol was more similar to that of triacylglycerol in PS, but the percentages of radioactivity in the LCPA derived by elongation of 20:4 increased dramatically in triacylglycerol with increasing stage of spermatogenesis (25→40→50%

TABLE 4

Incorporation of [1-^{14}C]Arachidonate into n-6 Fatty Acids and Lipid Classes by Enriched Germinal Cells as a Function of Cell Number and Volume[a]

Lipid class	Radioactivity (dpm/10^5 cells/100 μ^3)					
	20:4	22:4	22:5	24:4	24:5	Total RA
	Primary spermatocytes					
Total lipid	1163±379	112± 37	11± 2	35± 8	8± 2	1329± 423
Phospholipid	750± 24	43± 9	7± 2	23± 9	2± 1	826± 262
Triacylglycerol	266± 80	70± 25	4± 1	31± 15	4± 1	376± 120
Diacylglycerol	20± 3	2± 1	0.7± 0.2	1± 0.6	0.8± 0.3	26± 5
Methyl ester	39± 18	14± 6	1± 0.3	12± 0.7	2± 0.5	69± 35
Cholesteryl ester	28± 14	8± 5	1± 0.4	2± 1	2± 0.7	40± 27
	Round spermatids					
Total lipid	3659±466[b]	524± 93[b]	41±10[c]	334± 95[c]	52±13[c]	4645± 575[b]
Phospholipid	2567±439[b]	223± 36[b]	31±10[c]	295±183	25± 8[c]	3142± 411[b]
Triacylglycerol	601±142	270± 72[c]	21± 7	215± 81	27± 8[c]	1134± 295[c]
Diacylglycerol	87± 14[b]	19± 4[c]	2± 0.5[c]	6± 2	2± 1	118± 20[b]
Methyl ester	118± 29	47± 12[c]	4± 1	48± 9[c]	6± 2[c]	222± 54[c]
Cholesteryl ester	48± 10	15± 5	1± 0.5	12± 5	2± 0.6	78± 20
	Condensing spermatids					
Total lipid	4344±939[c]	961±208[b]	66±22[c]	602±179[c]	100±36[c]	6073±1286[b]
Phospholipid	2887±393[b]	347± 46[b]	52±13[b]	213± 69[c]	27± 6[b]	3538± 517[b]
Triacylglycerol	776±132[c]	516±164[b]	32± 6[b]	437±212[b]	58±27	1819± 519[c]
Diacylglycerol	127± 54	36± 17	4± 1[c]	19± 10	5± 2	196± 82
Methyl ester	177± 52[c]	95± 40	5± 2[c]	46± 7[c]	13± 8	424± 175
Cholesteryl ester	39± 13	12± 5	1± 0.7	20± 13	6± 4	72± 32

[a]Cell suspensions were incubated with 10 μCi [1-^{14}C]arachidonate for 22 or 44 hr. Lipids were extracted and fractionated by thin-layer chromatography and assayed for radioactivity by scintillation counting and gas radiochromatography. N = 5 in each case. Statistical analysis is by Student's t-test.
[b]Different from value for primary spermatocytes, $p < .01$.
[c]Different from value for primary spermatocytes, $p < .05$.

of radioactivity). There was no parallel shift of this magnitude in percentages of incorporation into diacylglycerol or phospholipid. This is consistent with biosynthesis of triacylglycerol from diacylglycerol by acylation of the *sn*-3 position with a high specificity for 22 or 24 carbon LCPA in the later stages of spermatogenesis. Fatty acid compositions also support this view. Diacylglycerol resembled phospholipid in fatty acid composition, whereas triacylglycerol was quite different in composition from each of the other lipids (Table 1). The 18 carbon fatty acids were notably higher in triacylglycerol of PS than in triacylglycerol of later stages (36% vs 26% and 22%) whereas, as previously noted, the LCPA were much higher in triacylglycerol of later stages, especially CS. Whereas most of the fatty acids had the same or a lower percentage in triacylglycerol than in diacylglycerol, 18:1, 22:4 and 22:5 were consistently higher in triacylglycerol than in diacylglycerol. The 18:1 showed the greatest increase in PS, whereas the 22 carbon fatty acids accounted for most of the increase in later stages. From stochiometric considerations, these observations are consistent with biosynthesis of triacylglycerol from diacylglycerol by acylation of the *sn*-3 position with either 18 carbon fatty acids or 22:5, the latter fatty acid predominating in later stages.

Lipids having the chromatographic mobilities of methyl esters and cholesteryl esters contained n-6 LCPA which were labeled with ^{14}C to a high specific radioactivity (Table 3). These fractions were not further characterized chemically. Although distribution of radioactivity in the methyl ester band was similar to that of triacylglycerol, specific radioactivities of LCPA in this fraction were the highest seen in any lipid class.

Temperature Effects

In order to assure that results were not affected by incubation of cells at temperatures slightly higher than the normal scrotal temperature of the mouse, a complete set of incorporation studies was carried out at 32 C. PS, RS, CS and whole testis incorporated [1-^{14}C]arachidonate into fatty acids and lipid classes in patterns and proportions which were not distinguishable from those of 37 C incubations (data not shown) after 22 hr.

TABLE 5

Specific Radioactivities of n-6 Fatty Acids and Lipid Classes from Whole Testes Incubated or Injected with [1-^{14}C]Arachidonate[a]

Lipid class	Specific radioactivity (dpm/μg fatty acid $\times 10^{-2} \pm$ SEM)									
	20:4		22:4		22:5		24:4		24:5	
	22 hr	44 hr	22 hr	44 hr	22 hr	44 hr	22 hr	44 hr	22 hr	44 hr
WT in vitro										
Total lipid	90± 3	153± 20	86±12	132± 12	2±0.7	3±1	46± 8	129±56	15± 4	22± 5
Phospholipid	56± 6	89± 10	53± 6	61± 11	1±0.4	1±0.1	33±10	55±23	12± 4	10± 2
Triacylglycerol	209±40	287± 43	159±35	228± 33	4±0.8	6±2	125±24	267±67	30± 4	56± 7
Diacylglycerol	70± 8	141± 28	94±14	212± 34	2±0.3	9±3	78± 8	135±25	29± 8	45±14
Methyl ester	96±10	371±110	91± 8	356±115	2±0.5	9±3	56± 8	250±88	7± 1	41±13
WT *in vivo*										
Total lipid	64± 5	53± 1	82±11	84± 4	6±1	12±0.6	55± 3	50± 1	38± 6	40±0.2
Phospholipid	49± 3	54± 1	63± 8	61± 7	6±0.8	11±0.6	31± 2	44± 0.3	32± 7	36±13
Triacylglycerol	120±14	88± 8	100±21	98± 15	5±2	9±1	44± 2	61± 8	39±12	41±2
Diacylglycerol	58±10	50± 0.5	49± 4	42± 0.2	7±1	12±0.8	29± 2	40± 4	32± 6	42±10
Methyl ester	90	69± 3	53	62± 7	6	11±0.3	24	49± 5	16	22± 0.6

[a]Whole testes were incubated (*in vitro*) or injected (*in vivo*) with 10 μCi [1-^{14}C]arachidonate. After 22 or 44 hr lipids were extracted, fractionated by thin layer chromatography and analyzed by gas radiochromatography. N = 6 for WT in vitro, 22 hr, 4 for 44 hr; N = 2 for WT in vivo 22 and 44 hr; N = 1 for values without SEM; dpm = disintegrations/min[a]

TABLE 6

Distribution of Radioactivity among Fatty Acids and Lipid Classes of Enriched Germinal Cell Suspensions and Whole Testis Incubated with [1-^{14}C]Arachidonate[a]

Cell type (N)	% Total radioactivity ± SEM				
	20:4	22:4	22:5	24:4	24:5
	Total lipid				
PS (6)	88 ± 1.5	6 ± 1.0	1 ± 0.0	3 ± 0.5	1 ± 0.1
RS (6)	81 ± 3.7	10 ± 1.6	1 ± 0.4	6 ± 1.6	1 ± 0.2
CS (6)	76 ± 3.4	13 ± 1.7	1 ± 0.2	8 ± 1.8	1 ± 0.2
WT in vitro (14)	78 ± 1.3	14 ± 0.8	2 ± 0.2	4 ± 0.4	2 ± 0.3
WT in vivo (4)	68 ± 1.9	13 ± 0.5	10 ± 2.2	3 ± 0.0	6 ± 0.3
	Phospholipid				
PS (6)	91 ± 1.2	5 ± 0.5	1.0 ± 0.0	3 ± 0.4	0.5 ± 0.2
RS (6)	87 ± 1.7	7 ± 1.0	1.2 ± 0.2	4 ± 0.7	0.8 ± 0.1
CS (6)	83 ± 1.8	10 ± 0.6	1.3 ± 0.3	5 ± 1.0	1.0 ± 0.0
WT in vitro (14)	84 ± 1.0	10 ± 0.5	1.4 ± 0.1	4 ± 0.3	1.2 ± 0.1
WT in vivo (4)	74 ± 1.6	9 ± 0.5	10 ± 1.7	3 ± 0.0	4 ± 0.2
	Triacylglycerol				
PS (6)	74 ± 3.4	16 ± 2.0	1.3 ± 0.3	7 ± 1.7	1.0 ± 0.2
RS (6)	60 ± 5.0	21 ± 2.2	1.7 ± 0.2	15 ± 3.5	2.0 ± 0.3
CS (6)	51 ± 5.0	26 ± 1.6	1.8 ± 0.3	19 ± 3.6	3.0 ± 0.3
WT in vitro (14)	74 ± 2.1	18 ± 1.2	1.5 ± 0.2	5 ± 0.4	2.4 ± 0.4
WT in vivo (4)	48 ± 2.2	31 ± 0.4	8 ± 1.5	4 ± 0.2	9 ± 0.7
	Diacylglycerol				
PS (5)	79 ± 5.0	12 ± 2.4	2 ± 0.5	5 ± 1.5	2 ± 0.9
RS (5)	75 ± 2.4	16 ± 1.5	2 ± 0.2	5 ± 1.2	2 ± 0.4
CS (5)	68 ± 4.1	19 ± 2.8	2 ± 0.5	8 ± 1.7	2 ± 0.2
WT in vitro (10)	73 ± 1.8	16 ± 1.0	3 ± 0.3	6 ± 0.6	3 ± 0.5
WT in vivo (4)	78 ± 1.6	6 ± 0.7	9 ± 1.4	2 ± 0.3	4 ± 0.6
	Methyl ester				
PS (4)	64 ± 5.9	15 ± 2.1	2 ± 0.3	17 ± 3.3	2 ± 0.5
RS (6)	58 ± 4.4	19 ± 1.0	2 ± 0.3	18 ± 2.8	2 ± 0.3
CS (5)	52 ± 5.4	22 ± 0.7	1 ± 0.2	23 ± 4.7	3 ± 0.5
WT in vitro (14)	66 ± 2.1	20 ± 0.9	1 ± 0.1	9 ± 0.7	4 ± 0.5
WT in vivo (4)	21 ± 4.4	28 ± 1.2	8 ± 1.2	14 ± 0.3	25 ± 1.9
	Cholesteryl ester				
PS (4)	74 ± 2.7	17 ± 1.9	2 ± 0.4	4 ± 0.5	2 ± 0.5
RS (5)	67 ± 5.6	17 ± 2.0	3 ± 0.4	12 ± 3.1	2 ± 0.7
CS (5)	60 ± 5.6	15 ± 0.9	2 ± 0.6	19 ± 4.9	4 ± 0.5
WT in vitro (10)	69 ± 2.3	21 ± 1.8	3 ± 0.5	5 ± 0.5	2 ± 0.5
WT in vivo (4)	25 ± 1.6	58 ± 1.3	6 ± 0.6	4 ± 0.3	9 ± 1.0

[a]Cell suspensions or whole testis were incubated with or injected intratesticularly with 10 μCi [1-^{14}C]arachidonate. After 22 or 44 hr, total lipids were extracted, separated by thin layer chromatography and analyzed by gas radiochromatography. N = number of experiments.

DISCUSSION

Whole mouse testis and suspensions of the various enriched germinal cell types (PS, RS, CS) incorporated [^{14}C]arachidonate into 20:4, 22:4, 22:5, 24:4 and 24:5 of phospholipid, triacylglycerol, diacylglycerol, methyl esters and cholesteryl esters. The distribution and magnitude of incorporation suggested that each of the germinal cell types is capable of making a major contribution to the metabolism of n-6 fatty acids by the testis. However, most of the radioactivity was incorporated into the elongation products, 22:4 and 24:4, in germinal cells, whereas only 1% of radioactivity was incorporated into 22:5, the major n-6 LCPA of germinal cells. This discrepancy may have been a function of in vitro incubation rather than cell composition, since whole testis in vitro also incorporated relatively little (2%) radioactivity into 22:5. The patterns of incorporation of all in vitro incubations were sufficiently different from those of whole testis in vivo injected with [^{14}C]arachidonate to indicate some disruption of the normal patterns of LCPA metabolism by removal from the mouse and isolation of the cells from their intratesticular environ-

TABLE 7

Distribution of Radioactivity among Lipid Classes of Enriched Germinal Cells and Whole Testis Incubated with [1-^{14}C]Arachidonate*

Lipid class	% Total radioactivity ± SEM (N)				
	PS (6)	RS (6)	CS (6)	WT in vivo	WT in vitro
Cholesteryl ester	3.1 ± 0.7	1.9 ± 0.4	0.9 ± 0.2	6.0 ± 0.4	1.3 ± 0.1
Methyl ester	4.1 ± 0.6	6.1 ± 1.3	6.0 ± 0.7	2.6 ± 0.2	8.0 ± 0.6
Triacylglycerol	25.8 ± 3.2	22.2 ± 5.0	25.6 ± 4.2	19.4 ± 2.9	59.6 ± 2.5
Unesterified fatty acid	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	1.2 ± 0.2
Diacylglycerol	2.0 ± 0.3	2.7 ± 0.4	2.9 ± 0.4	8.8 ± 2.1	2.2 ± 0.3
Phospholipid	64.8 ± 3.5	66.7 ± 6.6	64.2 ± 4.7	63.2 ± 4.6	27.7 ± 2.3

*Cell suspensions or whole testes were incubated with 10 μCi [1-^{14}C]arachidonate for 22 hr and washed free of exogenous radioactivity. WT in vivo were injected intratesticularly. Total lipids were extracted and separated by thin layer chromatography. N = number of experiments.

ment. Apparent metabolic quiescence of the germinal cells after 22 hr in vitro is also consistent with this view. However, absolute levels of incorporation into the various LCPA suggest that the same elongation and desaturation activities (3, 7-9) are present, both in vivo and in vitro, although in different proportions. The similarities between distributions of radioactivity among lipid classes in germinal cells and whole testis in vivo suggest that the normal pathways of complex lipid synthesis are intact in the isolated germinal cells. Both relative and absolute levels of incorporation suggest that the enzymatic elongation and desaturation activities necessary for biosynthesis (3) of 22:4, 22:5, 24:4 and 24:5 from 20:4 are more concentrated in the later stages of spermatogenesis, RS and CS. In similar studies reported earlier using [1-^{14}C]acetate as substrate (12), CS incorporated less radioactivity into LCPA than did PS. Since in this heterogeneous system, there are multiple substrates giving rise to each LCPA by elongation, the stoichiometry of acetate incorporation is unclear. Therefore, arachidonate incorporation would seem to be the better index of n-6 LCPA synthesis. In vitro studies of [^{14}C]acetate incorporation by testis have resulted in recovery of up to 30% of labeled fatty acid in the unesterified fatty acids (12,16,17). In contrast, LCPA are rapidly incorporated into complex lipid and very little label is found in unesterified fatty acids derived from [^{14}C]arachidonate or in 16 or 18 carbon fatty acids in which label would be indicative of catabolism of 20:4. This suggests a highly specific and tenacious conservation of LCPA by the germinal cells. Moreover, incorporation of radioactivity into LCPA is not likely to have resulted from elongation with [^{14}C]acetate derived from β-oxidation of arachidonate.

The relative (%) incorporations of radioactivity into 22:5 and other LCPA by in vitro incubations are not consistent with either the fatty acid compositions of the cell types involved or the distribution of radioactivity in whole testis in vivo. Most of the radioactivity appears in 22:4 rather than 22:5, the major LCPA in terms of mass. However, the absolute levels of incorporation (specific radioactivity) into 22:5 are of the same order of magnitude in germinal cells and whole testis, in vitro or in vivo. Although these comparisons must be made with caution due to the lack of data on specific activities of the precursor pools, similarities between the incorporation patterns of germinal cells which contain no Sertoli cells and whole testis in vitro which do contain Sertoli cells in intact tubules do not support a sequestering of 22:5 synthesis into Sertoli cells, as has been suggested by the experiments of Beckman and Coniglio in the rat (8). These investigators looked at distribution of radioactivity in lipids of Sertoli and germinal cells separated after intratesticular injection (in vivo) of [^{14}C]arachidonate. Their approach does not rule out the possibility that 22:5 accumulates in the Sertoli cell after synthesis in germinal cells.

It is clear that the characteristic and unique LCPA metabolism of the testis is to some degree a function of complex interactions between the various cell types present in the germinal epithelium. These interactions may ocur at the levels of LCPA biosynthesis, complex lipid synthesis, intracellular lipid transport and humoral, hormonal or compartmental influences regulating metabolism. These studies demonstrate that the isolated germinal cells of the mouse contain each of the metabolic activities necessary for synthesis of their major characteristic lipids, including LCPA. To what extent these activities play a major role in biosynthesis of 22:5 in vivo has yet to be determined.

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Severe Fatty Liver in Rats Fed a Fat-Free Ethanol Diet, and Its Prevention by Small Amounts of Dietary Arachidonate[1]

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ABSTRACT

Rats were fed ethanol and a fat-free diet for 30 days to determine whether dietary fat is needed for the development of fatty liver. The severity of fatty liver was similar to that of rats fed an isocaloric diet with 35% fat. Small amounts (29 mg/day) of dietary arachidonic acid prevented alcoholic fatty liver. Rats fed either the alcohol (AF) or control (CF) fat-free diets developed essential fatty acid deficiency (EFAD) as measured by the triene/tetraene ratio of liver and plasma lipids. Rats fed arachidonic acid (AA, alcohol and CA, control diets) did not develop EFAD. Although EFAD alone did not cause the development of fatty liver, the combination of dietary ethanol and EFAD did. The ratios of 16:1/16:0 and 18:1/18:0 in liver lipids indicated that desaturase enzymes were less active and lipogenesis was reduced in rats fed the AA diet compared to those fed the AF diet. In contrast, stimulated lipogenesis appears to have been the cause of fatty liver in rats fed the AF diet.
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It is well known that alcoholic fatty liver develops in rats fed high fat ethanol-containing diets (1-7). The triacylglycerols (TG) that accumulate in livers of these rats is of dietary origin (3,8) and the severity of fatty liver increases with the amount of diet fat consumed (7). Even alcoholic rats fed as little as 2% fat develop fatty liver (7), but whether rats consuming no fat develop alcoholic fatty liver has not been determined.

When dietary fat is not available, hepatic lipogenesis is stimulated. In the present study, we examined whether TG produced de novo would also accumulate due to alcohol consumption. Since dietary polyunsaturated fatty acids reduce hepatic lipogenesis, we also investigated the effect of supplementing the fat-free alcohol diet with small amounts of arachidonic acid (20:4) on the development of fatty liver.

MATERIALS AND METHODS

Animals and Diets

Sixteen male Sprague-Dawley rats weighing ca. 50 g were obtained from Hilltop Lab Animals, Inc. (Chatsworth, CA). Rats were housed individually in stainless steel cages and fed Wayne Lab Blox diet (Allied Mills, Chicago, IL) ad libitum for 4 days. Animals were divided into 4 groups of 4 each. Each rat was fed a Lieber/DeCarli liquid rat diet (Bio-Mix 711, Bio-Serv, Inc., Frenchtown, NJ) in which fat was substituted with an isocaloric amount of maltose-dextrins. The contents of the diets are summarized in Table 1. All alcohol diets (AF, AA) contained 50 g/l ethanol (Table 1). Control diets (CF and CA) were made isocaloric to the alcohol diets using maltose dextrins. Fat free diets (AF and CF) contained no additional fat. Diets containing 20:4 (AA and CA) had 1 g of the acid (purchased from Nu-Chek Prep, Inc., Elysian, MN) per liter of diet. This acid was 70% pure. The fatty acid composition of this supplement has been reported (3). Rats were individually pair-fed these diets to animals in the AF group for 30 days. Capped feeding tubes (Bio-Serv, Inc.) were used to minimize ethanol evaporation. Rats in the AF group were fed ad libitum. Diets were mixed according to suppliers' instructions, kept at 5 C under nitrogen and used within 72 hr. After storage for 3 days, diets were analyzed for their fatty acid content and composition as described below. No change was observed in either the content or composition of the fatty acids with storage of the AA or CA diets.

Quantitation and Analysis of Lipids

Rats were sacrificed, plasma was isolated from their whole blood and their livers were removed. Lipids from plasma and liver were isolated and various classes (cholesteryl esters, CE; phospholipids, PL; and TG) were isolated by thin layer chromatography (TLC) (4). Fatty acid methyl esters of various lipids were prepared and analyzed by gas liquid chromatography (GLC). These were quantitated using methyl pentadecanoate as an internal standard (3).

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Abbreviations: Diets are indicated as fat-free with ethanol (AF), fat-free without ethanol (CF), or similar diets with 0.9% of the calories as arachidonic acid with (AA) or without (CA) ethanol. The composition of these diets is described in the text and Table 1.

TABLE 1

Diet Compositions

Diet	Protein	Ethanol	Carbohydrate (g/l)	Fat	Salt	Vitamins
AF	42.2	50.0	112.8	-	10.0	5.0
AA	42.2	50.0	112.8	1.0	10.0	5.0
CF	42.2	0	202.4	-	10.0	5.0
CA	42.2	0	202.4	1.0	10.0	5.0

All components are given as g/l. A modified Lieber-DeCarli liquid diet was used in which fat was substituted using maltose-dextrins (1).

RESULTS

Rats fed the AF, AA, CF and CA diets gained an average of 11, 6, 21 and 27 g in the 30-day period. During this time, all rats consumed an average of 29.1 ml of diet per day. In an earlier study, rats of the same age (23-27 days) consumed an average of 45.1 ml of a liquid 35% fat diet with 34% ethanol (5). The fat-fed rats gained an average of 44.3 g in 30 days (5). The differences between these results must be attributed to the presence or absence of diet fat, since the diets were otherwise isocaloric to one another by volume and similar in every other respect.

More complete diet consumption and weight gain data are presented in Tables 2-4. From Table 2, each group of rats consumed the same amount of diet (they were pair-fed) during all of the 4 one-week intervals. Therefore, the amount of diet consumed by each rat reflects the desire or ability of rats in the AF group to eat. During the first week, they consumed the least food (23 ml/day). Later they ate at a more continuous level of ca. 30 ml/day. Therefore, the rats were in a steady state feeding situation at and near the end of the experiment. Rats fed the AA diet lost the most weight compared to the other groups in the initial week, but their rate of growth was the highest of all the groups at the end of the 30-day period. All rats gained weight continuously after the first week. However, rats fed the AF diet grew at only half the rate of the rats in the AA group throughout the 3rd and 4th weeks.

Some of the rats in the AF group consumed greater amounts of diet than others. Table 3 demonstrates that rat number 4 consumed the most and rat numbers 1 and 2 consumed the least diet in the first week of feeding. These rats maintained this pattern throughout the study. Corresponding to these results are the increases in body weight as shown in Table 4. Rats in the first pair gained the least weight (7.0 g/30 day), while those in the fourth pair gained the most (42.3 g/30 day).

TABLE 2

Weight Gain and Diet Consumption

	Diet Group				
Week	AF	AA	CF	CA	Diet Consumed per day (ml)
	(wt in g)				
I	−19.5± 4.5	−30.25± 4.6	−17.8± 4.9	−17.3± 10.0	23.0± 4.6
II	− 7.7± 9.7	−25.3 ±10.6	− 9.5± 10.7	− 8.0± 15.1	30.2± 5.7
III	7.3± 10.6	- 10.7 ±20.5	11.5± 16.9	16.0± 21.2	34.0± 8.1
IV	15.5± 12.0	5.3 ± 19.9	31.5± 14.3	27.5± 24.8	29.7± 3.4

Rats fed the AF diet were given food ad libitum. Rats fed the AA diet were given the amount of food consumed by the corresponding paired rats in the AF group on the following day. Rats in the CF and CA groups were also pair-fed this amount of food on subsequent days. All rats in the AA, CF and CA groups consumed all available diet while those in the AF group always had excess food available. Therefore, the amount of food consumed by each group of rats was identical and is shown in the last column of the table by weeks. The weeks of the experiment are as follows: I = 0-7 days, II = 8-14 days; III = 15-21 days; IV = 22-30 days. Rats were weighed daily and the values shown indicate weight gain from the time that the experimental diet was fed. All rats lost weight in the initial week of the experiment. Diet abbreviations are defined in Table 1. Values are mean ± SD.

TABLE 3

Diet Consumption for Rats of Each Pair by Week

	Pair Number			
Week	1	2	3	4
I	20.3±4.9	20.3±2.6	22.0±4.4	26.1±5.9
II	25.4±3.5	26.9±3.8	35.4±3.2	35.6±1.5
III	27.6±2.5	27.5±2.5	42.1±3.9	42.2±3.8
IV	30.9±4.4	30.8±4.4	37.0±8.8	37.9±8.6

Values are in ml of diet consumed per day ± SD. Each pair contained one rat of each diet group. Rats in each pair were fed isocaloric amounts of diet. Weekly periods are defined in Table 2.

TABLE 4

Weight Gain for Rats of Each Pair by Week

	Pair Number			
Week	1	2	3	4
I	−23.3± 6.7	−26.8± 6.4	−21.3± 5.3	−13.5± 8.7
II	−19.5±11.1	−22.0± 9.0	−11.0± 9.6	1.2± 9.4
III	− 8.8±13.6	−10.0± 8.8	15.0±20.1	27.5± 8.6
IV	7.0±12.0	9.8±14.3	31.3±24.7	42.3±10.4

Values are in g gained during the experimental period as in Table 2. Each value represents the mean ± SD. Weekly periods are defined in Table 2.

Variations in each of these values may have been partially due to the expiration of ethanol by rats in the AF and AA groups. Large deviations in the weight gain of rats in each diet group (Table 2) are related to variations in individual diet consumption and weight gains of the four pairs of rats (Tables 3 and 4).

The levels of hepatic total lipids (TL), TG, PL and CE from rats fed all 4 low-fat or fat-free diets are shown in Figures 1-4. Fatty liver developed only in rats fed the alcohol diet without added fat (AF). The level of hepatic lipids in rats fed a high fat alcohol diet for one month is independent of age (5). Therefore, levels of liver fat observed in this study can be compared to other results for larger rats fed higher levels of fat (3,4,7). Two characteristics of alcoholic fatty liver are elevated levels of TG and CE (1,4). High levels of hepatic PL have also been associated with alcoholic fatty liver in rats, but the difference between alcohol and control rats is usually small (3,4). These trends were also seen in this study. However, levels of hepatic CE were nearly twice as high in rats fed no fat (Fig. 4) as in rats fed 35% fat (1,4). Levels of hepatic PL in this study (Fig. 3) were the same as when 35% fat was fed (3). However, rats which consumed the CF diet accumulated less TL (Fig. 1) and TG (Fig. 2) than rats fed control high fat diets (3,4).

One g of 20:4 per liter of diet (0.9 calorie %) prevented alcoholic fatty liver (Fig. 2). Rats fed the AF diet (Fig. 2) accumulated higher levels of hepatic TG than rats fed alcohol diets containing 2, 5, 10, 15, or 25% fat (7). Yet rats fed 0.9% 20:4 had lower levels of hepatic TG than when rats were fed an alcohol diet containing 2% fat (7). Levels of hepatic TG, CE and TL in rats fed the AA diet was similar to those of rats fed control diets (Figs. 1-4).

Since the ratio of 20:3(n-9)/20:4(n-6) exceeded 0.4 in livers of rats fed AF and CF diets (Fig. 5), they had essential fatty acid deficiency (9). In the absence of dietary fat, it is known that hepatic lipogenesis is stimulated. However, both essential fatty acid deficiency (Fig. 5) and lipogenesis (10) are reduced when 20:4 is fed. While lipogenesis is inhibited by the type and amount of diet fat, polyunsaturated fat is the most effective (10,11). Of the dietary polyunsaturates, 20:4 is more effective than 18:2, or 18:3 in reducing the activity of fatty acid synthetase (12). Therefore, the mechanism by which fatty liver was prevented in rats fed the AA diet may have been mainly by decreased lipogenesis.

Predominately, palmitate is produced by fatty acid synthetase. Therefore, chain elongation and desaturation play a major role in the synthesis of other fatty acids of tissue lipids. Since nearly all the fatty acids in the rats fed fat-free diets are generated

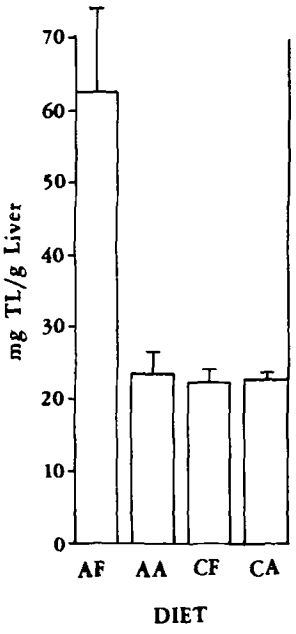

FIG. 1. Levels of hepatic fat from rats fed various diets for 30 days. Diet abbreviations are defined in Table 1. Rats were individually pair-fed these diets to the rats fed the AF diet for 30 days. Lipid levels were measured as the weight of their fatty acid methyl esters per wet weight of liver. Methyl esters were quantitated using GLC with methyl pentadecanoate as an internal standard (3). Total lipids (TL) of rats fed the AF diet were significantly more abundant than in rats fed either of the other diets with $p<0.01$ by the 2-tailed t-test.

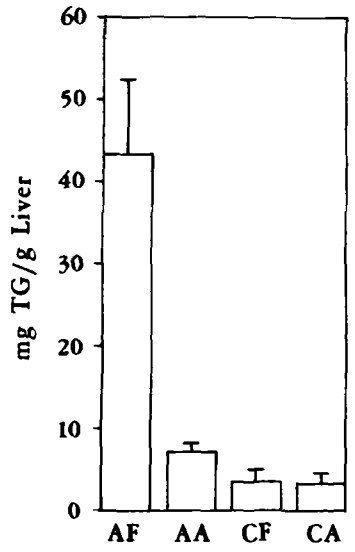

FIG. 2. Levels of hepatic triglycerides (TG) from rats fed various diets as defined in Table 1. Levels of TG fatty acid methyl esters were determined by GLC as described in Figure 1 after separating the TG fraction from total lipids by TLC. Levels of TG were significantly greater in rats fed the AF diet than in rats fed either of the other diets with $p<0.01$ by the 2-tailed t-test.

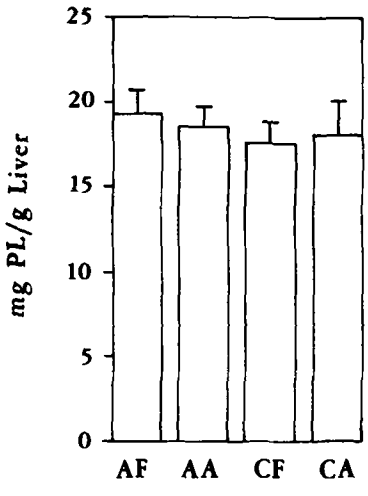

FIG. 3. Levels of hepatic phospholipids (PL) from rats fed various diets as defined in Table 1. Levels of PL fatty acid methyl esters were determined by GLC as described in Figure 1 after separating the PL fraction from TL by TLC. Levels of PL were not significantly different in livers of rats from any of the 4 groups.

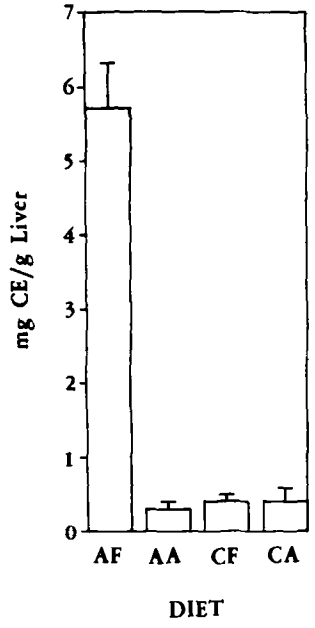

FIG. 4. Levels of hepatic cholesteryl esters (CE) from rats fed various diets as defined in Table 1. Levels of CE fatty acid methyl esters were determined by GLC as described in Figure 1 after separating the CE fraction from TL by TLC. Levels of CE were significantly greater in rats fed the AF diet than in rats in any of the 3 remaining groups with $p<0.001$ by the 2-tailed t-test.

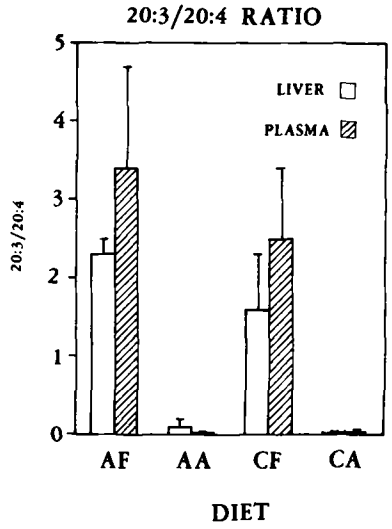

FIG. 5. Ratio of trienes to tetraenes from total lipids of liver and plasma from each of the diet groups as described in Table 1. Essential fatty acid deficiency was observed in rats fed the AF and CF diets.

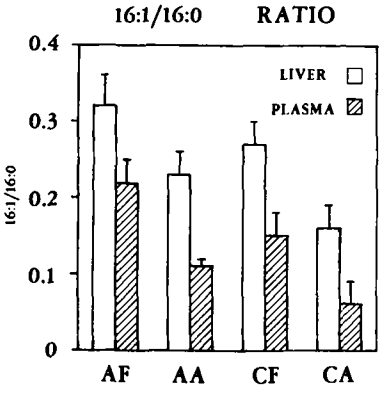

FIG. 6. Ratio of fatty acids 16:1/16:0 from liver and plasma phospholipids (PL) of rats fed each of the diets described in Table 1. Livers of rats fed the AA diet had a significantly lower ratio than rats fed the AF ($p<.05$) diet. Livers of rats fed the CA diet had a lower ratio than those fed either the AF ($p<.01$), or AA ($p<.05$) diets by the 2-tailed t-test. From rats fed the AF diet, plasma lipids had a significantly greater 16:1/16:0 ratio than plasma from rats fed the AA ($p<.01$), CF ($p<.05$), or CA ($p<.01$) diets. Plasma PL of rats fed the CA diet had a lower ratio than in rats fed the AA diet with $p<.05$ by the 2-tailed t-test.

by de novo synthesis, the ratio of unsaturated to saturated fatty acids provides a measure of desaturation activity in the liver. Dietary arachidonic acid reduced both the 16:1/16:0 (Fig. 6) and 18:1/18:0 ratios (Fig. 7) in both alcohol fed and

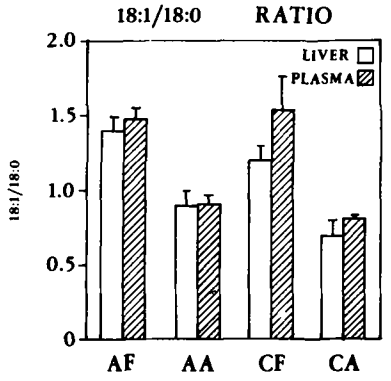

FIG. 7. Ratio of fatty acids 18:1/18:0 from liver and plasma phospholipids (PL) of rats fed each of the diets as described in Table 1 for 30 days. The ratio of 18:1/18:0 from PL of rat liver was significantly smaller in rats fed the AA diet than in those fed the AF ($p<.01$) or CF ($p<.01$) diets. Rats fed the CA diet also had a significantly lower ratio of 18:1/18:0 in liver PL than in rats fed the AF ($p<.001$), AA ($p<.05$), or CF ($p<.001$) diets. In plasma, 18:1/18:0 was significantly lower in rats fed the AA diet than in those fed the AF ($p<.001$) or CF ($p<.01$) diets. Also in plasma PL, this ratio was lower in rats fed the CA diet than in those fed the AF ($p<.001$), AA ($p<.05$) and CF ($p<.01$) diets by the 2-tailed t-test.

control rats. This indicates that desaturase enzyme activities may have been reduced by feeding 20:4 as we have suggested previously (3).

DISCUSSION

Rats in this study lost weight in the initial week of feeding (Table 2). Due to this loss, the net gain in weight for the 30-day period was low for all groups. After the first week, all rats gained weight continuously. Much of the essential fat needed may have been mobilized from adipose tissue during the initial week of the experiment. However, it is unlikely that these rats had enough residual adipose tissue during the second, third and fourth weeks to meet their growing needs. Therefore, lipogenesis was probably the major source of fat in each of these rats.

Although alcoholic fatty liver develops in rats fed high-fat diets, whether a fat-free ethanol diet would have a similar effect has not been investigated. The present study shows that it does (Fig. 2). The level of fatty liver that developed was nearly the same as in rats fed ethanol and a 35% fat diet (4). Control rats fed the fat-free diet without ethanol had lower levels of hepatic TG (3.6 mg/g) (Fig. 2) than rats fed a similar diet but with 35% fat (14.6 mg/g) (4). Levels of hepatic PL were the same in either study, whereas levels of CE were higher in livers of rats fed the AF diet (Fig. 4) than in rats fed

35% fat with ethanol (1,4). This higher level of hepatic CE may be due to a combination of effects: stimulated lipogenesis and reduced lipid mobilization from the liver (4).

Upon comparing the levels of hepatic TG from alcoholic rats fed 35% (4) and no fat (Fig. 2), the difference was 11.9 mg TG/g liver. When the same values were computed for controls (4) (Fig. 2), the resulting difference was 11.0 mg/g. This similarity may be consequential or it may indicate a tendency for alcoholic rats to maintain the same relative level of fatty liver regardless of the fat source.

It has been suggested that alcoholic fatty liver may develop due to a decrease in hepatic fatty acid oxidation (13). This decrease would be caused by an excess of NADH supplied during ethanol metabolism. Fatty acid oxidation is usually associated with starvation when animals have used glycogen reserves and must use stored fat to produce energy. In the present experiment, we fed our animals a diet in which fat was replaced with carbohydrate. Under these circumstances, glycogen stores should not be depleted and fatty acid oxidation minimal or absent. However, alcoholic fatty liver was still produced. The rats which developed fatty liver were not fasting because they were given free access to diet. Therefore, it is unlikely that fatty liver developed in these animals due to reduced fatty acid oxidation.

Rats fed ethanol along with a high-fat diet accumulate hepatic TG that originate from the diet. However, liver TG from rats fed a fat-free diet must be synthesized within the tissue. When a small amount of arachidonic acid was supplemented in the fat-free alcohol diet, the data from liver PL fatty acid composition suggests that desaturase activity was reduced (Figs. 6 and 7). Under other circumstances, lipogenesis and desaturase activity have been stimulated or reduced together (14). Therefore, it is likely that lipogenesis was stimulated upon feeding the AF and CF diets and that it was reduced upon feeding 20:4. Arachidonic acid was not as effective in preventing fatty liver when 35% fat was fed with the alcohol diet (3,4). This may have been due to minimal hepatic lipogenesis.

Fatty liver has been reported to develop in rats fed a (nonalcoholic) fat-free diet (15-20). However, in the present study, the amount of liver TG observed in rats fed the control, fat-free (CF) diet was not only low, but it was less (Fig. 2) than in control rats fed a similar diet with 35% fat (3,4). There are several reasons for the discrepancy between our low levels of liver TG in controls fed a fat-free diet and higher levels reported previously (15-19). The first is that our diets did not include sucrose or glycerol which were thought to have a significant influence on the development of fatty liver (15,16). Another reason is that we fed rats for a shorter period of time (30 days) than in studies where fatty liver developed in rats fed no fat (10-30 weeks) (15-17). Therefore, the absence of fat in the AF diet could not have alone been responsible for the high level of fatty liver observed.

In conclusion, fatty liver develops in rats fed ethanol, regardless of the level of dietary fat. When dietary fat is abundant, it is the predominant source for hepatic steatosis. When no fat is available from the diet, TG that are produced de novo accumulate. At both extremes (no diet fat or high fat diet), fatty liver is severe. When intermediate levels of polyunsaturated fat (2-25%) are fed with the Lieber-DeCarli diet, fatty liver is relatively low (7). It appears that when lipogenesis is responsible for the generation of a significant portion of the liver fat, dietary 20:4 reduces fatty liver.

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Potentiating Effect of 5,8,11-Eicosatrienoic Acid on Human Platelet Aggregation

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ABSTRACT

5,8,11-Eicosatrienoic acid (20:3ω9), a fatty acid increased in the platelet phospholipids of man and animals fed saturated fats, was either added to human platelets simultaneously with the aggregating agents, or incorporated into the platelet phospholipids by preincubation. 20:3ω9 markedly increased the response of platelets to all aggregating agents tested when added simultaneously with the agent, but solely to thrombin and ionophore, after incorporation into the platelet phospholipids. The potentiating effects of 20:3ω9 on thrombin aggregation do not appear to be related to prostaglandin formation, but rather to the production of a monohydroxy derivative through the lipoxygenase pathway.
Lipids 18:291-294, 1983.

INTRODUCTION

Several polyunsaturated fatty acids have been shown markedly to influence platelet functions. Arachidonic acid (20:4ω6) is a well known (1,2) inducer of platelet aggregation as a precursor of endoperoxides and prostaglandins. By contrast, dihomogammalinolenic acid (20:3ω6) and 5,8,11, 14,17-eicosapentaenoic acid (20:5ω3), also prostaglandin precursors, are inhibitors of platelet aggregation (3,4).

An increase in 5,8,11-eicosatrienoic acid (20:3ω9) in certain blood lipids has been reported in atherosclerotic patients (5), as well as in animals deficient in essential fatty acids (6,7) or fed saturated fats (8). In animals, the higher level of 20:3ω9 in the platelet phospholipids was associated with a higher susceptibility of platelets to thrombin-induced aggregation (7,8). A similar result was also observed recently in farmers from Great Britain and France (9).

The present study determined whether 20:3ω9 in platelets might be responsible for the platelet hyperactivity observed in men and animals fed a saturated fat diet, and, if so, through which mechanism.

MATERIALS AND METHODS

The human serum albumin (essentially fatty acid free), human thrombin and arachidonic acid utilized in these studies were obtained from Sigma Chemical Co. (St. Louis, MO). Collagen was purchased from Horm (Munich) and cation-ionophore A 23187 was given by Lilly Laboratories (Indianapolis). Unlabeled and [^{14}C]20:3ω9 were chemically synthesized according to the technique previously reported for 20:4ω6 (10). Silica Gel G plates for thin layer chromatography (TLC) were purchased from Merck (Darmstadt), and the various solvents and reagents from Prolabo (Paris).

Blood from human volunteers was collected with ACD (citric acid 0.8%, sodium citrate 2.2%, dextrose 2.45%) as the anticoagulant (1/9 in volume). Platelet-rich plasma was obtained by centrifugation (100 G × 15 min); platelets were isolated from their plasma also by centrifugation (900 G × 10 min) and, for studies reported in Figures 1 and 4, resuspended (300,000/μl) in a Tyrode without calcium, containing Hepes buffer (pH = 7.4) as previously described (11).

For incorporation of 20:3ω9 in the platelet phos-

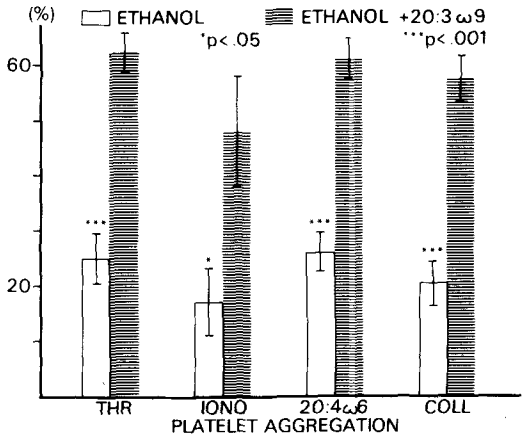

FIG. 1. Influence, on human platelets (resuspended in a Tyrode buffer) of 20:3ω9 (5×10^{-6} M), diluted in ethanol and added simultaneously with the following aggregating agents: thrombin (THR), 0.015 U/ml; ionophore (IONO) A 23187, 0.25×10^{-6} M; arachidonic acid (20:4ω6), 2×10^{-6} M; collagen (COLL), 0.5 μg/ml. Results expressed as percentage of platelet aggregation (mean ± SE of 5 determinations).

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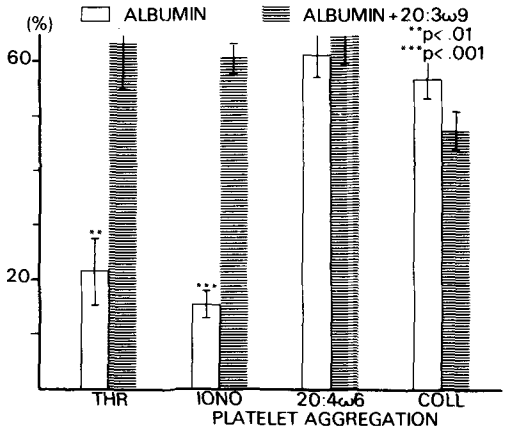

FIG. 2. Influence of 20:3ω9 incorporated into the platelet phospholipids (by a 2-hr incubation period of platelets with 20:3ω9 bound to albumin and resuspension in Tyrode/Hepes) on aggregation induced by the agents as in Figure 1.

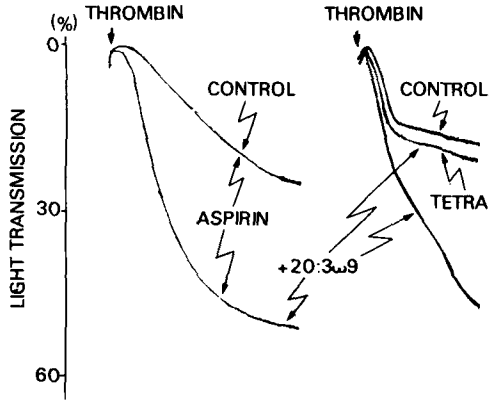

FIG. 3. Influence of aspirin (left tracings) at 2×10^{-4} M and of 5,8,11,14-heneicosatetraynoic acid (TETRA) (an inhibitor of platelet lipoxygenase) (right tracings) at 5×10^{-6} M, on the aggregation induced by thrombin (0.015 U/ml). Before testing the effect of aspirin and TETRA on thrombin aggregation, platelets were incubated for 2 hr in tyrode albumin not containing (controls) or containing 20:3ω9 to enrich platelets with this fatty acid.

pholipids (studies of Figures 2, 3 and Table 1), platelets were incubated for 2 hr in a shaker bath at 37C in a Tyrode/Hepes buffer containing albumin (3.5 g/l) and 20:3ω9 bound to albumin (12). The binding of 20:3ω9 to albumin in a molar ratio of 1 was done by overnight incubation at 37C of the fatty acid in a Tyrode/Hepes buffer/albumin (pH: 7.4) solution. Then, the platelet suspension was acidified (pH = 6.4) with citric acid and centrifuged at 700 G for 10 min. Finally, the platelets were resuspended in the Tyrode/Hepes buffer without albumin, to perform platelet aggregation or to analyze by gas liquid chromatography (GLC), fatty

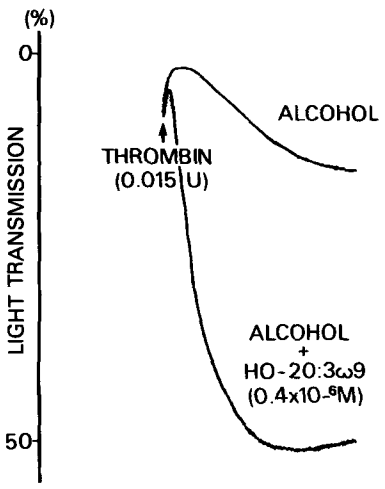

FIG. 4. One typical recording observed in 3 different series of the potentiating effect of monohydroxy-20:3ω9 (0.4×10^{-6} M, final concentration) diluted in ethanol, added simultaneously with thrombin. This response of human platelets to thrombin (0.015 U/ml) was performed under similar conditions to those reported in Figure 1.

acid composition of the platelet phospholipids after TLC separation (13).

Platelet aggregation was studied with a turbidimetric method (14). In the first series of studies (Fig. 1), 20:3ω9 was added simultaneously with the aggregating agent into the cuvette of the aggregometer. For these experiments, 20:3ω9 was dissolved in ethanol and 2 μl of this solution (0.5%) were added to 0.4 ml of the platelet suspension (5×10^{-6} M). In studies reported in Figure 3, aspirin at 4×10^{-3} M (20 μl) diluted in Tyrode/ethanol (9:1 in volume) was added to the platelet suspension (0.4 ml) 3 min before the aggregating agent. 5,8,11,14-Heneicosatetraynoic acid, a specific inhibitor of human platelet lipoxygenase (15), was added in ethanol and used at 5×10^{-6} M.

The metabolism of [^{14}C]20:3ω9 was studied after double extraction of lipids as reported recently (13). The lipid extract was analyzed by TLC. Monohydroxy-eicosanoic acids were separated from fatty acids by elution with hexane/diethyl ether/acetic acid (60:4:1, v/v). Prostaglandins and thromboxane B were separated by a second elution with diethyl ether/methanol/acetic acid (90:1:2, v/v), phospholipids staying at the origin (16). Finally, the different phospholipids were separated by a third elution with chloroform/methanol/acetic acid/water (85:15:14:4, v/v). After each elution, a quantitative radiochromatogram was performed. Thus, the amount of each compound and the incorporation into phospholipids could be calculated.

Finally, the monohydroxy-20:3ω9 was prepared

TABLE 1

Amounts of 20:3ω9 in Different Platelet Fractions Before and After Stimulation by Thrombin and Ionophore

Inductor	Free	Monohydroxy	Cyclooxygenase Products	PC	PE	PI + PS	Total phospholipids
None (Tyrode)	3.2 ± 0.90	0.17 ± 0.08	ND	11.53 ± 1.26	1.63 ± 0.31	3.51 ± 0.68	16.6 ± 1.8
Thrombin (0.1 U/ml)	2.99 ± 0.98	2.27[a] ± 0.45	ND	10.95 ± 0.98	1.46 ± 0.66	2.91 ± 0.23	15.3 ± 1.2
Ionophore A 23187 (10^{-6}M)	1.49 ± 0.42	5.78[b] ± 0.64	ND	9.56 ± 0.99	1.17 ± 0.16	2.16 ± 0.29	12.9 ± 1.2

[a] $p<0.005$.
[b] $p<0.001$.
Results expressed in nmol/10^9 platelets are mean ± SE of 5 experiments. ND = not detectable; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI + PS = phosphatidylinositol + phosphatidylserine.

from exogenous [^{14}C]20:3ω9 as follows: 20:3ω9 (final concentration 10^{-5} M) dissolved in ethanol was incubated for 10 min at 37C with a suspension of washed platelets as a source of enzymes. Lipids were extracted at pH = 3 by chloroform/ethanol (2:1). The monohydroxy derivative was separated and purified by two successive runs with hexane/diethyl ether/ acetic acid (80:20:1, v/v) as eluent.

RESULTS

As shown in Figure 1, 20:3ω9, added simultaneously with the aggregating agents, markedly potentiated platelet aggregation induced by all agents. In contrast to this, when platelets were preenriched with 20:3ω9 (Fig. 2), solely the aggregation to thrombin and ionophore was increased. The aggregations to arachidonate and collagen were not modified (Fig. 2).

[^{14}C]20:3ω9, when incubated with platelets, was predominantly incorporated into the glycerophospholipids (Table 1) since 83.4 ± 9.0% (mean ± SE of 5 experiments) of the 20:3ω9 was found in the glycerophospholipids, representing 16.6 nmol/10^9 platelets. Among the various fractions, it was in the phosphatidylcholine that the bulk (69.5%) of the radioactive 20:3ω9 was incorporated. GLC analysis of total phospholipid fatty acids of platelets incubated with or without 20:3ω9 confirmed the incorporation into phospholipids of this fatty acid. In this fraction, the level of 20:3ω9 passed from 0.2 to 1.9% (mean of 5 experiments). However, the increase found by GLC analysis was slightly lower than this observed by radioactivity determination. Incubation of 20:3ω9-rich platelets in the presence of thrombin or cation ionophore A 23187 induced the formation of a monohydroxy-20:3ω9. The formation of this substance was accompanied by a decrease of 20:3ω9, both free and bound to phospholipids.

As shown in Figure 3, aspirin added to the platelet suspension at a concentration (2×10^{-4} M) which completely inhibits prostaglandin formation from arachidonic acid did not modify the potentiating effect of 20:3ω9 on thrombin-induced aggregation. By contrast, this potentiating effect was completely suppressed by 5,8,11,14-heneicosatetraynoic acid (Fig. 3).

Finally, the monohydroxy-20:3ω9, added to a human platelet suspension simultaneously with thrombin as done for 20:3ω9 in the study reported in Figure 1, markedly increased the response of platelets to thrombin (Fig. 4), as observed with 20:3ω9. However, to achieve the same effect, the concentration of the hydroxy derivative was 0.4×10^{-6} M, whereas it was 5×10^{-6} M for the fatty acid. Besides, 5,8,11,14-heneicosatetraynoic acid was added at 5×10^{-6} M (as in the study of Figure 3) to human platelet suspensions at the same time that the monohydroxy-20:3ω9 and aggregation induced by thrombin as above. In 6 experiments, the increased response (mean ± SE) of platelets with the monohydroxy alone was 59.9 ± 9.4% whereas it was 61.2 ± 6.4% in the presence of heneicosatetraynoic acid.

DISCUSSION

The present experiments appear to confirm previous results suggesting that the fatty acid 20:3ω9 in the platelet phospholipids might be one of the factors responsible for the increased response of platelets to thrombin aggregation in animals and men on a saturated fat diet (8,9).

The marked potentiating effect of 20:3ω9 on the response to all agents, when added simultaneously with this agent, suggests that 20:3ω9 has to be present under its free form to increase platelet aggregation. Concordant with this hypothesis are the results obtained after incorporation of 20:3ω9 into the platelet phospholipids which have shown

that it was solely the aggregation to thrombin and ionophore which was increased. Thrombin and ionophore (17,18) are known to be able to release consistent amounts of polyunsaturated fatty acids from the platelet phospholipids, while collagen appears to be much less efficient in that respect (1). Arachidonic acid does not release the esterified fatty acids at all.

It seems that the increased response to aggregation of 20:3ω9-rich platelets may be explained by the monohydroxy derivative produced in substantial amounts (ca. 1 nmol/ml of platelet suspension) after stimulation by thrombin or ionophore. This monohydroxy derivative appears to be formed from 20:3ω9 both free and bound to phospholipids. By contrast, no cyclooxygenase products derived from 20:3ω9 could be detected. This was expected since cyclooxygenase needs a substrate with at least 3 double bonds at 8,11,14 positions on C_{20} fatty acids (19).

The observation that the potentiating effect of 20:3ω9 on platelet aggregation was not inhibited by aspirin at 2×10^{-4} M suggests that this effect was not due to an increase in prostaglandin formation. By contrast, 5,8,11,14-heneicosatetraynoic acid at a concentration (5×10^{-6} M), known to inhibit specifically the lipoxygenase pathway in human platelets (15), inhibited the potentiating effect of 20:3ω9 but not that of the monohydroxy-20:3ω9. Thus, it seems that lipoxygenase is involved in the potentiating effect of 20:3ω9. In addition, the monohydroxy derivative of 20:3ω9 appears to be at least 10 times as potent as the acid itself to increase in a similar way the response of platelets to thrombin. Consequently, the amount of the monohydroxy-20:3ω9 needed to induce the potentiation observed is consistent with the amount synthesized (1 nmol/ml of platelet suspension) in the presence of thrombin.

Another monohydroxy fatty acid (12-OH-20:4) has been found by other investigators (20,21) to play an essential role in the irreversible platelet aggregation induced in rat by arachidonic acid. Our present results indicate that a polyunsaturate fatty acid such as 20:3ω9, which apparently is not a prostaglandin precursor, may play an agonist role in platelet aggregation.

Finally, it seems of interest to underline the remarkably different effect on platelet functions of the 20:3 isomers. When tested under the present experimental conditions, instead of the potentiating effect described here for the ω9, 20:3ω3 presents a moderate, and 20:3ω6 a strong, inhibitory effect on platelet functions (12), in confirmation of the work performed by previous investigators (22).

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Lung Lipid Synthesis from Acetoacetate and Glucose in Developing Rats in vitro

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ABSTRACT

Acetoacetate (AcAc) and glucose were compared as energy sources and as precursors for lipid synthesis in the lungs of developing rats. Minced lung tissue was incubated with [3-^{14}C]AcAc or [U-^{14}C]glucose and the oxidation of each substrate to CO_2 or its incorporation into tissue lipids was quantified. The highest rates of oxidation were obtained during the first 5 days for AcAc and the first 2 days of life for glucose and oxidation of AcAc was 3-4 times greater than that of glucose at all ages. Throughout postnatal development, the rates of nonsaponifiable lipid, fatty acid and hence total lipid (chloroform/methanol extractable) synthesis from AcAc were 2-3 times those of glucose. The highest rates of total lipid synthesis from AcAc and glucose were observed at birth. Glucose was utilized for glyceride-glycerol synthesis at a higher rate than AcAc. Similar patterns of incorporation of AcAc and glucose into various lipid classes were noted. Of the total lipids synthesized from AcAc and glucose, respectively, phospholipid plus monoglyceride accounted for 64% and 77%, triglyceride 13% and 13%, diglyceride plus cholesterol 11% and 4%, fatty acids 9% and 4%, and cholesteryl esters 3% and 1%. At birth, the specific activities of all lipids except triglyceride derived from AcAc were greater than those from glucose. Rates of synthesis of all complex lipids declined with age. The results of these experiments demonstrate that AcAc is utilized more readily than glucose for energy production and lipid synthesis in developing rat lungs.

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De novo synthesis of fatty acid provides a source of lung lipids (1,2). This type of lipid synthesis is particularly important in the production of pulmonary surfactant necessary for alveolar stability (3,4). Inadequate production of surfactant can result in serious respiratory distress, especially in premature babies.

Studies of adult rats have demonstrated that several compounds, including glucose, glycerol, acetate, amino acids, pyruvate, lactate, and fatty acids, serve as substrates for the synthesis of lung lipids (1,2,5-10). In developing rats, the quantitative contribution of substrates other than glucose has not been established. It has been noted, however, that the relative contribution of glucose to total lipid synthesized de novo is higher in adult than in fetal and developing rat lungs (11). This suggests a greater dependency of the developing lung on other substrates. In addition to the earlier observations that ketone bodies are preferred substrates for lipid synthesis in the developing brain of rats (12-15), we and others have shown that ketone bodies administered to rats during various stages of development are incorporated readily into lung lipids, especially phospholipids (14,16,17). However, the quantitative contribution of ketone bodies to lung lipogenesis could not be assessed from these in vivo studies. In view of the increased availability of plasma ketone bodies in developing rats (18-20), the present study was undertaken to establish quantitatively the importance of AcAc compared to glucose as a precursor of lipids in the developing lung. The results show that ketone body is more readily utilized than glucose for production of lung lipids.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats obtained by previously described breeding procedures (21) were used in all experiments. Fetal rats of 22 days gestation were delivered by caesarean section and used for metabolic study immediately. Rats, 1-20-days old, were suckled by their dams until used for in vitro experiments. Adult male rats (200-250 g) were included for comparison.

Metabolic Studies

Rats were anesthesized with diethyl ether. Lungs were removed and placed in ice cold 0.9% saline. Tissues were dissected free of vascular tissue, blotted, weighed, and finely minced with scissors to less than 0.5 mm^3 cubes. The minced tissues were incubated immediately, usually within 3-5 min

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after the removal of the tissue from animals. Preliminary experiments indicated that minced lung tissue possessed higher metabolic activity than did tissues prepared by other methods, such as homogenization and tissue slicing.

The incubation system, in a final volume of 2 ml, consisted of Ca^{2+}-free Krebs-Ringer bicarbonate buffer, pH 7.4; 75-125 mg minced lung tissue; 33 mg bovine serum albumin; and 20 μmol [3-^{14}C] AcAc or [U-^{14}C]glucose containing 0.5 μCi radioactivity. Flasks (25 ml) were flushed with O_2/CO_2 (95:5%), stoppered, and incubated at 37C in a metabolic shaker (90 strokes/min). In a preliminary study, linear rates of CO_2 and lipid production were obtained with AcAc (2-20 mM) incubated for 0.5-3 hr and glucose (5-20 mM) for 0.5-2 hr. Thus, tissues were incubated for 2 hr in the presence of 10 mM substrate in all experiments. The pH of 7.2-7.4 was maintained in the medium during the 2 hr incubation. The reaction was stopped by adding 0.5 ml of 2 N H_2SO_4. Hyamine hydroxide (0.2 ml) was injected to filter paper suspended in a center well, and incubation was continued for an additional 20 min. After CO_2 collection, center wells were cut out and placed in scintillation vials containing 10 ml of aqueous counting scintillant. Flasks, in duplicate, containing no tissue were incubated to determine nonenzymatic CO_2 production. Tissue was separated from the incubation medium by centrifugation (1500×g, 10 min), washed twice with cold 0.9% saline, and homogenized in distilled water. Two 1-ml aliquots were transferred to tubes containing 20 ml of chloroform/methanol (2:1, v/v) for lipid extraction (22). After extraction, samples were evaporated to dryness under nitrogen.

The extracted lipids from one aliquot were resuspended in 500 μl of the chloroform/methanol mixture. An aliquot of 50 μl was used to determine the radioactivity in the total lipids synthesized from the labeled substrate. The remaining lipid suspension was saponified with 3 ml of 20% KOH in methanol for 2 hr. ^{14}C-labeled nonsaponfiable lipids and fatty acids were extracted with petroleum ether (bp 50-60) according to previously described procedures (13). The radioactivity in the aqueous phase represented glyceride-glycerol synthesized from labeled substrate (17).

The second extract was used to determine the radioactivity incorporated into the following lipid classes: phospholipid and monoglyceride (PL + MG), diglyceride and cholesterol (DG + C), free fatty acids (FFA), triglycerides (TG), and cholesteryl esters (CE). The lipids were separated by thin layer chromatography (TLC) as described elsewhere (21). After development, the TLC plates were exposed briefly to iodine vapors for visualization of lipids; identification was based on comparison with known standards. Lipid bands were scraped from the TLC plates directly into scintillation vials containing 10 ml of toluene scintillation fluid (4 g Omnifluor, 230 ml ethanol, 770 ml toluene).

The radioactivity of CO_2 and all lipid fractions was determined by a liquid scintillation counter (Model Mark III, Searle Analytic, Inc.).

The content of tissue DNA was measured by the method of Burton (23).

Chemicals

[U-^{14}C]Glucose, [ethyl-3-^{14}C]AcAc, and Omnifluor were purchased from New England Nuclear, Boston, MA. Before the experiments, [ethyl-3-^{14}C]AcAc was converted to [3-^{14}C]AcAc by the method of Krebs and Eggleston (24). Aqueous counting scintillant was purchased from Amersham Corp., Arlington Heights, IL. Lipid standards for TLC were obtained from Applied Science Laboratories, Inc., State College, PA. Organic solvents for lipid extraction were purchased from Fisher Scientific Co., Pittsburgh, PA. Bovine serum albumin (Fraction V) and other chemicals were purchased from Sigma Chemicals Co., St. Louis, MO.

Statistical Analysis

Data were analyzed by one-way analysis of variance. Comparisons of treatment means between substrates at each age were made by Student's t-test. Developmental comparisons of treatment means for each substrate were made by Duncan's New Multiple Range Test (25).

RESULTS

Oxidation of AcAc and glucose was compared by measuring CO_2 production from radiolabeled substrates. Throughout the developmental period, as well as in adult rats, AcAc was oxidized at a rate 3-4 times that of glucose (Fig. 1). Rates of AcAc oxidation during the first 9 days of postnatal life were higher than those for 20-day-old and adult rats. A relatively steady decline from 5 to 20 days was noted. Glucose oxidation was highest at birth and in 1-day-old rats, with a decline at 5 days of age ($P < 0.05$). This lower level of oxidation was maintained throughout development and in adulthood.

Rates of AcAc incorporation into total lipids (chloroform/methanol extractable) were significantly greater than those from glucose at each age studied except 1 day ($P < 0.05$) (Fig. 2A). The highest rates of lipid synthesis, 2701 ± 299 and 1601 ± 139 nmol substrate incorporated/g tissue/2 hr from AcAc and glucose, respectively, were observed at birth. After one day, lipid synthesis from

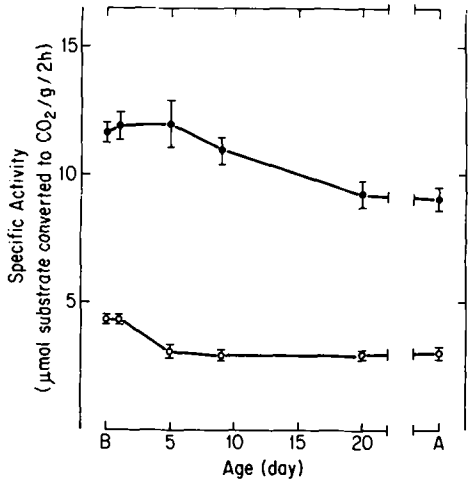

FIG. 1. CO_2 production from [3-^{14}C]acetoacetate (●---●) and [U-^{14}C]glucose (O—O) in newborn, suckling and adult rat lungs. Minced tissue was incubated for 2 hr at 37C in 2 ml of Ca^{2+}-free Krebs-Ringer bicarbonate buffer containing 33 mg bovine serum albumin and 20 μmol (0.5 μCi) of [3-^{14}C]acetoacetate or [U-^{14}C]glucose. Each point represents the mean ± SEM for 4-6 samples. B denotes newborn rats at birth and A denotes adult male rats fed ad libitum.

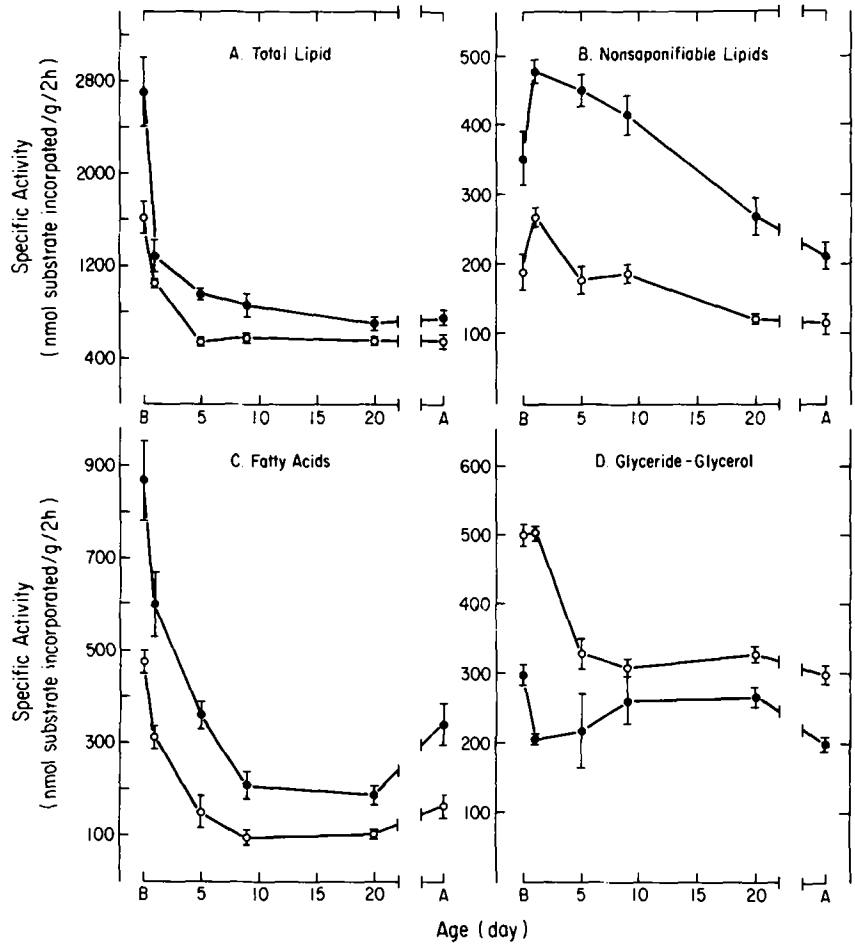

FIG. 2. Synthesis of lipids from [3-^{14}C]acetoacetate (●—●) and [U-^{14}C]glucose (O—O) in lungs of newborn, suckling and adult rats. Experimental details were as described in Figure 1. Each point represents the mean ± SEM for 4-6 samples. B denotes newborn rats at birth and A denotes adult male rats fed ad libitum.

AcAc and glucose had declined, respectively, to 47% and 65% of values at birth. Following a further decline at 5 days of age, no differences in synthetic rate were observed among 5, 9, 20 day old and adult rats.

Lung lipids were fractionated into nonsaponifiable lipid, fatty acids, and glyceride-glycerol. Nonsaponifiable lipid and fatty acid synthesis from AcAc was ca. 2-3 times that from glucose at all ages investigated (Figs. 2B and 2C). The highest rate of nonsaponifiable lipid synthesis from glucose (268 ±13 nmol glucose incorporated/g tissue/2 hr) occurred at 1 day of age; there were no statistically significant differences in synthetic rates observed among the other age groups in developing rats. In contrast, AcAc incorporation into nonsaponifiable lipid was maintained at a high level at 1, 5 and 9 days of age (415 to 477 nmol AcAc incorporated/g tissue/2 hr). The rate of AcAc incorporation was moderate at birth, and the low adult level was reached at 20 days of age. Rates of fatty acid synthesis from the two substrates were highest at birth, decreased sharply throughout the remaining suckling period, and subsequently increased moderately in adults (Fig. 2C).

Glyceride-glycerol synthesis from glucose was greater than from AcAc at all ages except 9 days (Fig. 2D). The synthesis from glucose was highest at birth and day 1, and declined rapidly to the adult level by day 5. In contrast, glyceride-glycerol synthesis from AcAc remained at a relatively constant low level at all ages.

Throughout development, lipids synthesized from AcAc were distributed as follows: 23-46% nonsaponifiable lipids, 24-57% fatty acids, and 16-37% glyceride-glycerol. With glucose as the substrate, the distribution was 16-32% nonsaponifiable lipids, 16-41% fatty acids, and 43-59% glyceride-glycerol (Fig. 2).

Chloroform/methanol extractable lipids were analyzed by TLC for distribution into various lipid classes. The percentage distribution of lipids synthesized from AcAc and glucose in lungs of 5-day-old rats, presented in Figure 3, is representative of other ages investigated.

A similar pattern of incorporation into complex lipids was observed for both substrates. The PL + MG fraction represented 64% and 77% of lipids synthesized from AcAc and glucose, respectively. As determined by TLC using a solvent system of chloroform/acetone/glacial acetic acid (88:12:0.05, v/v/v), this fraction consisted of at least 80% PL. Of the remaining lipids synthesized from AcAc and glucose, respectively, TG accounted for 13% and 13%, DG + C for 11% and 4%, FFA for 9% and 4%, and CE for 3% and 1%.

To determine the quantitative incorporation of AcAc and glucose into each lipid class during

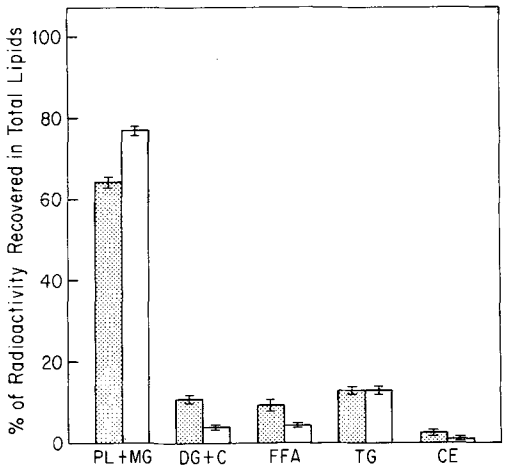

FIG. 3. Percent distribution of [3-^{14}C]acetoacetate (▨) and [U-^{14}C]glucose (□) in lipids synthesized in lungs of 5-day-old rats. Minced tissue was incubated as described in Figure 1. Total lipids were extracted with chloroform/methanol (2:1, v/v) and separated by TLC into the following classes: phospholipid and monoglyceride (PL + MG), diglyceride and cholesterol (DG + C), free fatty acids (FFA), triglycerides (TG), and cholesteryl esters (CE). Each bar represents the mean ±SEM of 5 samples.

postnatal development, specific activities were calculated. For all lipid classes except CE, the highest levels of lipid synthesis from glucose occurred at birth (Table 1). The synthetic rate for each lipid class declined rapidly after birth and reached the adult rate by 1 or 5 days of age.

A decline in complex lipid synthesis from AcAc with age was also noted (Table 2). Synthesis of PL + MG decreased by 50% between birth and 1 day of age, and by 33% between 1 day and 5 days of age. Thereafter, incorporation remained constant at the adult level. Synthesis of DG + C decreased significantly from birth to 5 days of age. A decline in FFA synthesis was observed during the early suckling period, with a subsequent increase at 20 days of age and in adult rats to the level observed at 1 day of age. Incorporation into TG was greatest at birth and declined by 50% at 1 day of age. The level of TG synthesis remained constant throughout the suckling period and then declined further in 20-day-old and adult rats. CE synthesis was greatest during the early suckling period, decreasing to the adult level at 20 days of age.

The rate of PL + MG synthesis from AcAc at birth was 1793 ± 128 nmol AcAc incorporated/g tissue/2 hr, which was significantly greater ($P < 0.05$) than the rate from glucose (1237 ± 102 nmol glucose incorporated/g tissue/2 hr). TG synthesis from AcAc was greater than from glucose

TABLE 1

Incorporation of [U-^{14}C] Glucose into Lipids in Lungs of Developing Rats

Age (days)	Lipid classes				
	PL+MG[a]	DG+C	FFA	TG	CE
	(nmol glucose incorporated/g tissue/2hr)				
Birth	1237±102[b]	102±11[b]	92±7[b]	167±19[b]	12±2[b]
1	819± 17[c]	57± 5[c]	51±4[c]	106± 7[c]	10±2[b,c]
5	418± 12[d]	23± 1[c]	23±2[c]	71± 3[d]	6±1[b,c,d]
9	458± 23[d]	32± 4[c]	21±3[c]	61± 2[d]	8±1[b,c,d]
20	415± 21[d]	35± 2[c]	42±4[c]	52± 3[d]	5±1[c,d]
Adult	431± 43[d]	27± 5[c]	38±6[c]	47± 4[d]	2±1[d]

[a]Abbreviations for lipid classes are PL+MG, phospholipid and monoglyceride; DG+C, diglyceride and cholesterol; FFA, free fatty acids; TG, triglyceride; CE, cholesteryl ester.
[b]Values are means±SEM for 4 or 5 samples. All values within a column not sharing the same superscript are significantly different ($P<0.05$).

in 5-day-old rats; whereas synthesis from glucose exceeded that from AcAc in 20-day-old and adult rats. AcAc was utilized to a greater extent than glucose for FFA, DG+C and CE synthesis at all ages investigated.

To correlate the developmental changes in lipogenic capacity, tissue DNA content was determined. The rat lungs contained 10.3±0.5, 9.7±0.2 and 9.9±0.2 μg DNA/mg wet tissue at 6 hr, 9 days and 15 days of age, respectively.

DISCUSSION

Acetyl CoA (AcCoA) serves as an immediate precursor for lipid synthesis (26). Catabolism of glucose and AcAc produces equal amounts of AcCoA; therefore, the rate of lipogenesis expressed in nmol or μmol of substrate incorporated/g tissue/2 hr permits a quantitative comparison of glucose and AcAc as lipid precursors. The results of the present study demonstrate that AcAc is more rapidly utilized than glucose for synthesis of various lipids and for energy production in postnatal and adult rat lungs. Throughout the entire postnatal stage, the rates of synthesis of fatty acids and nonsaponifiable lipids from AcAc were 2-3 times those from glucose.

Moreover, the rates of AcAc and glucose utilization for energy and lipid production were greater during the first 24 hr of age than the remaining suckling period and adulthood. This developmental pattern cannot be attributed to changes in tissue composition during growth. We have found that the DNA content of the lung did not change during the suckling period. Also, lung protein and water contents remain constant in suckling rats, although the protein level in adults is 20-30% higher than in young rats (10).

Glucose is a physiological substrate for lung lipids in adult mammals (1,2,5-10). Although preformed fatty acids in the circulatory system are readily incorporated into complex lipids (1,8), fatty

TABLE 2

Incorporation of [3-^{14}C] Acetoacetate into Lipids in Lungs of Developing Rats

Age (days)	Lipid classes				
	PL+MG[a]	DG+G	FFA	TG	CE
	(nmol acetoacetate incorporated/g tissue/2hr)				
Birth	1793±128[b]	205±31[b]	213±28[b]	209±22[b]	18±1[b]
1	906±118[c]	147±15[c]	123±13[c]	105±10[c]	33±4[c]
5	606± 33[d]	101± 8[d]	87± 5[d]	118± 5[c]	32±2[c]
9	554± 97[d]	116±17[c,e]	85±11[d]	96±22[c]	31±6[c]
20	452± 39[d]	93±11[d]	106±10[c]	37± 4[d]	9±1[d]
Adult	507± 50[d]	103±20[d,e]	130±13[c]	23± 2[d]	6±1[d]

[a]Abbreviations for lipid classes are PL+MG, phospholipid and monoglyceride; DG+C, diglyceride and cholesterol; FFA, free fatty acids; TG, triglyceride; CE, cholesteryl ester.
[b]Values are means ± SEM for 4 or 5 samples. All values within a column not sharing a common superscript are significantly different ($P<0.05$).

acids synthesized de novo from glucose account for 30-60% and 20-40% of total lipids synthesized in adult and suckling rats, respectively (1,5,11). The preferential use of AcAc for fatty acid synthesis indicates the quantitative importance of ketone bodies for complex lipid production in the developing lung. Indeed, the specific activity of complex lipids, particularly phospholipids, synthesized from AcAc is higher than from glucose at birth. The predominant synthesis of phospholipids from AcAc observed throughout the entire suckling period in the present experiments is consistent with earlier findings in our in vivo study (16). The highest rate of phospholipid synthesis from AcAc, observed at birth, coincides with increased capacity for de novo fatty acid synthesis measured by ^3H_2O incorporation (11). This further substantiates the importance of AcAc as a lipid precursor at a time of increased de novo lipogenesis, and when phospholipids are needed for pulmonary surfactant. The mechanism of the enhanced rate of AcAc incorporation into lipids is still not understood, but may in part be attributed to increased activities of cytoplasmic AcAcCoA synthetase and AcAcCoA thiolase as discussed in our earlier report (16).

While AcAc is utilized better than glucose for fatty acid synthesis, more glucose in incorporated into glyceride-glycerol. Throughout postnatal development, glyceride-glycerol synthesized from glucose accounted for 43-59% of total lipids, whereas that from AcAc was only 16-37% of the total. In the lung, as well as other tissues, glucose is converted to α-glycerol phosphate via dihyroxyacetone phosphate by α-glycerol phosphate dehydrogenase during the glycolytic process (27). To the contrary, there is no known pathway for the synthesis of α-glycerol phosphate from AcAc. The reason for the persistent detection of [3-^{14}C]AcAc label in glyceride-glycerol in the present in vitro study and in our earlier in vivo experiment (16) is not clear. Although recycling of the label through metabolic pathways, e.g., recycling of [^{14}C]AcCoA through the Krebs cycle, may be a possibility, the physiological significance of AcAc conversion to glycerol could not be established. It is possible that glucose and AcAc are complementary to each other as substrates for the synthesis of glycerol lipids, such as phospholipid, with glucose providing the glycerol moiety and AcAc providing fatty acid. It is perhaps significant in this regard that 10 mM exogenous glucose introduced into the incubation medium enhanced the incorporation of [3-^{14}C] AcAc into total lipids (data not shown).

It should be stressed that a rebound of fatty acid synthesis from both AcAc and glucose was noted in adult rats after a sharp decline throughout the suckling period. A similar developmental change in lipogenic capacity has been reported (10,11). This change may be associated with the nutritional status of the animals. As already discussed, both dietary fatty acid and that synthesized de novo contribute to lung lipids. For suckling rats, milk, which has a high fat content (28), could serve as a major source of lipid and lead to the decrease in de novo fatty acid synthesis observed immediately after birth. By contrast, the adult rats in our experiments, as well as those used by Maniscalco et al. (11), consumed laboratory rat chow, a high carbohydrate diet. De novo synthesis of fatty acids from AcAc and glucose was probably increased due to the lower availability of exogenous fatty acids.

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Conversion of Cyclolaudenol to 24α- and 24β-Ethylsterols in the Cucurbitaceae

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ABSTRACT

[2-^{3}H]Cyclolaudenol was converted into α-spinasterol, 24β-ethylcholesta-7,25-dien-3β-ol and 24β-ethylcholesta-7,22,25-trien-3β-ol by seedlings of *Cucurbita maxima*. As 24-methylenecycloartanol is the obligatory precursor of 24-ethylsterols, it can be assumed that the transformation of cyclolaudenol to 24-methylenecycloartanol must have occurred. These results lead us to postulate the existence, in the Cucurbitaceae family, of an enzymatic system capable of isomerizing Δ^{25}alkylsterols into $\Delta^{24(28)}$sterols. *Lipids* 18:302-305, 1983.

INTRODUCTION

Sterols of most higher plants and algae are C_{28} and C_{29} compounds in which the additional group at C-24 is derived from methionine (1).

The mechanism of the reaction can differ in various classes of algae, fungi and higher plants, thus leading to intermediates differing in the position of the olefinic bonds and the final configuration assumed by the C-24 alkyl group (2). For example, in Chrysophyte algae and higher plants, a 24-ethylidenesterol (**12**) is produced, which is then reduced to give either 24α-ethyl (**16**) or 24β-ethyl (**14**) sterols (3-5). In algae of the order Chlorococcales, the experimental evidence is consistent with the production of 24β-methyl-25-methylene intermediates, such as cyclolaudenol (**3A**), which are then reduced giving the corresponding 24β-methylsterols (**4**) (5-8). In Cucurbitaceae, two alternative pathways have been suggested for the biosynthesis of either 24α-ethyl- or 24β-ethylsterols (9,10).

Recently, the cooccurrence of 24-methylenecycloartanol (**5A**) and cyclolaudenol (**3A**) in maize (11) has led to the suggestion that higher plants may generally give two products of alkylation from cycloartenol (**1A**): the 24-methylene derivative (**5A**), which is the precursor of both 24α-methyl (**7**) and 24-ethylsterols, and cyclolaudenol (**3A**), potential precursor of 24β-methylsterols (**4**).

Labeled cyclolaudenol (**3A**) and cycloeucalenol were detected in *Polypodium vulgare* after administrating $^3H/^{14}C$ mevalonate (12). In *Trebuxia* spp. (Clorococcaceae), it was demonstrated that 31-norcyclolaudenol could be converted only into 24β-methylsterols but never into 24β-ethylsterols (13). If the pathway for the synthesis of 24β-isomers in higher plants is similar to that in *Trebouxia*, cyclolaudenol (**3A**), while it could give 24β-methylsterols, could not be further metabolized into 24-ethylsterols (11).

In the present paper, we describe, for the first time, the transformation of cyclolaudenol (**3A**) into both 24α-ethyl and 24β-ethylsterols in *Cucurbita maxima* seedlings.

EXPERIMENTAL

General

Methods (infrared, mass spectrometry, thin layer chromatography, radio-thin layer chromatography, and liquid scintillation counting) are generally as previously described (10).

C. maxima var. True Hubbard seeds were obtained from Fratelli Ingegnoli (Milan, Italy). Chemical purity of cyclolaudenol, kindly supplied by Dr. A.S. Narula, was tested by TLC-$AgNO_3$, GLC-MS and OsO_4 treatment (7).

[2-^{3}H]-Cyclolaudenol

[2-^{3}H]Cyclolaudenol (**3A**, sp act 5.9 mCi/mmol) was prepared from the corresponding 3-ketone derivative by alumina-catalyzed exchange with ^3H_2O (14), followed by $NaBH_4$ reduction of [2-^{3}H]cyclolaudenone.

In order to determine the chemical purity of [2-^{3}H]cyclolaudenol (**3A**), an aliquot of labeled **3A** (2.1×10^6 dpm) was diluted with carriers (**3A**) (~ 2 mg) and (**5A**) (~ 2 mg), acetylated and treated with OsO_4 in pyridine at room temperature overnight (7). The crude mixture was then treated with $Na_2S_2O_5$ for 2 hr under stirring, extracted with Et_2O and chromatographed on silica gel ($CHCl_3$/MeOH, 98:2). TLC radioscanning and liquid scintillation counting of the triol monoacetates showed the label was associated almost entirely with 9β,19-cyclo-4,4,14α,24ξ-tetramethyl-5α-cholesta-3β,25ξ,26-triol 3-acetate (**17**, 2.0×10^6 dpm, 99.4% of the diol fraction), whereas the label associated with 9β,19-cyclo-4,4,14α,24ξ-tetramethyl-5α-cholesta-3β-24ξ, 28-triol 3-acetate (**18**, 8000 dpm, 0.4% of the diols fraction) was negligible.

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Test to Exclude the Cooccurrence of Cyclolaudenol and 24-Methylenecycloartanol in *C. maxima* Seedlings

C. maxima seeds were germinated at 25C under light on moist filter paper in Petri dishes. A solution of 7.6×10^6 dpm of [2-^{14}C]acetate (10 ml, sp act 50 mCi/mmol) was fed to 10 five-day-old seedlings under continuous light for 24 hr. After incubation, the seedlings were homogenized, extracted with boiling 80% aqueous ethanol, saponified and the neutral lipids extracted with petroleum ether. TLC on silica gel (cyclohexane/ethylacetate, 85:15) gave a 4,4-dimethylsterol fraction, which was acetylated (C_5H_5N-Ac_2O) to the acetates (3 mg, 65×10^5 dpm). After argentation TLC ($CHCl_3$ free from EtOH) of the acetates, a band corresponding (Rf, GLC) to 24-methylenecycloartanyl acetate was isolated, counted (1.1×10^5 dpm) and hydroxylated with OsO_4 following the above described procedure.

Analytical TLC ($CHCl_3$/MeOH, 94:6) of the crude reaction mixture showed only one spot, visualized by vanillin, corresponding to 9β,19-cyclo-4,4,14α,24ξ-tetramethyl-5α-cholesta-3β,24ξ, 28-triol 3-acetate (**18**).

An amount (6×10^4 dpm) of the oxidized fraction, diluted with cold (**17**) (2 mg) and (**18**) (2 mg) was chromatographed ($CHCl_3$/MeOH, 96:4) and bands corresponding to carriers were scraped off and counted: 9β,19-cyclo-4,4,14α,24ξ-tetramethyl-5α-cholesta-3β,24ξ,28-triol 3-acetate (**18**) 5.6×10^4 dpm and 9β,19-cyclo-4,4,14α,24ξ-tetramethyl-5α-cholesta-3β,25ξ,26-triol 3-acetate (**17**), 230 dpm.

Lack of radioactivity in the compound (**17**), derived from cyclolaudenol (**3A**), excluded the occurrence of cyclolaudenol (**3A**) in *C. maxima* seedlings.

In vivo Experiments

C. maxima seeds were germinated at 25C under light in Petri dishes on moist filter paper. On the 5th day after seed germination, labeled cyclolaudenol (22×10^6 dpm) in acetone solution (0.5 ml) was applied to cotyledons of 35 seedlings as previously described (9). The seedlings were kept under alternate lighting for 7 days, then extracted with boiling ethanol. Unsaponifiable lipids were separated on TLC (cyclohexane/EtOAc, 85:15) to give desmethysterols (1.6×10^6 dpm) and 4,4-dimethylsterols (4×10^6 dpm) which were acetylated (C_5H_5N-Ac_2O). Multiple argentation TLC ($CHCl_3$ free from EtOH) of the steryl acetates gave the labeled sterols (Table 1) which were identical (GLC and GLC-MS) with authentic standards (15). Recrystallization of the individual steryl acetates from $CHCl_3$/MeOH led to samples of constant specific activity: **15B**, 14,200, 14,750, 14,400 dpm mg^{-1}; **10B** 11,200, 11,000 dpm mg^{-1}; **11B** 71,400, 72,090, 71,850 dpm mg^{-1}.

TABLE 1

Radioactivity Incorporated into Sterols after Incubation of 5-day-old Seedlings of *C. maxima* with [2-^{3}H]Cyclolaudenol (10 μCi)

Sterols	^{3}H dpm[a]	%[b]
α-Spinasterol (*15B*)	2.44×10^5	19.3
24β-Ethyl-cholesta-7,25-dien-3β-ol (*10B*)	0.68×10^5	5.3
24β-Ethyl-cholesta-7,22,25-trien-3β-ol (*11B*)	9.52×10^5	75.3

[a]Determined as pure acetates.
[b]Percentage relative to the sterol fraction only.

α-Spinasteryl (**15B**) acetate (10.7 mg, 138,000 dpm) was saponified and treated with CrO_3/pyridine complex (16) to give the corresponding 3-keto derivative **15C** (6.15 mg, 71,700 dpm), which was purified by TLC (cyclohexane/EtOAc, 90:10). The only radioactive peak, corresponding to 3-keto-α-spinasterol, was scraped off (2.67 mg), counted (40,850 dpm) and treated with 5% NaOH in ethanol at reflux for 24 hr. Petroleum extract of the reaction mixture was subjected to TLC (cyclohexane/EtOAc, 90:10) and the recovered ketone **15C** (1.70 mg) was counted (250 dpm, 147 dpm mg^{-1}, 99% loss of the tritium). An aliquot of the previously separated 4,4-dimethylsterol fraction (1×10^6dpm) was purified by argentation TLC ($CHCl_3$ free from EtOH) after acetylation. The 4,4-dimethylsteryl acetates (6×10^5dmp), after dilution with **3A** and **5A** acetates, were treated with OsO_4 overnight under the described conditions giving the mixture of diols **17** and **18** which were separated by TLC ($CHCl_3$/MeOH, 98:2) and counted in a liquid scintillator: **17**, 3.7×10^5 dpm; **18**, 150 dpm.

RESULTS AND DISCUSSION

[2-^{3}H]Cyclolaudenol (**3A**) was efficiently converted into both 24α (**15B**) and 24β (**10B** and **11B**) ethylsterols by *C. maxima* seedlings (Table 1); recovered radioactive 4-desmethylsterols constituted almost one-third of the total sterol fraction (4,4-dimethyl- and 4-desmethylsterols). The conversion yields are comparable with those reported for the incubation of [24-^{3}H]-[26,27-^{14}C]cycloartenol (**1A**) with *C. maxima* seedlings (9). The labeled cyclolaudenol (**3A**) was unambigously tested, before the incubation experiments, in order to exclude the co-presence of 24-methylenecycloartanol (**5A**), which is known to be the precursor of the 24-ethylsterols (2).

Our data indicate that *C. maxima* may contain an enzyme system which can isomerize the C-25(26) double bond in **3A** giving the C-24 methylene

derivative **5A** (Fig. 1), which is the obligatory precursor of the C_{29} phytosterols (2,11). On the other hand, the lack of 24-methylsterols (15) and cyclolaudenol (see Experimental) in *C. maxima* seedlings could suggest that **3A** is not the physiological substrate of the supposed $\Delta^{25}\rightarrow\Delta^{24(28)}$-sterol isomerase.

It can be presumed that the true role of the above-cited enzyme is that of protonating the Δ^{25}-24β-ethylsterol (**10B**), giving rise to the 24α-ethylsterol via the carbocations **9** and **8**. If such an enzyme is operative, the Cucurbitaceae may possess a system capable of transforming the 24β-ethylsterol into 24α-ethylsterol, e.g., (**10B**)$\rightarrow$(**15B**). In *Bryonia dioica*, we showed that active synthesis of both 24α- and 24β-ethylsterols depends on the development stage of the plant (10). In *Cucurbita pepo* (17), it has been shown that in the adult plant only 24α-alkylsterols are present, whereas the seeds contain both 24α- and 24β-alkylsterols.

Taking the abovementioned findings into account, it could be envisaged that, in some Cucurbitaceae, the 24β-ethylsterols may be progressively transformed into the 24α-ethylsterols during the maturation of the tissues via a $\Delta^{25}\rightarrow\Delta^{24(28)}$ isomerase.

The anomaly of the Cucurbitaceae among the higher plants could be further exemplified if we consider their sterol composition, which comprises sterols present both in algae and in more evolved plants. A striking example of this peculiarity is the recent isolation in *B. dioica* roots of stigmasta-7,E24(28)-dien-3β-ol(**19B**) which had never been found in the plant kingdom, whereas the very

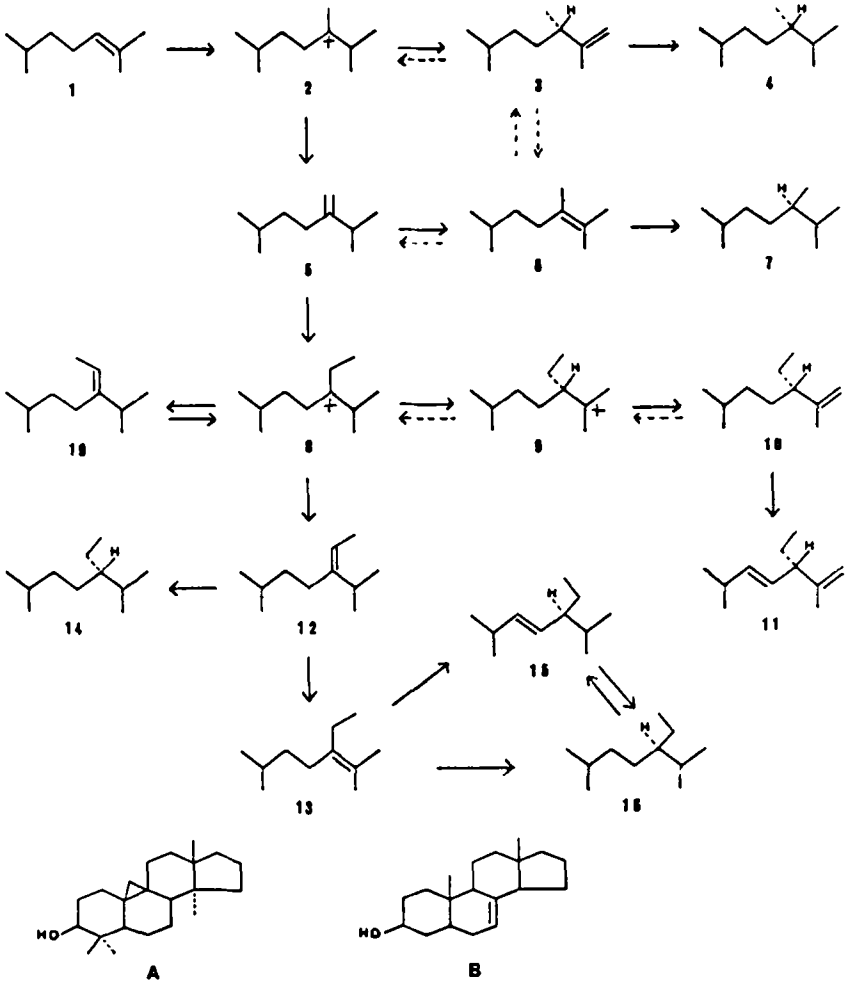

FIG. 1.

similar fucosterol (stigmasta-5,E24(28)-dien-3β-ol) is predominant in the marine brown algae (18,19).

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Increases in Hyperlipoproteinemia, Disturbances in Cholesterol Metabolism and Atherosclerosis Induced by Dietary Restriction in Rabbits Fed a Cholesterol-Rich Diet

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ABSTRACT

The influence of dietary restriction on cholesterol transport and metabolism was investigated in rabbits given standard or cholesterol-rich diets (0.2 g cholesterol/ kg body weight daily) either *ad libitum* or with 50% caloric ration. Dietary restriction which has only a slight influence in control rabbits markedly aggravated the disturbances induced by exogenous cholesterol. With limited feeding, control rabbits presented a moderate increase in plasma cholesterol, whereas marked aggravation of hypercholesterolemia was observed in cholesterol-fed rabbits. Analysis of the lipoprotein profile showed that the excess of plasma cholesterol with the restricted cholesterol-rich diet corresponded to an increase in the concentration of very low density lipoprotein (VLDL) and low density lipoproteins (LDL) without any additional changes in the composition of these lipoproteins. No significant change appeared in the high density lipoprotein (HDL) concentration. The parameters of cholesterol metabolism were determined, from the curves of [^{3}H] cholesterol radioactivity decrease, using a two-pool model. The increase in cholesterol turnover rate induced by the cholesterol-rich diet was accentuated by dietary restriction, whereas rabbits on standard restricted diet showed a slight decrease. The large increase in the size of both pools A and B in cholesterol-fed rabbits was even more pronounced with limited feeding. Dietary restriction induced additional accumulation of cholesterol in the aortic wall and the grade of the lesions was also aggravated.
Lipids 18:306-312, 1983.

INTRODUCTION

In our previous experiments on hyperlipoproteinemia induced by semisynthetic diets, rabbits spontaneously reduced their food intake (1, 2). Numerous works on the nutritional origin of cardiovascular diseases report the influence of various dietary components, while few studies deal with the importance of total caloric intake. Currently, the influence of dietary restriction on hypercholesterolemia and atherogenesis is not clear. In humans, improved lipoprotein profiles were observed during weight reduction induced by dietary restriction (3-7). Studies carried out in species which develop spontaneous atherosclerosis showed the beneficial effect of a low calorie diet. The incidence of atherosclerosis can be reduced by dietary restriction started early in life in genetically obese rats (8) as well as in white Carneau pigeons (9). Conversely, experimental atherosclerosis induced by cholesterol-rich diet in the chick was not prevented by dietary restriction (10).

The aim of the present study was to investigate the influence of dietary restriction on cholesterol transport and metabolism as well as on the grade of atherosclerosis in rabbits on standard or atherogenic diets.

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MATERIALS AND METHODS

Animals and Diets

Four groups of 8 New Zealand male rabbits, average weight at the start of experimentation 2.9 kg, received the dietary treatment described in Table 1 for 3 months. Cholesterol intake was adjusted to body weight in both cholesterol and restricted-cholesterol groups. This was performed using diets containing 5% cholesterol with which the daily amount, necessary to provide 0.2 g/kg, was calculated for each rabbit and the dietary ration complemented with standard diet.

Cholesterol Turnover

Body weight and plasma cholesterol (11) were

TABLE 1

Dietary Treatment

	Standard diet[a] (g/ day)	Cholesterol[b] (g/ kg/ day)
C	160	-
RC	80	-
Ch	160	0.2
R.Ch	80	0.2

[a]UAR– Rabbit chow diet.
[b]Cholesterol was incorporated in the chow diet in the crystalline form.
C = control; R = restricted; Ch = cholesterol.

measured weekly. When these parameters became stabilized, each rabbit was injected, via the marginal vein of the ear, with 25 μ Ci of [1,2^{3}H]cholesterol (CEA-Saclay, France) prepared as described by Goodman and Noble (12). The injection of labeled cholesterol was carried out after 5 and 6 weeks of experiment in control and cholesterol groups, respectively; blood samples were collected from the opposite ear daily for the following 7 days and then weekly for 50 days. After specific extraction (12), radioactivity was measured in a Packard liquid scintillation counter using toluene (0.4% PPO and 0.01% POPOP) as scintillator.

Kinetic parameters of cholesterol turnover were calculated from the curves of specific plasma radioactivity decrease using a two-compartment model according to Goodman and Noble (12).

Lipoprotein Analysis

Animals were fasted overnight before exanguination and the blood collected over EDTA (1 mg/ml).

Lipoprotein fractions: VLDL ($d < 1.006$), LDL ($1.006 < d < 1.063$), HDL_2 ($1.063 < d < 1.125$), HDL_3 ($1.125 < d < 1.21$) were separated on a KBr density gradient in a Beckman SW 41 rotor using a modification of the technique described by Chapman et al. (13). The density of the bottom of the tube was increased by replacing the solution of density 1.24 by one of 1.30 to reduce contamination by protein of the denser fractions. The gradients were then constructed and centrifuged as previously described (13). HDL_3 was purified by additional centrifugation at density 1.21 (40.000 rpm for 48 hr).

Determination of lipoprotein compositions and quantitative analysis were performed as previously described (2,14).

Tissues Analysis

The liver, intestine and aorta were removed and weighed.

The extent of the lesions of the arch and thoracic aorta were determined from photographs by planimetry. The artery samples were then pulverized in 0.25 M sucrose, 1 mM tris buffer (pH 7.2) for the determination of N-acetyl-glucosaminidase activity (15) and protein content (16). The remaining homogenates were lyophilized and extracted in a chloroform methanol (2:1, v/v) mixture for cholesterol determination (17).

The cholesterol content was also determined in liver and in aliquots of the whole intestine homogenate (17) after extraction with chloroform methanol.

Statistics

All values are expressed as means ± SEM of 8 rabbits. Statistical significance was determined using Student's t-test.

RESULTS

Body Weight

Changes in body weights during the experimental period are presented in Figure 1. Rabbits on restricted diets presented a weight reduction which took place principally in the first weeks of the experiment. No significant changes were observed with cholesterol supplementation of the diets.

Plasma Cholesterol and Lipoproteins

Dietary restriction induced only a slight increase in plasma cholesterol in control rabbits, whereas it markedly enhanced the hypercholesterolemia induced by exogenous cholesterol (Fig. 2). Plasma cholesterol was doubled in rabbits on restricted cholesterol-rich diets compared to the cholesterol group.

The rise in plasma cholesterol was associated with changes in lipoprotein level (Table 2) and composition (Table 3) and also in the percentage distribution of cholesterol between the lipoprotein fractions (Table 4).

Profound modifications in lipoprotein profile were observed in both groups receiving cholesterol. Cholesterol-rich diets induced a marked increase in VLDL and LDL concentration but no significant changes were seen in the level of HDL_2 and HDL_3. Marked alterations in VLDL and LDL composition were observed in cholesterol-fed rabbits. These lipoproteins showed an increase in their cholesterol content and a decrease in triglycerides and

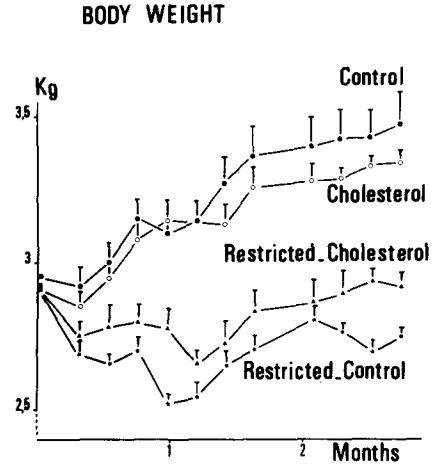

FIG. 1. Changes in body weight during the experimental period.

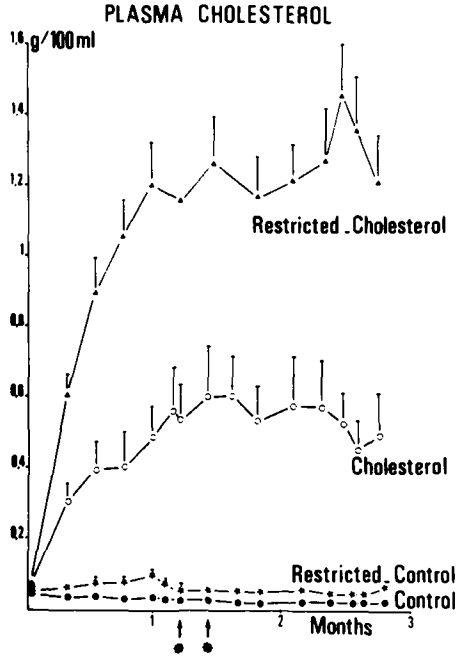

FIG. 2. Influence of dietary restriction on cholesterolemia in rabbits fed either standard or cholesterol-rich diets. * Injection of [^{3}H]-cholesterol after 5 and 6 weeks of experiment in controls and cholesterol groups, respectively.

TABLE 2

Influence of Dietary Restriction on Lipoprotein Levels in Rabbits Fed either Standard or Cholesterol-Rich Diets

	VLDL	LDL	HDL_2	HDL_1
C	62 ± 14^a	22 ± 4.2^c	69 ± 13	101 ± 40
RC	45 ± 25^a	54 ± 10^b	101 ± 12	84 ± 11
Ch	849 ± 145^b	183 ± 39^c	94 ± 22	118 ± 34
R.Ch	1883 ± 305^c	357 ± 47^d	82 ± 9.7	91 ± 23

(mg/100 ml plasma); means ± SEM of 8 rabbits.
^{a,b,c,d}Superscript letters indicate intergroups statistical difference, means not sharing a common letter are significantly different ($P<0.05$).
C = control; R = restricted; Ch = cholesterol.

TABLE 3

Influence of Dietary Restriction on Percent Composition of Lipoproteins in Rabbits Fed Either Standard or Cholesterol-Rich Diets

		Proteins	Triglycerides	Phospholipids	Cholesterol Esters	Free Cholesterol
VLDL	C	15.0 ± 1.2^a	54.3 ± 2.4^a	16.3 ± 0.4^a	10.7 ± 2.4^a	3.7 ± 1.1^a
	RC	15.5 ± 0.9^a	45.0 ± 3.5^a	17.6 ± 0.9^a	16.6 ± 1.2^b	5.3 ± 0.9^a
	Ch	8.6 ± 1.0^b	5.0 ± 0.9^b	19.0 ± 0.9^{ab}	55.2 ± 1.2^c	12.2 ± 1.3^b
	R.Ch	7.6 ± 0.4^b	4.8 ± 0.6^b	24.4 ± 1.8^b	48.5 ± 2.6^d	14.7 ± 1.3^b
LDL	C	26.3 ± 2.8^a	19.1 ± 1.9^a	24.5 ± 2.2^{ab}	24.4 ± 3.3^a	5.7 ± 1.2^a
	RC	31.0 ± 1.5^a	16.4 ± 1.8^a	27.2 ± 3.0^b	17.1 ± 2.2^a	8.3 ± 1.5^a
	Ch	19.7 ± 1.3^b	2.6 ± 0.5^b	20.0 ± 0.6^a	43.0 ± 2.6^b	14.7 ± 1.6^b
	R.Ch	18.2 ± 1.3^b	3.0 ± 0.5^b	19.2 ± 1.5^a	42.7 ± 3.2^b	16.9 ± 0.8^b
HDL_2	C	50.4 ± 2.6	11.4 ± 1.5^a	24.7 ± 2.0^{ab}	9.2 ± 1.0^a	4.3 ± 0.6^a
	RC	45.2 ± 0.9	11.8 ± 0.8^a	27.4 ± 1.6^a	9.8 ± 1.3^{ab}	4.8 ± 1.0^a
	Ch	51.5 ± 3.4	4.8 ± 0.8^b	20.8 ± 1.5^b	16.1 ± 3.2^b	6.8 ± 1.2^a
	R.Ch	47.7 ± 1.7	4.7 ± 0.8^b	21.6 ± 0.9^b	14.6 ± 1.8^b	11.4 ± 0.7^b
HDL_1	C	63.8 ± 5.5	4.8 ± 1.1^a	20.7 ± 3.2^{ab}	8.4 ± 1.2	2.3 ± 0.4^a
	RC	58.2 ± 4.2	7.1 ± 3.1^{ab}	20.7 ± 1.2^b	11.3 ± 0.8	2.7 ± 0.3^a
	Ch	63.1 ± 7.7	1.3 ± 0.2^b	18.1 ± 3.5^{ab}	13.1 ± 3.3	4.4 ± 1.0^{ab}
	R.Ch	59.1 ± 5.8	5.8 ± 2.5^{ab}	14.0 ± 1.3^a	15.3 ± 4.4	5.8 ± 2.5^b

Means ± SEM of 8 rabbits.
^{a,b,c,d}Superscript letters indicate intergroups statistical difference, means not sharing a common letter are significantly different ($P<0.05$).
C = control; R = restricted; Ch = cholesterol.

TABLE 4

Influence of Dietary Restriction on the Distribution of Cholesterol Between the Lipoproteins in Rabbits Fed either Standard or Cholesterol-Rich Diets

	VLDL	LDL	HDL_2	HDL_3
C	23.0 ± 3.68^a	22.5 ± 3.00	26.8 ± 3.75^a	27.7 ± 3.00^a
RC	18.5 ± 3.82^a	27.6 ± 2.25	33.0 ± 3.00^a	20.9 ± 1.80^a
Ch	81.6 ± 2.51^b	15.2 ± 2.56	1.9 ± 0.38^b	1.3 ± 0.25^b
R.Ch	81.1 ± 2.67^b	16.1 ± 2.20	1.8 ± 0.44^b	1.0 ± 0.14^b

(Percent of total plasma cholesterol); means ± SEM of 8 rabbits.

[a,b]Superscript letters indicate intergroups statistical difference, means not sharing a common letter are significantly different ($P<0.05$).

C = control; R = restricted; Ch = cholesterol.

proteins. The HDL_2 also presented an enrichment in cholesteryl esters. The dietary restriction accentuated the rise in apoB-containing lipoproteins, but had only a slight influence on their composition. No additional changes were observed except a slight increase in free cholesterol of HDL_2 and HDL_3 and a decrease in cholesterol esters of VLDL.

Quantitative and qualitative alterations of the lipoproteins induced by cholesterol feeding resulted in profound disturbance of cholesterol transport. The participation of HDL in cholesterol transport was markedly reduced, whereas the proportion associated with VLDL increased from 23 to 81% total cholesterol. This distribution was altered to the same extent in both groups receiving cholesterol, restricted or not.

The increase in plasma cholesterol observed in control rabbits with limited feeding corresponded to an increase in LDL concentration and an enrichment of VLDL in cholesteryl esters.

Cholesterol Metabolism

The kinetic parameters of cholesterol metabolism are presented in Table 5. The metabolic clearance fraction was markedly delayed in all groups. The increase in the metabolic turnover rate (PR_A) induced by cholesterol feeding was accentuated by dietary restriction, whereas rabbits on standard restricted diet showed a significant decrease.

The mass of pool A was higher in control rabbits on restricted diet but no changes occurred in the mass of pool B. Cholesterol-fed rabbits presented an increase in both pools A and B which was markedly accentuated by dietary restriction.

Changes in the Cholesterol Content of Various Tissues

Dietary restriction has no effect on the cholesterol content of the liver or intestine in control rabbits, as shown in Table 6. Therefore, in this group, the increase in pool A mainly corresponded in hypercholesterolemia.

A marked increase in hepatic and intestinal cholesterol concentration was observed in cholesterol-fed rabbits. This was aggravated by dietary restriction principally in the intestine.

TABLE 5

Influence of Dietary Restriction on the Kinetic Parameters of Cholesterol Turnover in Rabbits Fed either Standard or Cholesterol-Rich Diets

	M_A	M_B	PR_A	MCF
	(mg/kg body wt)		(mg/kg/day)	(%/day)
C	266 ± 15.5^a	633 ± 65.2^a	52.7 ± 2.10^a	3.94 ± 0.4^a
RC	349 ± 13.9^b	614 ± 35.5^a	38.6 ± 1.90^b	1.39 ± 0.1^b
Ch	1235 ± 84.5^c	991 ± 58.6^b	90.6 ± 2.16^c	0.40 ± 0.1^c
R.Ch	1597 ± 83.3^d	1617 ± 150^c	123 ± 4.30^d	0.29 ± 0.05^c

M_A: Mass of cholesterol in pool A; M_B: minimum mass of cholesterol in pool B assuming that cholesterol synthesis in tissues of pool B is negligible; PR_A: production rate of cholesterol in pool A; MCF: metabolic clearance fraction; means ± SEM of 8 rabbits.

[a,b,c,d]Superscript letters indicate intergroups statistical difference, means not sharing a common letter are significantly different ($P<0.05$).

C = control; R = restricted; Ch = cholesterol.

TABLE 6

Influence of Dietary Restriction on the Changes in Cholesterol Content of Liver and Intestine in Rabbits Fed either Standard or Cholesterol-Rich Diets

	Liver (mg/kg body wt)	Intestine
C	60 ± 2.7[a]	53 ± 1.1[a]
RC	66 ± 2.2[a]	59 ± 2.5[a]
Ch	1144 ± 145[b]	97 ± 6.3[b]
R.Ch	1448 ± 195[b]	140 ± 9.2[c]

Means ± SEM of 8 rabbits.

[a,b,c]Superscript letters indicate intergroups statistical difference, means not sharing a common letter are significantly different ($P<0.05$).

C = control; R = restricted; Ch = cholesterol.

Atherosclerosis

Cholesterol feeding resulted in dramatic atherosclerosis in the arch as well as in the thoracic aorta (Table 7). According to the changes in pool B, marked accumulation of cholesterol occurred in the arterial wall which was accentuated by dietary restriction. A concomitant increase in the activity of a lysosomal enzyme: N-acetyl glucosaminidase (NAGA) was observed.

The extent of the lesion area was doubled when cholesterol feeding was associated with dietary restriction. No spontaneous lesions were observed in either group receiving the standard diet.

DISCUSSION

Dietary restriction had a moderate influence in control rabbits but markedly aggravated the disturbances induced by cholesterol feeding.

Total starvation is well known to result in hypercholesterolemia, as shown in man (18) and rabbit (19-22). In contrast, limited feeding may have a beneficial effect. Restriction of dietary intake is generally accompanied by a decrease of serum cholesterol and triglyceride concentration in normal and obese humans (3-7). The beneficial effect of dietary restriction was also reported in genetically obese rats (8) and in the White Carneau pigeon (9), reducing the incidence of spontaneous atherosclerosis. The present experiment shows that dietary restriction has an adverse effect when associated with cholesterol feeding. An increased hypercholesterolemia with a semipurified diet containing casein was previously observed in young rabbits when a restricted feeding regime was applied (23). The effect of dietary restriction was less marked in adult rabbits (23), but in this experiment with a semipurified diet, the daily food intake in the group fed ad libitum was low. A slight increase in experimental atherosclerosis with dietary restriction was also observed in chicks (10). Therefore, the influence of dietary restriction seems to be different in endogenous or exogenous hypercholesterolemia.

Emaciation induced by dietary restriction in control rabbits is accompanied by a slight hypercholesterolemia which corresponds to an increase in LDL and an enrichment in cholesteryl esters of VLDL without changes in the ratio HDL cholesterol/total cholesterol. Fasting hypercholesterolemia previously observed in rabbits also corresponded to an increase in LDL (21).

Changes in the lipoprotein profile in cholesterol-fed rabbits: increase in apo B-containing lipopro-

TABLE 7

Influence of Dietary Restriction on the Changes in Cholesterol Content, NAGA Activity and Visual Grading Atherosclerosis of Aorta in Rabbits Fed either Standard or Cholesterol-Rich Diets

		Arch	Thoracic
Cholesterol (mg/100 g wet weight)	C	235 ± 35[a]	152 ± 10.5[a]
	RC	206 ± 14[a]	169 ± 7.5[a]
	Ch	844 ± 154[b]	472 ± 110[b]
	R.Ch	2557 ± 300[c]	1031 ± 243[c]
NAGA (mU/mg protein)	C	0.40 ± 0.026[a]	0.40 ± 0.020[a]
	RC	0.38 ± 0.021[a]	0.31 ± 0.015[b]
	Ch	0.94 ± 0.13[b]	0.56 ± 0.073[c]
	R.Ch	2.60 ± 0.29[c]	0.72 ± 0.078[c]
Lesions (% surface area)	Ch	39 ± 8.6[a]	9 ± 3.3[a]
	R.Ch	78 ± 2.7[b]	17 ± 2.1[b]

Means ± SEM of 8 rabbits.

[a,b,c]Superscript letters indicate intergroups statistical difference, means not sharing a common letter are significantly different ($P<0.05$).

C = control; R = restricted; Ch = cholesterol.

teins, mainly the VLDL, and alterations in their composition have been extensively reported (24-27). The results presented here are in complete agreement with these observations. Aggravation by dietary restriction of the hyperlipoproteinemia induced by exogenous cholesterol is spectacular. It is noted that the excess of plasma cholesterol corresponds to an increase in VLDL and LDL without additional changes in their composition. In cholesterol-fed rabbits, it is well known that the increase in β migrating VLDL represents an accumulation of chylomicron remnants (28) which result from saturation of the hepatic lipoprotein receptors (29). In the present experiment, the increase in the fraction $d < 1.006$ is markedly accentuated by dietary restriction, yet further investigations will be needed to elucidate if this increase corresponds to chylomicron remnants or to VLDL of intestinal and/or hepatic origin. The difference observed in restricted animals could be caused either by a more rapid inhibition of the hepatic uptake mechanism or by an increased production of lipoproteins. In fact, in the rabbit, a moderate increase in β VLDL results in saturation of the hepatic receptors. When the β VLDL cholesterol is above 1.4 mg/dl, the removal mechanism begins to be saturated and the fractional clearance rate decreased (29). Thus, overproduction of chylomicron or VLDL is probably responsible for the excess accumulation.

The values obtained for kinetic parameters of cholesterol metabolism in control and cholesterol-fed rabbits are consistent with data previously reported (30, 31). In control rabbits, there is no dietary intake of cholesterol; therefore, the production rate in pool A represents the endogenous synthesis. The results show a reduction in endogenous synthesis with the standard restricted diet. Similarly, fasting rabbits exhibited hypercholesterolemia and decreased fecal steroid excretion (22). The proportional relationship between the daily quantity of cholesterol synthesized and the energy expenditure was first reported in the rat (32). Our data are in agreement with this observation. In addition, the simultaneous rise in plasma cholesterol confirms the model system reported by Chevallier et al. (33) where a reduction in synthesis results in an increase of cholesterolemia.

Conversely to standard diets, cholesterol feeding induced an increase in metabolic turnover rate which is accentuated by dietary restriction. Rabbits have been shown to be deficient in control mechanisms to prevent the very large increase in total body cholesterol observed with cholesterol feeding (30). This is markedly aggravated in restricted animals in spite of a similar intake of exogenous cholesterol. The fact that dietary restriction reduces endogenous synthesis in control rabbits suggests that the adverse effect observed in cholesterol-fed rabbits corresponds to increased intestinal absorption of cholesterol. Nevertheless, an interaction between cholesterol feeding and dietary restriction is not excluded.

The aggravation of atherogenesis by dietary restriction is remarkable. Rabbits on the restricted cholesterol-rich diet present a rise in the activity of NAGA, a lysosomal enzyme well known to increase during atherosclerosis (15). The accumulation of cholesterol in the aortic wall and the grade of atherosclerosis are markedly accentuated when cholesterol feeding is associated with dietary restriction. This increased cholesterol accumulation in the aortic wall could be facilitated by limitation of storage in the adipose tissue. Assuming that the difference in body weight occurring with dietary restriction corresponds to a reduction in the mass of adipose tissue which represents an important part of pool B (34), the deposition in other tissues of pool B could be accentuated.

The relationship between the concentration of VLDL and LDL and the extense of atherosclerosis is noted. The atherogenic effect of apo B-containing lipoproteins has been widely reported (35,36). The appearance of abnormal lipoproteins in experimental atherosclerosis (24-27) as well as in familial hypercholesterolemia (37,38) and the fact that they stimulate the proliferation of aortic smooth muscle cells (39) and also the accumulation of cholesteryl ester in macrophages (40,41) have been the focus of recent research. The present experiment, where increased concentration occurred without additional changes in composition, suggests the primordial importance of the quantitative aspect of the lipoprotein profile.

In conclusion, dietary restriction, which has only little influence in control rabbits, markedly aggravates the disturbances of cholesterol transport and metabolism as well as atherogenesis when associated with cholesterol feeding. Study of the turnover provides interesting information concerning the distribution of cholesterol between the different pools, but additional investigations will be needed to explain the definite mechanism by which such accumulation of cholesterol occurs.

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Stereospecificity of Different Lipases[1]

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ABSTRACT

The stereospecificity of 4 lipases towards enantiomeric alkyldiacylglycerols and alkylmonoacylglycerols was investigated. No stereospecific breakdown of the former substrate was observed for lipases from pancreas, *Rhizopus arrhizus*, *Pseudomonas fluorescens*, or bile salt-stimulated lipase from human milk. All lipases degraded 2-oleoyl-3-tetradecyl-*sn*-glycerol faster than 1-tetradecyl-2-oleoyl-*sn*-glycerol. Among X-1-acyl-3-alkylglycerol isomers, 1-acyl-3-alkyl-*sn*-glycerol was preferentially attacked by the 3 first mentioned lipases. Possible mechanisms and metabolic implications for these stereospecificities are discussed.
Lipids 18:313-318, 1983.

INTRODUCTION

It is generally recognized that the first degradation of triacylglycerol by lipases involves ester bonds at one or both of the primary positions of glycerol (1). Since the lipase first studied, pancreatic lipase, did not discriminate between positions 1 and 3 (2), little interest was devoted to the possible stereospecificity of different lipases. This was also due to the lack of suitable substrates and methods for the assessment of stereospecificity. More recently, it was demonstrated that lipoprotein lipase (3,4) and the heparin-releasable hepatic lipase (5) preferentially attack position 1, whereas tongue lipase prefers position 3 of triacylglycerol or alkyldiacylglycerol (4). Several lipases did not show any stereospecificity in attacking triacylglycerol or its analogues, such as pancreatic lipase (4) and liver lysosomal lipase (5). Our studies have now been extended to other lipases with unknown stereospecificity. The bile salt-stimulated lipase of milk was studied to compare its specificity to that reported for milk lipoprotein lipase. Furthermore, 2 microbial lipases have been investigated. The specificity of the 3 enzymes was compared to that of pancreatic lipase.

EXPERIMENTAL

Materials

1,2-Di[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol, 1-tetradecyl-2,3-di[^{14}C]oleoyl-*sn*-glycerol, 2-[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol, 1-[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol, 1-tetradecyl-2-[^{14}C]oleoyl-*sn*-glycerol, and 1-tetradecyl-3-[^{14}C]oleoyl-*sn*-glycerol were prepared as described previously (5). They were purified by thin layer chromatography (TLC). At intervals, the purification was repeated to minimize the concentration of acyl migration products. The following lipase preparations were used: lipase from *Rhizopus arrhizus* in ammonium sulfate suspension (ca. 13750 U/mg) (obtained from Boehringer, GFR), a highly purified lipase from *Pseudomonas fluorescens* (obtained from Amano Pharmaceutical Co., Nagoya, Japan (6)), bile salt-stimulated lipase isolated from human milk as described previously (7) and purified pancreatic lipase and colipase obtained from Dr. T. Wieloch, Department of Physiological Chemistry, University of Lund (8).

Lipase Assays

^{3}H- and ^{14}C-labeled substrates were mixed to obtain racemic mixtures of alkyldiacylglycerol or alkylmonoacylglycerol. Two mg of these mixtures were taken to dryness in a centrifuge tube. Two ml of acacia in water (15 mg/ml) was added, and the mixture was sonicated twice at 0C for 5 min, and 0.1 ml was then added to lipase assay mixtures (0.25 ml total volume). The assay mixture for bile salt-stimulated lipase contained 0.1 M Tris-HCl (pH 9.0), sodium taurocholate (8 mg/ml), 0.2 M sodium chloride, and bovine serum albumin (16 mg/ml). The assay mixture for other lipases was 0.1 M Tris-HCl (pH 7.4), 0.1 M sodium chloride and bovine serum albumin (16 mg/ml). To incubations with pancreatic lipase, 10-fold more colipase than pancreatic lipase was added. The amount of lipases added was varied to give different degrees of hydrolysis. For *R. arrhizus* lipase, the desired lipolysis was obtained by 5-250 ng of the enzyme preparation. The incubations were shaken for 30 min at 37C, and lipids were then extracted and separated by TLC (5). Radioactivity was measured by liquid scintillation counting, and counting efficiency was monitored using the external standard ratio method.

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[1]A preliminary account of this work was given in Abstract 457 of the ISF/AOCS World Congress, New York, 1980.

RESULTS

Lipolysis of Alkyldiacylglycerol

In the first series of experiments, different amounts of lipases were incubated with equal amounts of [^{14}C]- and [^{3}H]alkyldiacylglycerol, and the appearance of ^{14}C and ^{3}H in different breakdown products was compared. Lipase from *P. fluorescens* degraded the 2 alkyldiacylglycerol isomers at equal rates, as evidenced by the constant ^{14}C/^{3}H ratio in the substrate even at 90% hydrolysis (Fig. 1). In fatty acid, ^{14}C/^{3}H was 0.6-0.7 and in alkylmonoacylglycerol the ratio was well above unity, especially at extensive lipolysis. The deviations of ^{14}C/^{3}H from unity indicate that the proportions of the breakdown products varied markedly for the 2 alkyldiacylglycerol isomers. [^{14}C]Alkylmonoacylglycerol accumulated more than [^{3}H]alkylmonoacylglycerol, which instead was split to fatty acid and alkylglycerol to a higher degree. This indicates that a stereospecificity was exerted at the alkylmonoacylglycerol level, although no specificity vs enantiomeric alkyldiacylglycerols could be detected.

Similar experiments were performed with 3 other lipases (Table 1). No significant change in ^{14}C/^{3}H of alkyldiacylglycerol during varying degrees of hydrolysis could be observed. In the breakdown products, small but reproducible changes in ^{14}C/^{3}H were observed but they cannot be taken as definite evidence for existing stereospecificities. The increase of ^{14}C/^{3}H to 1.41 in alkylmonoacylglycerol after incubation with *R. arrhizus* lipase suggests, however, such a specificity at the alkylmonoacylglycerol level. It was clearly less marked than that observed for *P. fluorescens* lipase (Fig. 1).

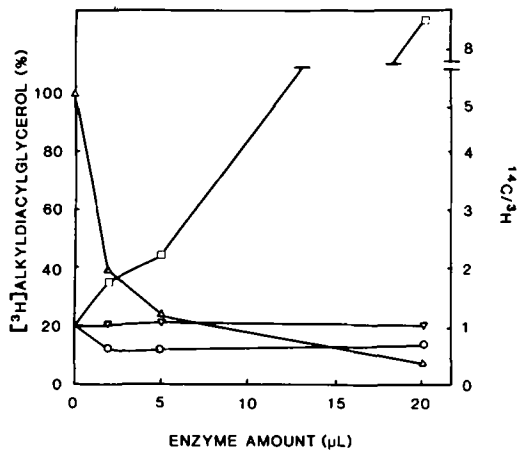

FIG. 1. Lipolysis of racemic alkyldiacylglycerol by lipase from *P. fluorescens*. 25 μg of 1,2-di[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol plus 1-tetradecyl-2,3-di[^{14}C]oleoyl-*sn*-glycerol was incubated for 30 min with different amounts of lipase. Symbols: △, amount of remaining substrate ([^{3}H]alkyldiacylglycerol, %); ^{14}C/^{3}H ratio in alkyldiacylglycerol ▽, alkylmonoacylglycerol □, and fatty acid o. The ^{14}C/^{3}H ratio in the substrate was set at unity. Data are means of two observations.

Lipolysis of X-1-Alkyl-2-acylglycerol

Since the experiments indicated that lipases attacked alkylmonoacylglycerols stereospecifically, the next series of experiments were performed with 2-[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol and 1-tetradecyl-2-[^{14}C]oleoyl-*sn*-glycerol, which are the expected intermediates in the incubations described in Table 1 and Figure 1. Figure 2 shows that lipase from *P. fluorescens* degraded the former isomer several times more rapidly than the other isomer.

TABLE 1

Hydrolysis of Alkyldiacylglycerol Isomers by Different Lipases[a]

Lipase	^{3}H-Labeled substrate remaining (%)	^{14}C/^{3}H		
		Alkyldiacyl-glycerol	Alkylmono-acylglycerol	Fatty acid
Pancreatic lipase	82.5	1.04	0.82	0.91
	42.8	1.05	0.99	0.87
Bile salt-stimulated lipase	71.4	1.06	1.01	0.80
	56.3	1.08	1.02	0.87
R. arrhizus lipase	52.0	1.10	0.93	0.84
	35.5	1.02	1.11	0.88
	13.2	0.90	1.41	0.73

[a]Different amounts of lipases were incubated with a racemic mixture of 1,2-di[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol and 1-tetradecyl-2,3-di[^{14}C]oleoyl-*sn*-glycerol as described under Experimental. The ^{14}C ^{3}H ratio of the substrate was set at unity. Data are means from 2 incubations.

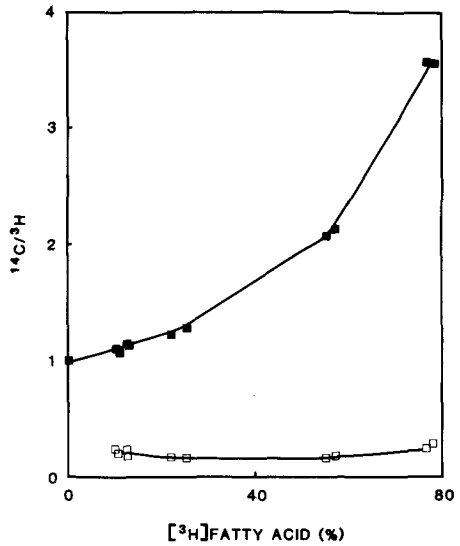

FIG. 2. Lipolysis of *rac*-1-alkyl-2-acylglycerol by lipase from *P. fluorescens*. Equal amounts (50 μg) of 2-[^{3}H] oleoyl-3-tetradecyl-*sn*-glycerol and 1-tetradecyl-2-[^{14}C] oleoyl-*sn*-glycerol were incubated with different amounts of lipase for 30 min. Symbols: $^{14}C/^{3}H$ ratio in alkylmonoacylglycerol ■; and fatty acid □. The $^{14}C/^{3}H$ ratio in the original substrate was set at unity.

The $^{14}C/^{3}H$ ratio of the liberated fatty acid was below 0.3 at varying degrees of hydrolysis. This supports the stereospecificity described in Figure 1. Similar stereospecificities were observed for *R. arrhizus* lipase and pancreatic lipase, whereas the specificity for bile salt-stimulated lipase was less marked (Table 2). This pattern is somewhat surprising, since the data in Table 1 did not indicate any marked stereospecificity for these lipases.

The substrates contained less than 5% of X-1-alkyl-3-acyl isomers, and control incubations without enzyme showed that acyl migration was negligible under the incubation conditions used. Therefore, it can also be concluded that the lipases used can attack the ester bond at the secondary hydroxyl group directly, and that previous acyl migration is not necessary.

Lipolysis of X-1-Alkyl-3-acylglycerol

The experiments were performed to elucidate the stereospecific degradation of the possible acyl migration products from the substrate mentioned above. The degradation of X-1-alkyl-3-acylglycerol should also be compared to that of alkyldiacylglycerol, since the former compound deviates from the latter only in lacking the 2-acyl group.

The ^{3}H-labeled isomer was degraded more rapidly by pancreatic lipase (Fig. 3). A similar but less marked specificity was found for lipase from *P. fluorescens*, and for *R. arrhizus* lipase (Table 3). No stereospecificity was detected for bile salt-stimulated lipase. All the specificities observed were evident at different degrees of hydrolysis. In control experiments, where enantiomeric alkylacylglycerols were hydrolyzed by KOH in methanol/water, the $^{14}C/^{3}H$ ratio in free fatty acids was 0.93-1.04, which substantiates the expectations for a nonstereospecific hydrolysis.

DISCUSSION

Triacylglycerols in food and in the body consist of a large number of isomers with different stereostructure and fatty acid composition. Preferential lipolysis of a triacylglycerol molecule may be due to a stereospecificity or fatty acid specificity of the lipase. Using triacylglycerol as substrates, it is

TABLE 2

Hydrolysis of *X*-1-Alkyl-2-acylglycerol Isomers by Different Lipases[a]

Lipase	^{3}H-Labeled substrate remaining (%)	$^{14}C/^{3}H$	
		Alkylmonoacylglycerol	Fatty acid
Pancreatic lipase	84-94	1.06 - 1.22	0.23 - 0.44
Bile salt-stimulated lipase	55-84	1.06 - 1.12	0.61 - 0.89
R. arrhizus lipase	69-89	1.06 - 1.45	0.17 - 0.38
P. fluorescens lipase	23-88	1.11 - 3.58	0.17 - 0.29

[a]Lipases were incubated with a racemic mixture of 2-[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol and 1-tetradecyl-2[^{14}C]oleoyl-*sn*-glycerol, as described under Experimental. The amount of lipases were adjusted to give varying degrees of lipolysis. The $^{14}C/^{3}H$ ratio of the substrate was set at unity. The data for each lipase represent ranges from 6-10 observations.

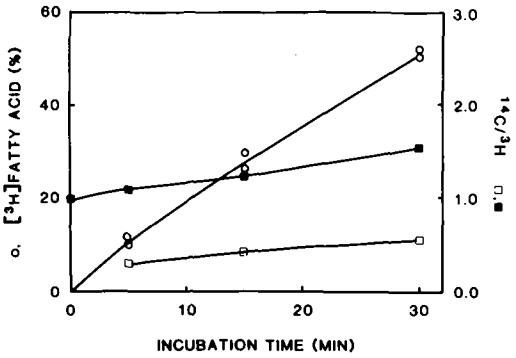

FIG. 3. Lipolysis of *rac*-1-alkyl-3-acyl-glycerol by pancreatic lipase. Equal amounts (50 μg) of 1-[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol and 1-tetradecyl-3-[^{14}C]oleoyl-*sn*-glycerol were incubated for different time periods with pancreatic lipase and colipase. Symbols: o ^{3}H in fatty acid; $^{14}C/^3H$ ratio in alkylmonoacylglycerol ■, and fatty acid □. The $^{14}C/^3H$ ratio in the original substrate was set at unity.

difficult to discriminate between these types of specificity. In the alternate approach (4,5) used in this paper, enantiomeric alkyldiacylglycerols or alkylmonoacylglycerols are used. The acyl moiety was the same in both enantiomers, thereby excluding effects due to fatty acid specificity and the physical form of the substrate.

The present study confirms previous observations that pancreatic lipase has no stereospecificity vs any of the primary positions of triacylglycerol or alkyldiacylglycerol (2,4,9). This also holds for bile salt-stimulated lipase and lipase from *R. arrhizus* and *P. fluorescens* (Table 4), which has not been studied previously.

Instead, another type of stereospecificity was discovered, namely that lipases which degrade alkyldiacylglycerol without stereospecificity can attack 2-acyl-3-alkyl-*sn*-glycerol faster than 1-alkyl-2-acyl-*sn*-glycerol. An indication for such a specificity was obtained previously for pancreatic lipase but alkylmonoacylglycerols were not tested as substrates (4). In the previous study, crude pancreatin was used, but the present experiments were performed with purified enzyme. A similar specificity at the diacylglycerol level was observed for lipoprotein lipase (4,10) and the heparin-releasable hepatic lipase (5) but, in these cases, a stereospecificity was also observed towards triacylglycerols or alkyldiacylglycerols.

The mechanism behind the stereospecificity vs alkylmonoacylglycerol remains unknown. The enzymes must have attacked the ester bond of the secondary alcohol directly, since the hydrolysis of 1,3-diacylglycerol analogues, which are the possible products after acyl migration, was less stereospecific. Noda et al. found (11) that 2,3-dioleoyloxybutane and 1-hexadecyloxy-3-oleoyloxybutane were degraded slowly by pancreatic lipase, and that 1-hexyloxy-2-octanoyloxypropane resisted hydrolysis. Under our conditions, X-1-alkyl-3-acylglycerol was hydrolyzed at least 20-fold faster than X-1-alkyl-2-acylglycerol by pancreatic lipase and *R. arrhizus* lipase, ca. 10-fold faster by *P. fluorescens* lipase but less than 2-fold faster by bile salt-stimulated lipase.

This study also demonstrates that 1-acyl-3-alkyl-*sn*-glycerol is degraded faster than its enantiomer by pancreatic lipase, and lipases from *P. fluorescens* and *R. arrhizus* (Table 3). A similar specificity was noted for bovine milk lipoprotein lipase (4) but no clear stereospecificity was noted in the degradation of 1,3-diacylglycerols by pancreatic lipase (10).

It has been demonstrated that pancreatic lipase is more easily bound to a triacylglycerol surface

TABLE 3
Hydrolysis of *X*-1-Alkyl-3-alkylglycerol Isomers by Different Lipases[a]

Lipase	^{3}H-Labeled substrate remaining (%)	$^{14}C/^3H$	
		Alkylmono-acylglycerol	Fatty acid
Pancreatic lipase	38-92	1.05 - 1.78	0.22 - 0.67
Bile salt-stimulated lipase	45-86	0.87 - 1.05	0.82 - 1.10
R. arrhizus lipase	57-95	1.01 - 1.19	0.58 - 0.87
P. fluorescens lipase	75-90	1.04 - 1.10	0.52 - 0.84

[a]Different amounts of lipases were incubated with a racemic mixture of 1-[^{3}H]oleoyl-3-tetradecyl-*sn*-glycerol and 1-tetradecyl-3[^{14}C]oleoyl-*sn*-glycerol. The $^{14}C/^3H$ ratio of the substrate was set at unity. The data for each lipase represent ranges from 9-18 observations.

TABLE 4
Stereospecificity of Different Lipases vs Alkylacylglycerols

Lipase	Preferred position in X-1-alkyl, 2,3-diacylglycerol	Preferred isomer among 1-alkyl,2-acyl-*sn*-glycerol (1A2E) and 2-acyl, 3-alkyl-*sn*-glycerol (2E3A)	Reference
Lipoprotein lipase (milk, post-heparin plasma, adipose tissue)	1	2E3A	4
Heparin-releasable hepatic lipase	1	2E3A	5
Hepatic lysosomal lipase	Non-spec	—	5
Pancreatic lipase	Non-spec	2E3A	2, this study
Lingual lipase	3	—	4
Bile salt-stimulated milk lipase	Non-spec	2E3A	This study
R. arrhizus lipase	Non-spec	2E3A	This study
P. fluorescens lipase	Non-spec	2E3A	This study

than to a trialkylglycerol surface (12), indicating the importance of the ester carbonyl groups in the substrate in this process. The carbonyl group at the secondary carbon of glycerol is probably important for the interaction of substrate with lipoprotein lipase, since no stereospecificity for this enzyme was observed when it was incubated with *rac*-1,2-dialkyl-3-acyl-glycerol (13). Another example of the role of the substituent at the secondary carbon is the fact that *R. arrhizus* lipase does not hydrolyze *sn*-2-phosphatidylcholine but is active towards the dimethylester of 1,3-diacylglycerol-2-phosphoric acid (14). In spite of this, it is clear that a 2-acyl group in the substrate is not always necessary for stereospecificity, since X-1-alkyl-3-acylglycerols can be degraded with specificity by some lipases.

The general conclusion from this study is that different lipases degrade alkyldiacylglycerols and alkylmonoacylglycerols with varying degrees of stereospecificity. Whether the stereospecificities observed are due to the interactions of the substrate with the active center of the enzyme remain to be studied.

The stereospecificity of lipases may have metabolic implications. The formation of 2,3-diacyl-*sn*-glycerol by lipases (3-5,15) may hinder the formation of phospholipids from lipolysis products, since only 1,2-diacyl-*sn*-glycerol is utilized in phospholipid synthesis. Alkyldiacylglycerol found in nature is the 1-alkyl-2,3-diacyl isomer, which is hydrolyzed slower than its enantiomer by lipoprotein lipase and hepatic lipase. The metabolic significance of this remains unclear. Also, analogues of the natural glycerides may be degraded and/or synthesized in the animal body and, using newly developed techniques, stereospecific handling of enantiomeric glycerides has been demonstrated also in the intact organism (16,17).

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Bromine Levels in Tissue Lipids of Rats Fed Brominated Fatty Acids

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ABSTRACT

Rats have been fed diets containing either 0.8% brominated corn oil or 0.5% of the ethyl ester of 9,10-dibromostearate or 9,10,12,13-tetrabromostearate. The brominated compounds were incorporated into semisynthetic diets and animals were observed after feeding periods of 5 days to 3 months. With the exception of adipose tissues, the highest concentration of lipid-bound bromine was observed after 5 days on the experimental diets. It was also observed that feeding of the dibromostearate resulted in the highest levels of lipid-bound bromine in heart, whereas the tetrabromostearate tended to result in higher levels of lipid-bound bromine in liver. Brominated corn oil produced more substantial changes than the brominated fatty acid esters, inducing fatty livers and enlarged yellow hearts. Microscopic examination indicated myocardial degeneration and occasional early necrosis.
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INTRODUCTION

Brominated vegetable oils have been used for many years as food additives and more recently as flame retardants (1); however, studies of the toxicological properties of these compounds have been limited. Depressed growth and enlarged heart and liver were observed in young male rats fed diets containing 2.5% brominated cottonseed oil (2,3). Lipid accumulated in liver and microscopic examination of the heart showed fatty degeneration involving the entire myocardium. These observations were essentially confirmed by Gaunt et al. (4) who fed rats diets containing 0.8% brominated maize oil. Some kidney and thyroid involvement was also noted in the latter study.

Lipid-bound bromine was detectable in most tissues of rats fed brominated oil (4-6). A maximum concentration in adipose tissue was attained after a 13-week feeding period. Restricting food intake increased the bromine concentration in adipose tissue dramatically, indicating that the brominated acids and/or metabolites were being mobilized at a rate slower than conventional fatty acids.

The major brominated components of a brominated oil would be the di- and tetrabromo stearates derived from addition of the bromine across the double bonds of oleic and linoleic acids. Some dibromopalmitate and hexabromostearate may also be present, depending on the levels of palmitoleic and linolenic acids in the original oil. Some sterols, for example, may also be brominated. At this point the contribution of these components to the toxicological effect of a brominated oil has not been defined.

This study was designed to compare the uptake and distribution of bromine from brominated oil and brominated fatty acids. In addition, some gross toxicological effects were monitored.

METHODS

Preparation of Brominated Oil

Oleic acid, for the synthesis of 9,10-dibromostearic acid, was isolated from olive oil by saponification and formation of urea adducts of the resulting free fatty acids (7,8). The purified oleic acid was found, by gas liquid chromatography (GLC) of methyl esters, to contain less than 2% linoleic acid and no linolenic acid.

Linoleic acid, for the synthesis of 9,10,12,13-tetrabromostearic acid, was isolated from safflower oil using similar procedures and contained less than 1% oleic acid and only a trace of linolenic acid. Corn oil (60% linoleic acid, 25% oleic acid and 1% linolenic acid) was used for the preparation of the brominated corn oil and as the oil in the control diet.

The oils were brominated by adding liquid bromine to a stirred solution of the oil in ether, at such a rate that the temperature could be maintained near 0C with a salt/ice bath. Under these conditions, substitution reactions were minimized (9). Stirring at 0C was continued for 1 hr after a bright orange solution was obtained (indicating a slight excess of bromine). Excess bromine was removed by successive washes with 2% aqueous solutions of sodium sulfite (acidified with HCl), sodium thiosulfate, and twice with water. The solutions were then dried over sodium sulfate and the solvent removed in a rotary evaporator. Bromine levels were determined by neutron activation and corresponded to those predicted from addition of the bromine to the double bonds.

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To minimize any possible gastrointestinal irritation that might occur by feeding free fatty acids for long periods of time and to reduce precipitation of the free acid in the intestine, the brominated free fatty acids were fed as the ethyl esters. These were prepared by refluxing in 95% ethanol with sulfuric acid added as a catalyst (10) and evaporating water and ethanol with a variable reflux condensor to drive the reaction. The mixture was refluxed until analysis showed less than 5% free fatty acid remaining (11). The solution was then neutralized with aqueous potassium carbonate, extracted with hexane, and the organic phase dried over sodium sulfate. The ethyl esters were recovered after removal of the hexane in a rotary evaporator.

Brominated corn oil was a light brown, very viscous oil and, after several days at room temperature, some crystallization was observed. Ethyl dibromostearate was a slightly cloudy oil with a greenish tinge and remained liquid on standing. Ethyl tetrabromostearate was a clear, slightly orange-brown oil which became a cream-colored solid after several days at room temperature.

Diet and Animals

Four powdered semisynthetic diets (Table 1) were prepared, identical in all respects except for the oil content. The control diet contained 10% corn oil, the brominated corn oil diet (BCO) contained 0.8% BCO and 9.2% corn oil, the ethyl dibromostearate diet (DBS) and ethyl tetrabromostearate diet (TBS) each contained 0.5% of the brominated fatty ester and 9.5% corn oil. The BCO was fed at a concentration of 0.8%, a level sufficient to produce lesions, according to Gaunt et al. (4).

TABLE 1

Composition of Experimental Diet

Ingredient	Percent by weight
Casein[a]	22.0
Cerelose[b]	63.0
Fat[c]	10.0
HMW salts[d,e]	4.0
Vitamin pre-mix[f,g]	1.0

[a]The Borden Chemical Co., Dominguez, CA.
[b]Corn Products Co., New York, NY.
[c]Corn oil—control diet.
[d]Nutritional Biochemicals Corp., Cleveland, OH.
[e]Zinc was added at a level of 6 mg/kg as zinc acetate.
[f]Provided as mg/100 g of diet, the following: thiaminehydrochloride, 0.4; riboflavin, 0.8; pyridoxine hydrochloride, 0.50; D-calcium pantothenate, 4.0; inositol, 20; menadione, 0.40; folic acid, 0.40; niacin, 4.0; choline dihydrogen citrate, 424; biotin, 0.03; B_{12}, 0.02.
[g]Vitamins A, D and E were added in ethanol solution to provide per 100 g of diet .875 IU of vitamin A, 125 units of vitamin D and 3.64 mg of D-α-tocopherol acetate.

Given the uncertainties of composition and extent of bromination, 65-70% of the mass of the brominated corn oil could be attributed to the tetrabromostearate. Thus, using a level of 0.5% TBS would approximate that present in the 0.8% BCO diet and give some indication as to whether the effects of the BCO could be attributed to its major brominated constituent. Feeding a comparable level of the DBS provides a comparison with the tetrabromo counterpart; however, this expedient represents a compromise in that it would represent a decrease of 20% in the bromine level but an increase of 35% in the moles of fatty acid.

Litter mate groups of weanling rats (50-70 g) from a random bred Wistar colony were placed on each of the 4 diets to give a total of 3 males and 3 females for each diet at each time period. Diets were fed ad libitum, for periods of 5 and 10 days (short-term animals) and for 1, 3 and 6 months (long-term animals).

The 5- and 10-day groups were removed from their lab chow diets and placed on the powdered synthetic control diet 24-48 hr prior to beginning the experimental diet to adapt to the new food form and to give more consistent intake of brominated oil intakes during these short feeding periods. Animals were housed in individual hanging wire cages, with food intakes routinely monitored, and body weights recorded weekly. Animals were sacrificed at the end of their feeding period by asphyxiation with carbon dioxide. Heart, liver, kidneys, adipose tissue (mesenteric), skeletal muscle, testes and brain were removed, weighed and frozen for subsequent analysis. To obtain some preliminary histological data, tissue samples from animals from each feeding period were fixed in formalin. Sections were stained with hematoxylin eosin for histopathological examination.

Analytical

Total lipids were extracted from tissue samples with chloroform/methanol (2:1, v/v) (12), and lipid content determined by evaporating a 100 μl aliquot of the extract in a tared aluminum pan, using a Perkin-Elmer microbalance for weighing. Methyl esters were analyzed by gas chromatography using a 200 ft stainless steel capillary column coated with ethylene glycol succinate.

Lipid extracts were analyzed for lipid-bound bromine by instrumental neutron activation analysis (NAA) at OSU's TRIGA reactor facilities. Lipid (5-50 mg) was weighed (to 0.01 mg) into thin aluminum foil pans and placed in polyethylene activation vials which were then heat sealed. Standards of pure dibromostearic acid were prepared in the same manner. Samples were activated for 1 hr at one megawatt in the rotating rack assembly. Activated lipids were then stored overnight in the

reactor to allow short half-life isotopes to decay, especially ^{24}Na and ^{27}Al, to minimize background radiation. Samples and standards were counted 1-7 days after activation for ^{82}Br ($t_{1/2} = 35.3$ hr) by accumulation of the 777 keV peak using a 30 cc Ge(Li) detector and a 2048 channel analyzer. Geometry of sample and standard were comparable and analyzer dead time was kept below 10% by allowing the hotter samples to decay. Sufficient counts (i.e., 10,000+) were accumulated above background radiation to ensure a less than 1% counting error. The largest error in the entire NAA procedure was from sample weighing and ranged from ± 1 to 25 ppm, depending upon sample activity.

Data were analyzed by standard analysis of variance techniques using the natural logarithm of the response variables (13). Differences between treatment and control means averaged over time were tested using Dunnett's procedure (14). When treatment and control means were compared at a given time interval, a protected least significant difference procedure was used with only probability levels less than .01 considered significant.

RESULTS

Growth and Food Intake

The brominated oils, especially the BCO, initially affected the overall response of the animals. Animals were lethargic and food consumptions were lower than controls. However, in 7-10 days, the animals adapted to the diets and thereafter no significant differences in growth or food efficiencies were observed. With the exception of the 5- and 10-day groups, food intakes were comparable among diet groups and differences in total intake of brominated oil thus reflected differences in levels of brominated compounds in the diet (Table 2). As will be discussed later, the time sequence of symptoms noted above corresponds to changes in bromine levels at the organ level.

Necropsy

Fatty livers were observed in all the animals fed BCO, in a few of the animals fed the brominated esters, and in one control (3 month, female). Hearts from many of the animals fed the BCO were enlarged, yellow colored, and firm to hard in texture; when cut, the edges of the cut bulged, indicating edema. The incidence of this gross lesion was 53% in females and 33% in males. The condition was most evident in the 5-day groups of males and the 3- and 6-month groups of females (Table 3).

Histopathology

Microscopic examination revealed myocardial degeneration and occasionally early necrosis in the long-term animals fed BCO. Evidence of early heart lesions was seen in the male rats fed DBS for 5 days, but the changes were mild and not conclusive. Liver hyperplasia, cardiac edema, and testicular degeneration and necrosis were seen in the animals fed TBS for 6 months. However, no pronounced differences among the dietary groups were revealed by histological examination.

TABLE 2

Total Grams of Brominated Oils Consumed

Feeding period	BCO[a]	DBS[b]	TBS[b]
Females			
5 days	0.36 ± 0.03	0.37 ± 0.14	0.23 ± 0.01
10 days	0.78 ± 0.06	0.49 ± 0.12	0.57 ± 0.03
1 month	2.90 ± 0.70	2.11 ± 0.49	2.06 ± 0.09
3 months	9.56 ± 0.51	6.31 ± 1.11	7.00 ± 0.16
6 months	21.57 ± 4.01	15.16 ± 0.14	14.39 ± 0.12
Males			
5 days	0.36 ± 0.06	0.21 ± 0.01	0.23 ± 0.03
10 days	0.97 ± 0.05	0.60 ± 0.05	0.57 ± 0.03
1 month	3.62 ± 0.30	2.16 ± 0.16	2.85 ± 0.47
3 months	10.57 ± 0.96	7.10 ± 0.64	7.57 ± 0.51
6 months	26.79 ± 2.86	17.12 ± 3.31	17.25 ± 1.21

[a]0.8% of diet as brominated corn oil (BCO).
[b]0.5% of diet as the ethyl ester of either dibromo- (DBS) or tetrabromostearate (TBS).

TABLE 3

Incidence of "Discolored" Hard Hearts in Animals Fed Brominated Corn Oil

	Female	Male
5 day	0/3	3/3
10 day	1/3	0/3
1 month	1/3	0/3
3 month	4/5	1/3
6 month	3/3	1/3
Total	9/17	5/15

Organ Weight and Lipid Accumulation

Some differences in the toxic effects of the brominated components were indicated by effects on liver size and lipid content (Table 4). Feeding female rats BCO produced liver enlargement (Table 5). Increased liver size was also indicated in female rats fed DBS or TBS for the 5- or 10-day interval; however, the difference was not significant for TBS. All male rats fed BCO had enlarged livers; however, the effect was only slight with the DBS and seen only in the 5- and 10-day groups fed the TBS. The BCO-induced liver enlargement was

TABLE 4

Effect of Brominated Corn Oil (BCO), Dibromostearate (DBS) and Tetrabromostearate (TBS) on Liver Size, and Heart and Liver Lipid Content– Analysis of Variance Data

Source of variation	Liver size		Liver lipid		Heart lipid	
	M	F	M	F	M	F
Time	$p<<.001$	$p< .005$	$p<.05$	$p<.01$	$p<.005$	ns
Diet	$p<<.001$	$p<<.001$	$p\simeq.001$	$p\simeq.025$	$p<.001$	$p<.001$
Time and diet	$p\simeq .01$	$p\simeq .025$	ns	$p\simeq.025$	ns	$p<.05$
Treatment vs control means						
BCO	$p< .01$	$p< .01$	$p<.01$	ns	$p<.01$	$p<.01$
DBS	$p< .01$ (day 5)	$p< .01$ (days 5 & 10)	ns	ns	ns	ns
TBS	$p< .01$ (day 5)	ns	ns	$p<.01$ (day 90)	ns	ns

TABLE 5

Liver Enlargement in Rats Fed Brominated Oils: Grams Tissue · 100 g of Body Weight

Diet	5 days	10 days	1 month	3 months	6 months
Females					
CO[a]	5.40 ± 0.24	5.35 ± 0.20	5.14 ± 0.44	4.41 ± 0.26	4.21 ± 0.45
BCO	6.89 ± 0.59	7.43 ± 0.30	7.11 ± 1.11	6.09 ± 0.30	5.47 ± 0.16
DBS	6.47 ± 0.57	6.51 ± 0.37	5.88 ± 0.60	4.60 ± 0.51	4.73 ± 0.24
TBS	6.32 ± 0.44	6.15 ± 0.26	5.41 ± 0.86	4.86 ± 0.22	4.07 ± 0.31
Males					
CO	5.23 ± 0.51	5.70 ± 0.22	5.45 ± 0.45	3.81 ± 0.26	3.62 ± 0.22
BCO	7.51 ± 0.06	7.38 ± 0.26	6.98 ± 0.72	4.67 ± 0.05	4.20 ± 0.38
DBS	6.25 ± 0.25	6.30 ± 0.12	5.86 ± 0.73	3.78 ± 0.29	3.89 ± 0.25
TBS	6.38 ± 0.19	6.33 ± 0.26	5.59 ± 0.70	3.96 ± 0.10	3.63 ± 0.07

[a]Corn oil, CO; brominated corn oil, BCO; dibromostearate, DBS; and tetrabromostearate, TBS.

greater in males and decreased significantly with longer feeding periods. In females, liver enlargement did not decrease steadily with time in animals fed the BCO but did with the brominated esters. No increase in liver size was noted in animals of either sex fed the TBS for 6 months. Only the BCO produced any consistent increase in liver lipid content (Table 6).

Cardiac enlargement (10%) was seen only in the animals fed BCO. A slight effect was observed in animals fed DBS for 10 days. Cardiac lipids were elevated relative to the control in all animals fed BCO and this differential was greatest in the 5-day groups, decreasing thereafter (Table 6).

Animals developing gross heart lesions when fed the BCO all showed enlarged hearts and increased cardiac lipid content when compared to their litter mate controls.

Lipid-Bound Bromine

Feeding brominated corn oil or brominated fatty acids produced elevated bromine levels in lipids of all tissues examined (Fig. 1)—bromine levels in lipids of tissues from control animals ranged from 10 to 20 ppm. The highest concentrations in heart and liver were found in the 5-day groups, with the exception of the heart lipids from males fed TBS for 3 months (Fig. 2). A similar response was observed in other tissues analyzed, with the exception of adipose tissue which showed higher bromine concentrations at three months. Animals fed BCO had higher lipid bromine concentrations than those fed either of the brominated esters, except for the heart lipids of the long-term animals fed DBS.

A maximum bromine concentration in lipid from heart and liver was observed at 5 days. After decreasing, bromine concentrations then increased again to a maximum at 1-3 months (Fig. 2). These curves illustrate differences in response of heart and liver tissues to the di- and tetrabromostearates. With liver lipids, animals fed TBS have higher lipid bromine concentrations than the animals fed the

TABLE 6

Lipid Accumulation in the Hearts and Livers of Rats Fed Brominated Oils
Milligrams of Lipid per Gram of Tissue

Diet	5 days	10 days	1 month	3 months	6 months
Heart tissue					
Females					
CO[a]	22.7±1.3	26.8±4.0	28.5± 4.6	28.3±4.3	29.1±1.2
BCO	32.2±5.8	33.3±2.4	32.2± 3.8	38.9±0.8	34.6±3.8
DBS	24.3±4.0	30.0±2.8	35.0±15.9	30.4±5.2	34.7±3.9
TBS	23.8±0.9	29.2±6.2	23.8± 2.6	26.2±6.7	34.6±5.6
Males					
CO	28.2±3.4	26.5±2.6	23.3±1.6	24.2±1.6	26.5±3.1
BCO	40.8±7.1	30.4±1.2	27.6±6.6	35.6±0.8	32.3±1.6
DBS	28.6±3.9	29.2±3.8	27.1±4.0	25.4±1.5	28.1±4.7
TBS	28.7±4.2	26.5±2.1	23.6±5.1	27.1±3.0	27.4±1.9
Liver tissue					
Females					
CO	37.0±1.3	41.2± 2.8	43.2±2.4	46.8±8.0	37.0±1.0
BCO	42.5±1.8	43.7±10.9	49.0±4.8	38.5±5.1	45.3±6.4
DBS	34.4±1.8	37.8± 0.9	40.1±7.6	41.8±6.5	37.4±1.2
TBS	37.1±3.2	37.8± 4.2	45.1±3.4	35.8±2.0	41.4±4.7
Males					
CO	38.0±1.9	34.3±4.7	40.3±6.1	41.1±3.2	39.9±1.6
BCO	47.3±9.0	38.6±1.5	47.8±4.6	46.6±4.6	51.4±9.4
DBS	39.4±2.4	35.1±1.5	32.5±3.7	45.6±16.7	41.9±4.8
TBS	40.5±5.7	35.7±2.9	35.5±2.7	39.6±2.9	44.2±6.3

[a]Corn oil, CO; brominated corn oil, BCO; dibromostearate, DBS; and tetrabromostearate, TBS.

DBS; in cardiac lipids, the reverse is true. In the males fed DBS for one month or longer, the bromine concentrations in heart lipids are even greater than those found in males fed BCO for comparable periods.

DISCUSSION

Subsequent experiments in our laboratory (15) have demonstrated that most of the bromine in the tissue lipids of rats fed diets containing brominated oils can be attributed to the presence of brominated fatty acids and their shorter chain metabolites. With the intake of brominated compounds relatively constant, the observation of a sharp maximum in tissue lipid bromine concentration after 5 days' exposure suggests an adaptive response.

The rapid decline in lipid bromine concentration in liver and heart, presumably due to the metabolism of the brominated fatty acids, compares with a similar decrease in erucic acid in rats fed clofibrate which induces the proliferation of peroxisomes with their β-oxidative system (16,17). High fat diets resulting in an elevated influx of fatty acids or the accumulation of fatty acids not metabolized by the mitochondrial pathway induce an increase in the capacity of the peroxisomal system (18,19). Atypical long-chain fatty acids as well as *trans* isomers of unsaturated acids are oxidized at significant rates in the peroxisomal pathway, and consequently one might postulate that brominated acids may also be metabolized by this route. Further investigations would be needed to confirm this suggestion. It has been reported that dibromopalmitate is not oxidized by the β-oxidative system of mitochondria (20).

There is a marked difference between animals fed DBS and TBS in the distribution of bromine in tissue lipids. With DBS, bromine concentrations are, for the most part, lower in liver lipids and higher in other tissues (particularly heart) analyzed. Just the reverse is true with TBS, with highest levels of bromine being found in liver lipid. It would appear that tetrabromostearate is not incorporated into lipoproteins to the same extent as the dibromostearate.

Rats fed brominated corn oil developed enlarged, hard, yellow hearts and enlarged livers. Crude lipid content of heart and liver were also elevated. (Whether the increase in tissue lipid involves increased levels of triglyceride or a simple increase in lipid mass from the substitution of brominated fatty acids for nonbrominated acids has not been resolved.) The myocardial degeneration and focal necroses seen in previous experiments with brominated oils (4) was not observed in this study. The

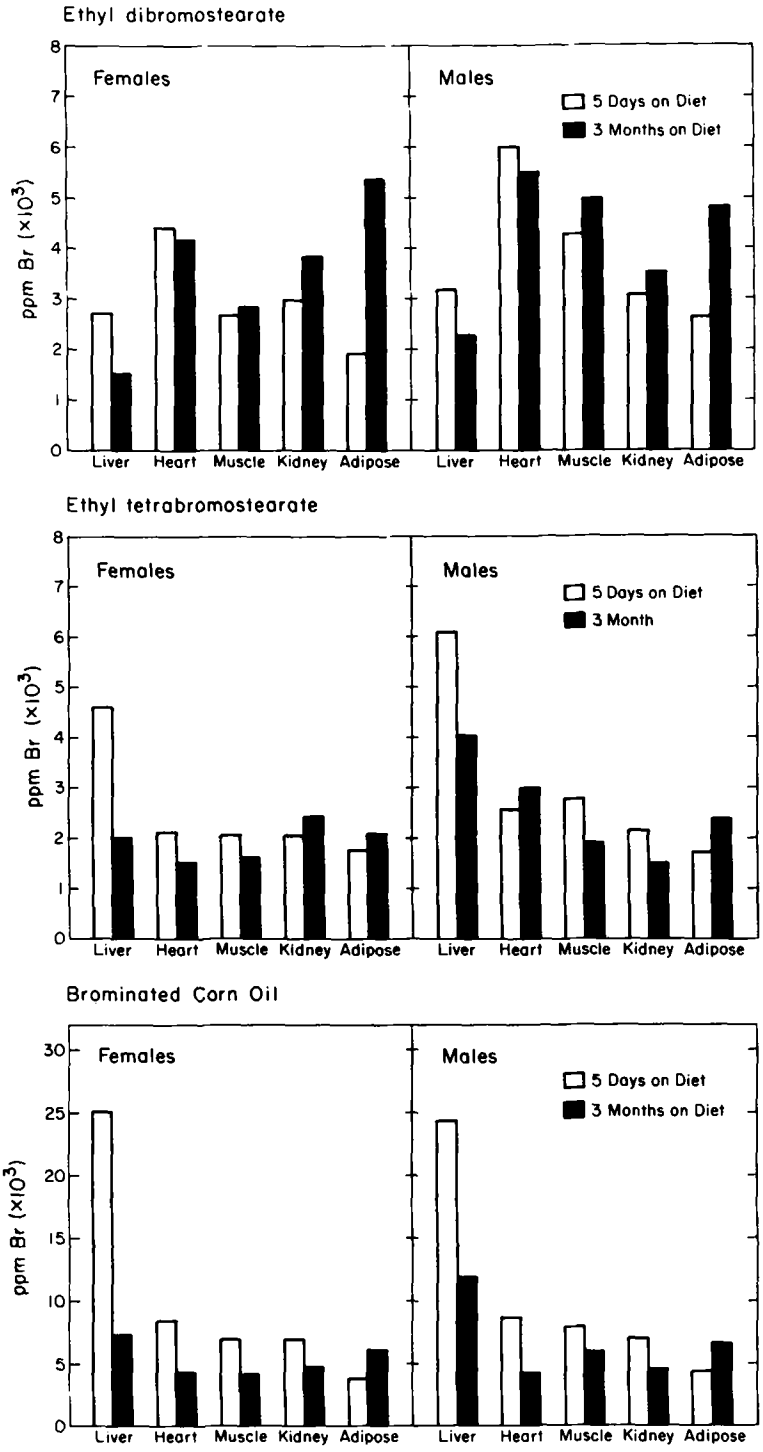

FIG. 1. Tissue levels of lipid bromine in rats fed ethyl dibromostearate, ethyl tetrabromostearate, and brominated corn oil.

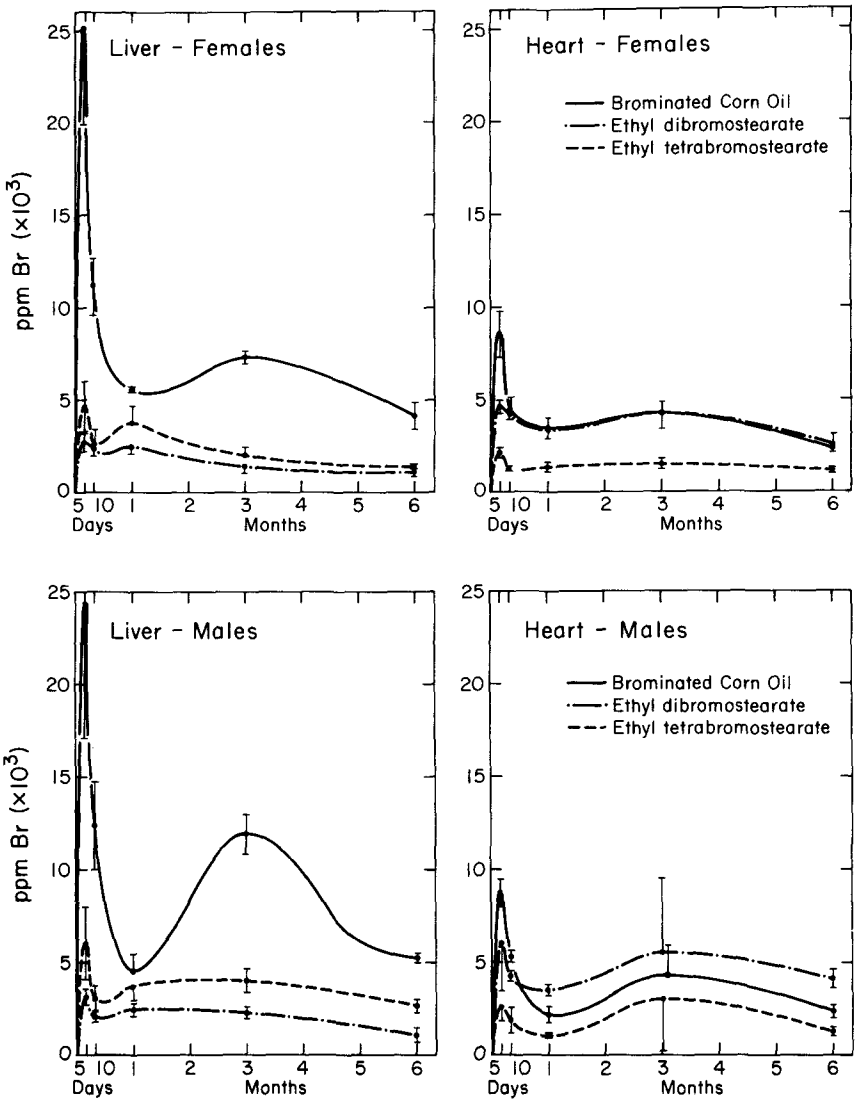

FIG. 2. Lipid bromine levels in heart and liver as a function of type of brominated compound and time of feeding.

reason for this difference is not apparent. It could involve differences in response with different rat strains or differences in composition of the brominated oils, some studies being carried out with a commercial product (6).

It is also of interest that feeding equivalent levels of the major constituent of the brominated corn oil, the tetrabromostearate did not produce a response comparable to that observed in animals fed the brominated corn oil. This may be due to better absorption with the brominated corn oil or to the effect of other brominated constituents.

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Toxicology of Brominated Fatty Acids: Metabolite Concentration and Heart and Liver Changes

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ABSTRACT

Rats were fed for 35 days diets containing 2% of either brominated corn oil (BCO), monoglyceride of dibromostearate (DBS), monoglyceride of tetrabromostearate (TBS) or a mixture of the two monoglycerides (BMG) which provided proportions of brominated acids comparable to that of the BCO. Hearts from all animals fed BCO were yellow colored and firm in texture. Myocardial cellular degeneration, mild to moderate edema and occasional small necrotic foci were observed. Hearts from animals fed DBS showed moderate edema and some slight necrosis. All diets produced an increase in lipid content of heart. Animals fed the experimental diets developed enlarged livers and showed elevated liver lipid content. The tetrabromostearate appeared to be the more active in producing these changes, in particular a severe intracellular fatty degeneration. Shorter-chain (C-16, C-14) metabolites of di- and tetrabromostearate were identified and the concentration of brominated fatty acids in heart, liver and adipose tissue determined and found to account for 80% of the bromine detected in these tissues by neutron activation analysis. TBS accumulated in liver while the highest concentration of DBS was observed in heart lipids. Although the concentrations of brominated acids in heart and liver lipids were comparable in rats fed BCO or BMG, BCO produced the more pronounced effects. This differential could be due to additional active components in BCO or to a variation in response associated with changes in the location of the fatty acid on the glycerol molecule.

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INTRODUCTION

In a previous study, the feeding of ethyl di- and tetrabromostearates to weanling rats failed to produce a toxic response equivalent to that seen when brominated corn oil (BCO) was fed at a similar dietary level (1). However, it was observed that bromine from dibromostearate was accumulating in cardiac lipids, and bromine from tetrabromostearate was accumulating in hepatic lipids. Maximum concentrations of bromine in tissue lipids were observed after only 5 days on experiment. These observations indicated that mobilization and/or metabolism of brominated fatty acids was occurring and differences existed in these processes for the di- and tetrabromostearates.

The differences in toxic response between BCO and the brominated fatty acid ethyl esters was attributed largely to absorption, with the ethyl esters resulting in lower doses being received than originally anticipated. To remedy this situation and assess better the toxicological effects of the brominated fatty acid, the monoglycerides were synthesized.

Our objectives in this study were: (a) to characterize further the effects of the dibromostearate (DBS) and the tetrabromostearate (TBS); (b) to duplicate the toxic response of the animal to the BCO with a mixture of the brominated monoglycerides (similar in DBS and TBS levels to the BCO); and (c) to isolate and identify any brominated metabolites that might be accumulating in the tissue lipids.

METHODS

Preparation of Brominated Oils

Brominated corn oil was prepared as previously described (1) by the direct addition of liquid bromine to the dissolved oil which contained 60.9% linoleate and 24.7% oleate. Monoglycerides of di- and tetrabromostearic acids were prepared starting with the isolation of oleic (Δ9-octadecenoic) and linoleic (Δ9,12 octadecadienoic) acids from olive and safflower oils, respectively. Following saponification, the soaps were extracted successively with 50% (1×) and 30% (3×) ether in hexane to remove unsaponifiable matter. The efficiency of this step was confirmed by the appearance of a single spot for the acids and a trace residue of other material with thin layer chromatography on silica gel. Urea adduction was carried out on the free acids using a modification of the method of Swern and Parker (2) and the efficiency of the process monitored by gas chromatography of the methyl esters.

The monoglycerides were prepared by heating a 1:1 mixture of fatty acid and glycerol under

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vacuum with constant stirring to a temperature which maintained the production of water but avoided excessive bumping of the oil (i.e., 150-160 C for oleic acid and 175-200 C for linoleic acid). When the calculated amount of water for the given sample of monoglyceride was removed, the monoglyceride was sampled and analyzed for free fatty acid (3). Once the level of free fatty acid was found to be 1% or less, the esterification reaction was discontinued.

Thin layer chromatography (TLC) of the monoglycerides confirmed that the majority of the oil was monoglyceride with traces of di- and triglyceride. Gas liquid chromatography (GLC) analysis of the methyl esters of the monoglycerides showed the glycerol monooleate to be 81% oleate, less than 1% linoleate and the remaining 18% saturates. The glycerol monolinoleate was found to be 98% linoleate and 1.4% oleate. The monoglycerides were then brominated as described (1).

The bromine content of the BCO, DBS and TBS was determined by neutron activation analysis to be 47.5%, 32.4% and 53.9%, respectively. These data compare reasonably well with predicted values of 45%, 27% and 48%, considering the error involved in both the neutron activation analysis (± 5% considering both counting and sample handling) and fatty acid analysis.

Diets and Animal

The brominated oils were dissolved in corn oil and incorporated into powdered semisynthetic diets (1) at a level of 2%, replacing 2% of the 10% corn oil normally added. The control diet contained 10% corn oil. Animals were also raised on rations containing 3% corn oil or 3% glycerol monooleate or glycerol monolinoleate to provide a low-fat reference and evaluate the effect of feeding a monoglyceride.

In addition to the experimental diets containing the BCO, DBS and TBS, a mixture of the DBS and TBS (labeled the brominated monoglyceride mixture:BMG), representative of the BCO, was added to the experiment. Computed as the free acid, the brominated monoglyceride mixture and the brominated corn oil provided per kg of diet 4.1 g and 4.9 g of the dibromostearate and 13.6 g and 10.7 g of the tetrabromostearate, respectively.

Five male Wistar rats (~100 g), obtained from Charles-River of California, were placed on each diet. Animals were housed individually in hanging wire cages and fed ad libitum for 35 days. Both the dietary level and the feeding period were chosen to produce significant toxicological changes in cardiac and hepatic tissues (4-6). Food intakes were monitored and body weights recorded weekly.

At the end of the feeding period, the animals were sacrificed by asphyxiation with CO_2; heart, liver and kidneys were quickly removed, weighed and samples taken for frozen and formalin fixed sections. The remaining heart, liver and kidney tissues and samples of adipose tissue, skeletal muscle and testes were frozen for subsequent lipid extraction, lipid bromine analysis and fatty acid analysis.

Histopathology

Samples of liver, heart and kidney were fixed in formalin and sections stained with hematoxylin-eosin to evaluate degenerative and/or necrotic changes. Other sections were stained with osmium tetroxide, a fat fixative, to visualize fatty infiltration and degeneration. The latter procedure was only partially effective due to inadequate penetration into the tissue block. Fatty infiltration was confirmed using an Oil-Red-O stain with frozen sections.

Analytical

Lipids were extracted by the method of Bligh and Dyer (7). Aliquots of the crude lipid extract which had been made to volume were then weighed to determine crude lipid content, prepared for neutron activation analysis as previously described (1) or methylated with methanolic HCl for GLC analysis of normal and brominated fatty acids.

The methylated portion was analyzed for fatty acids using a 200 ft, 0.03 in. id stainless steel capillary column coated with EGS at 160 C with helium as the carrier gas. A flame ionization detector was used. Under these conditions, the monobrominated but not the polybrominated fatty acids are detected.

A 2-3 mg portion of the methyl esters in hexane was applied to a 500 micron alumina GF TLC plate (Analtech) which had previously been activated for 1 hr at 150 C. Plates were developed with hexane in the continuous mode, with a narrow opening at the top of the tank (R.R. Lowry and I.J. Tinsley, personal communication). Additional hexane was added as needed during the 2-hr development. These conditions gave 4 bands with an increasing degree of resolution from the bottom to the top of the plate. Band no. 1 (near solvent front) contained the C-16 through C-22 saturated fatty acids; band no. 2 contained the monoenes palmitoleic and oleic acids; band no. 3 contained C-16 and C-18 dienes; and band no. 4 (just above origin), linolenic acid, arachidonic acid, and C-22 unsaturated acids, as well as the di- and tetrabrominated fatty acids. On occasion, plates with better separation have been obtained where the fourth band separates, yielding a band containing the dibrominated fatty acids and linolenic acid, and a fifth band (near the origin) which contains the tetrabrominated fatty acids and

arachidonic acid. Experiments with standard mixtures indicate that monobromo acids would chromatograph with the dibromo acids if present. The 22 carbon unsaturated acids distribute between the bands according to their degree of unsaturation. Since debromination yielded fatty acids with only one or two double bonds, the presence of the more unsaturated fatty acids did not present a problem in subsequent analysis.

The bands were detected with iodine vapor, and the fraction containing the brominated acids scraped from the plates and extracted 3 times with diethyl ether. The extracts were combined and concentrated, and a few micrograms removed for gas liquid chromatography to determine any carryover of mono- and diunsaturates, since the procedure is very sensitive to loading and atmospheric conditions.

The brominated esters were debrominated (8) by first removing all solvent, adding ~ 100 mg of zinc powder, 3 ml of anhydrous methanol, and one drop of 5% HCl (g) dissolved in anhydrous methanol, and heating at 80 C for 2 hr. The debrominated esters were recovered by adding 3 ml each of hexane, diethyl ether and water, shaking, centrifuging briefly and then removing the organic layer. The aqueous layer was reextracted with diethyl ether in hexane (50/50, v:v), and the organic layers combined and concentrated. The resulting mixture of unsaturated methyl esters was analyzed by GLC and the amounts of BFA determined by comparison of peak areas of these unsaturated fatty methyl esters to an external standard (methyl stearate). A sample of lipid from rat liver spiked with dibromostearate gave a recovery of $91.1 \pm 2.7\%$.

When appropriate, data were analyzed using a standard one-way analysis of variance of the logarithms of the response variables. Differences in treatment means were tested using the Newman-Keuls method (9).

RESULTS

Dietary Response and Brominated Oil Intakes

As expected, feeding the brominated oils at a 2% level had more effect on the animals than the 0.8% level of BCO and the 0.5% level of the brominated fatty acid ethyl esters used in the previous study (1). Animals were initially lethargic and consumed less food than the controls but, as in the previous study, in 7-10 days the animals appeared to adapt to the brominated oils, and activity and food consumption increased. However, the greater toxicity of the brominated oils fed at the 2% level was apparent through the feeding period. Intermittent diarrhea and soft feces were a problem with many of the animals fed the oils containing the tetrabromostearic acid and there was occasionally some weight loss, but this generally amounted to only a few grams and was recovered by the next weighing. Animals fed brominated oils were also less well groomed and had coarser fur than controls.

Food efficiencies (Table 1) were slightly higher in the animals fed the brominated oils. Total food and thus brominated oil intakes are similar for all diets. Intakes of the brominated oils and estimated intakes of the di- and tetrabromostearic acids are given. There was no overall effect of any of the brominated oils on growth. Animals fed the monoglycerides of either oleic or linoleic acid responded normally and data from these animals are not reported.

Necropsy

All the animals fed diets containing a brominated oil had fatty livers with those from the animals fed the oils containing TBS showing the more pronounced response. The animals fed BCO had the largest livers, which, in fact, were easily palpable before sacrifice and generally weighed twice as much as those from control animals. The livers

TABLE 1

Food Efficiencies and Brominated Oil Intakes

Diet	Food efficiency: g weight gain / 100 g food consumed	Brominated oil intake: Total[a] (g)	as DBS[b] (g)	as TBS[b] (g)
CO[c]	28 ± 2	-	-	-
BCO	32 ± 2	15.6 ± 1.5	3.2	10.6
BMG	29 ± 3	15.4 ± 1.2	3.7	8.3
DBS	31 ± 2	14.9 ± 0.9	10.9	(<0.15)
TBS	32 ± 3	16.5 ± 1.9	(0.20)	14.4

[a]As glycerol esters.
[b]As free fatty acid, dibromostearate, DBS and tetrabromostearate, TBS.
[c]Corn oil control, CO; brominated corn oil, BCO; and brominated monoglyceride mixture, BMG.

from animals fed the BMG and TBS appeared slightly less fatty than those of the animals fed BCO and were not as large. DBS produced the mildest response and it was only obvious when they were compared to control animals fed 3% corn oil, indicating that the condition might have been the result of the amount of fat in the diet rather than the DBS.

The gross heart lesion observed previously (1), characterized by a firm to hard texture, edema, and a pale yellowish coloration, was more prevalent in this study. All animals fed the BCO exhibited the lesion, while 70% of those fed the TBS, 50% fed the BMG, and only 30% fed the DBS had the same texture and appearance.

Kidney and all other tissues appeared normal and all animals had sufficient abdominal adipose deposits, indicating that the 2% dietary level was not toxic enough, under these experimental conditions, to cause wasting and severely alter the metabolism of the animal.

Organ Enlargement and Tissue Lipid Increases

The effects of the various diets on organ size and lipid concentrations are shown in Table 2. When the increases are expressed relative to the 10% corn oil control, the data indicates differences in the response of cardiac and hepatic tissues to the various brominated oils.

Heart size was highest in animals fed diets containing either DBS or BCO; however, the increase compared to the controls was not significant. No cardiac enlargement was observed with the animals fed TBS and BMG. All diets produced an increase in the lipid content of the heart—the BMG mixture producing the least effect.

Feeding BCO produced dramatic increases in both liver size and lipid concentration. TBS and BMG produced somewhat milder responses, with the DBS giving the least amount of hepatic enlargement and the mildest lipidosis.

The enlargement and lipid accumulation data support the observations made at the necropsy and illustrate two other important results of this experiment. The first point is that, as seen in the previous study (1), total organ enlargement is not always accounted for by increases in tissue lipids, especially in the case of the DBS diet. The second point is that the BMG mixture is not producing a toxic response equivalent to the BCO, indicating the possible influence of a toxicant in the BCO other than brominated fatty acids.

Histopathology

Histopathological examination of heart and liver tissues revealed intracellular fatty degeneration in all the tissues from the animals fed the brominated oils. Cellular degeneration, necrosis, and inflammation were a little more obvious in this study than in the previous one, but overall the lesions were mild, similar among diets, and not sufficiently definitive to attribute lesions to the di- or tetrabromostearic acids. No significant lesions were reported in kidney tissue with any of the experimental diets.

Cardiac tissue exhibited fatty degeneration, throughout the myocardium, in the form of very fine droplets often in linear arrays paralleling the contractile fibers. The greatest fatty degeneration was seen with the BCO and DBS, and occasionally the DBS appeared to have a greater effect than the BCO; however, staining was poor with the osmium

TABLE 2

Relative Organ Size and Lipid Content[a]

Diet	g/100 g body wt	mg lipid/g tissue
Heart		
CO[g]	$0.45 \pm 0.08^{c,d}$ (1.00)[b]	26.9 ± 1.0^{c} (1.00)
BCO	0.52 ± 0.07^{d} (1.16)	46.8 ± 6.1^{e} (1.74)
BMG	$0.45 \pm 0.04^{c,d}$ (1.00)	37.8 ± 3.8^{d} (1.41)
DBS	0.53 ± 0.04^{d} (1.17)	$42.9 \pm 2.6^{d,e}$ (1.59)
TBS	0.42 ± 0.04^{c} (0.93)	$41.1 \pm 4.3^{d,e}$ (1.53)
Liver		
CO	4.09 ± 0.24^{c} (1.00)	44.9 ± 11.6^{c} (1.00)
BCO	9.39 ± 0.75^{f} (2.30)	83.0 ± 13.5^{d} (1.85)
BMG	7.86 ± 0.47^{e} (1.92)	68.2 ± 6.0^{d} (1.52)
DBS	7.10 ± 0.41^{d} (1.74)	50.5 ± 3.8^{c} (1.26)
TBS	8.25 ± 0.24^{e} (2.01)	77.2 ± 10.6^{d} (1.72)

[a]Mean and standard deviation: n = 6.

[b]Relative to corn oil control.

[c,d,e,f]Mean values with the same superscript are not significantly different ($p < .01$).

[g]Corn oil control, CO; brominated corn oil, BCO; brominated monoglyceride mixture, BMG; monoglyceride of dibromostearate, DBS; and monoglyceride of tetrabromostearate, TBS.

tetroxide and interpretation of sections often difficult. Sections stained with Oil-Red-O had to be prepared to confirm the presence of large amounts of fat, especially in the cardiac tissue from animals fed the DBS which seemed particularly resistant to the osmium tetroxide stain. Not all sections from all animals were stained with the Oil-Red-O, so assessment of the relative influence of the diets on myocardial lipidosis must be done from the increased lipid levels rather than the histology.

The animals fed the BCO appeared to have the greatest incidence of myocardial cellular degeneration, necrosis, and inflammation. Fiber degeneration, mild to moderate edema, and occasional small foci of necrosis and macrophage infiltration were seen in 5 of the 6 animals examined. The DBS caused mild to moderate edema in all 7 animals examined, fiber degeneration and some slight necrosis in 3 animals, but no inflammatory response was observed in any of the sections. Most of the animals fed TBS exhibited mild cardiac edema, 2 showed signs of fiber degeneration, and one animal exhibited necrosis and macrophage infiltration. No edema, necrosis or inflammatory response were observed in any of the cardiac sections from animals fed the BMG diet.

In hepatic tissue, all animals fed the TBS-containing oil exhibited severe intracellular fatty degeneration, the degree of effect being BCO > TBS > BMG. Animals fed the DBS showed only mild fatty degeneration which appeared to be slightly greater than that seen in the 10% corn oil controls. The fatty degeneration was in the form of fine to very large droplets, occasionally with portal distribution. The fat content of the hepatic tissue was often severe enough, especially with the BCO, to make fixing and sectioning difficult.

Mild degeneration, an occasional necrotic cell, and some inflammation was seen in liver sections from 3 of the animals fed the BMG and DBS, and one fed the BCO. All animals but one fed the TBS had swollen cells and nuclei, indicating the beginning of degenerative changes.

Lipid-Bound Bromine

All tissue lipid from animals fed brominated oils contained high concentrations of bromine (Table 3). Noteworthy in the distribution of the lipid bromine is the fact that the tetrabromostearic acid-containing oils produced the highest concentrations in hepatic lipids and the DBS diet produced the highest concentrations in cardiac lipids. Cardiac lipid bromine concentrations in the animals fed the TBS-containing diets are one-half or less those encountered in the cardiac lipids of the animals fed the DBS. It is also noteworthy that the animals fed the BMG mixture had higher cardiac lipid bromine concentrations than those fed the BCO.

Fatty Acid Analysis

Fatty acid composition of the cardiac and hepatic lipids from the animals fed the brominated oils did not show any obvious differences from controls that could not be accounted for by the increased triglyceride levels or membrane proliferation.

Analysis of the isolated brominated fatty acid methyl esters by GLC of the debrominated unsaturates resulted in the identification of C-14 and C-16 mono- and diunsaturated fatty acids. The retention times of the latter did not correspond to those of C-14 and C-16 Δ9-unsaturated acids. Ozonolysis of the debrominated products (10,11) showed that the double bonds in the C-14 acids were in the Δ5 and Δ8 positions; in the C-16 acids, the double bonds were found to be in the Δ7 and Δ10 positions; and in the C-18 acids, the double bonds were found to be in the original Δ9 and Δ12 positions. The positions of the double bonds in the shorter-chain unsaturated acids produced by debromination confirmed that the derivatives were β-

TABLE 3

Bromine Content of Tissue Lipids
(μmol Br/g lipid)

Diet	Tissue					
	Heart	Liver	Adipose	Kidney	Muscle	Testes
Corn oil	0.26±.17[a]	0.17±.07	0.05±.01	0.13±.08	0.19±.04	0.17±.13
BCO[b]	123±16	759±116	172±59	185±34	203±19	62.9±23.2
BMG	192±23	644±136	203±72	107±21	168±27	69.5±36.7
DBS	482±63	196± 24	245±21	132±29	193±38	39.8±13.4
TBS	97.0±18.1	818±175	143±45	164±40	127±12	52.6±18.7

[a]Mean and standard deviation: n=3 for corn oil, n=5 for brominated compounds.
[b]Brominated corn oil, BCO; brominated monoglyceride mixture, BMG; monoglyceride of dibromostearate, DBS; and monoglyceride of tetrabromostearate, TBS.

oxidation products of the di- and tetrabromostearic acids in the form of the di- and tetrabromomyristate (C14) and palmitate (C16). The amounts of these metabolites found in lipids of heart, liver and adipose tissue of the animals fed the various brominated oil diets are shown in Figures 1 and 2. On the average, 80% of the bromine detected by neutron activation is accounted for by these compounds.

No shorter-chain intermediates were detected. However, the volatility of an unsaturated methyl laurate makes the loss of this ester a distinct possibility during our preparative and concentrating steps, especially if only small amounts were present. As noted previously, methyl esters of monobrominated fatty acids may be detected along with the conventional fatty acids on a stainless steel capillary column. No such derivatives were detected in tissue lipids of rats fed the brominated compounds.

Metabolites represented from 17 to 30% of the total brominated fatty acids isolated from the various tissues. The greatest proportion of metabolites appeared to be accumulating in cardiac lipids and in the hepatic lipid of the animals fed the DBS. The dibrominated fatty acids accumulated in cardiac lipids, and tetrabrominated fatty acids accumulated to a greater extent in hepatic lipids. The ratio of di- to tetrabrominated acids in the cardiac lipids of the animals fed the BCO and BMG mixture is ca. 3:1; this represents a 10-fold increase over the same ratio in hepatic lipids.

Rats ingesting diets containing BCO and BMG show comparable amounts of brominated fatty acids in tissue lipids. This is of interest considering the differing response to these two diets.

Metabolite distribution varies from tissue to tissue. The dibromomyristic acid concentrations are lower than the dibromopalmitic acid concentrations in cardiac and adipose lipids, whereas the reverse is true in liver lipid. Concentrations of the tetrabromomyristate are higher than the tetrabromopalmitate in lipids of all tissues analyzed. This observation further accentuates the fact that the di- and tetrabromo derivatives are being metabolized quite differently.

DISCUSSION

In this assessment of the toxicity of the di- and tetrabromostearic acids, it has been found that the dibromostearic acid had a greater effect on cardiac tissue than the tetrabromostearic acid, with the converse being true in hepatic tissue. The DBS appears to be metabolized and/or released more readily from the liver than the TBS. The amounts

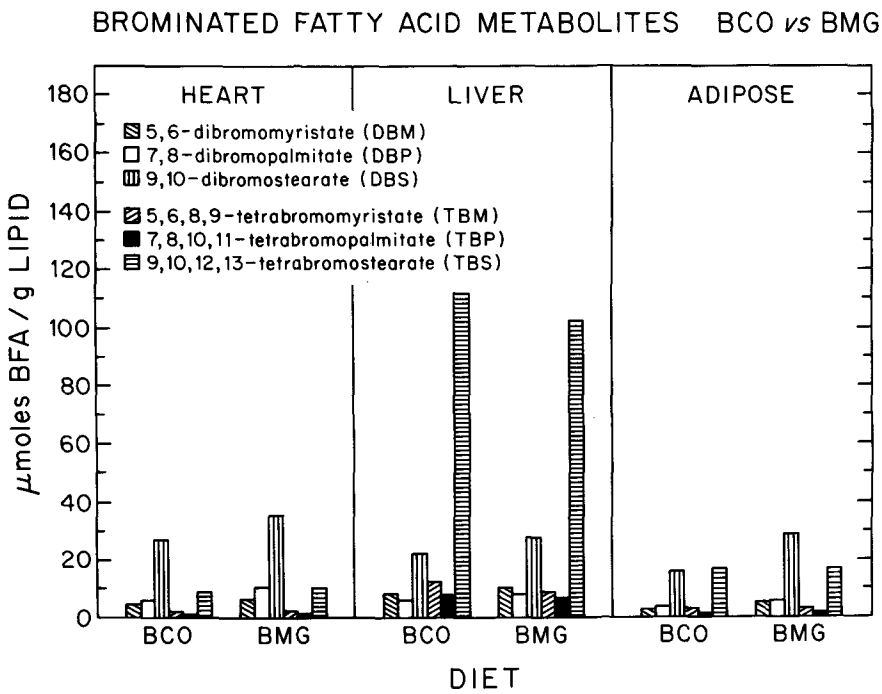

FIG. 1. Concentrations of dibromo- (DB) and tetrabromo- (TB) myristate (M), palmitate (P) and stearate (S) in tissue lipid from rats fed brominated corn oil or a mixture of the monoglycerides of dibromostearate and tetrabromostearate.

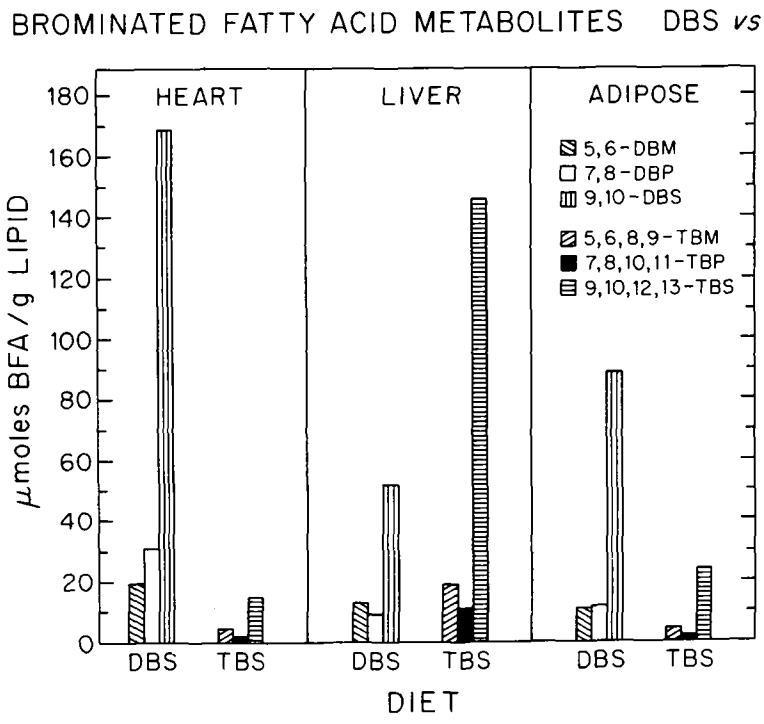

FIG. 2. Concentrations of dibromo- (DB) and tetrabromo- (TB) myristate (M), palmitate (P) and stearate (S) in tissue lipid from rats fed monoglycerides of dibromostearate or tetrabromostearate.

of tetrabromo derivatives which were transported and/or incorporated into the cardiac lipids were small compared to the amounts of dibromo derivatives that accumulated, supporting previous findings made in our laboratory (1).

Overall, the toxic response of the animals fed diets containing the BCO is greater than that observed in animals fed the other brominated oils. This might be explained by the presence of small amounts of some other brominated compound, such as a brominated sterol or the small amount of hexabromostearic acid (1%) derived from linolenic acid.

Another consideration is the position of the brominated acid on the glycerol. In corn oil, the majority of the linoleic acid is found in the 2-position on the triglyceride molecule and thus, with BCO, the majority of the TBS would be absorbed as the 2-monoglyceride after lipolysis in the intestine. The synthetic monoglycerides, however, would have the majority of the BFA in the 1- and 3-position, since migration from the 2-position is a common occurrence during esterification reactions (12). Thus, a much smaller proportion would be absorbed as the 2-monoglyceride. That the distribution of fatty acids on the glycerol molecule can be a factor has been demonstrated by Hung et al. (13). In feeding studies with natural and randomized high and low erucic rapeseed oils, substantial differences in response were observed. Differences in toxicity encountered in this study between the BCO and BMG mixture diets, despite similar intakes of brominated acids and the similar BFA concentrations produced in tissue lipids, may also indicate that there are differences in distribution and/or metabolism between the 1- and 3-monoglycerides and the 2-monoglycerides.

The shorter-chain metabolites of the brominated stearates isolated from the tissue lipids indicates that these acids are being metabolized by β-oxidation. The two cycles of β-oxidation compares with that observed with erucic acid (14). Failure to demonstrate β-oxidation of dibromopalmitic acid by mitochondrial preparations (15) would provide additional evidence to that presented in our previous study (1) that BFA are metabolized to some extent by peroxisomes. Further studies with isolated peroxisomes are required to confirm this suggestion.

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COMMUNICATIONS

Asymmetric Distribution of Decanoate in Triacylglycerol Synthesized in vitro by Mammary Glands of Lactating Mice

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ABSTRACT

Slices, prepared from the mammary glands of lactating mice, were incubated with either [1-^{14}C]acetate, [U-^{14}C]glucose, or [1-^{14}C]decanoate. From all 3 substrates, radioactivity in the synthesized lipids was found mainly in triacylglycerols (TG). When acetate or glucose served as substrate, decanoate (C_{10}) accounted for 24% of the fatty acids in TG. Hydrolysis of the TG by pancreatic lipase yielded [^{14}C] fatty acids which had relatively more C_{10} (38%) than did either of the other hydrolysis products mono- or diacylglycerol (14-17%). However, when TG produced by slices from C_{10} were hydrolyzed, the acid was found to be esterified equally at the C-1, C-2 and C-3 of glycerol. Thus, when fatty acids are synthesized de novo and are converted to TG by gland slices, C_{10} is predominantly located in the C-3 position, a finding in accord with the situation in milk TG, although such preferential incorporation does not occur when the free acid is presented to the tissue slices.
Lipids 18:335-338, 1983.

INTRODUCTION

A major portion (ca. 98%) of the lipid in milk is present in the form of triacylglycerol (TG). Non-ruminant milk TG contain appreciable amounts of medium chain length fatty acids which are predominantly found acylated to the C-3 position of glycerol (1). The medium chain length fatty acids are synthesized by the mammary glands during lactation while the long chain saturated, mono- and polyunsaturated fatty acids found in the milk are principally derived from the circulating blood lipids (1). When slices prepared from the mammary glands of lactating mice were incubated with [1-^{14}C]acetate and glucose, labeled lipids composed mainly of TG were produced which contained decanoate (C_{10}) at a level of ca. $^1/_4$ of the total fatty acids (2,3). In the present study, to determine whether the pattern of radiolabeling in vitro was what would be expected from observations made in vivo in milk, we investigated the position of C_{10} on the glycerol moiety of TG through the use of pancreatic lipase hydrolysis.

MATERIALS AND METHODS

Female C_3H mice which were actively lactating and suckling at least 6 pups for 17 days were killed by cervical fracture. Their mammary glands were excised and sliced by procedures described previously (2). Slices (100 mg) were incubated for 2 hr at 37 C in 2 ml Krebs-Henseleit bicarbonate buffer (pH 7.4) containing [1-^{14}C]acetate (2.5 mM) and glucose (10 mM) with 95% O_2 and 5% CO_2 as gas phase. In some experiments, acetate was omitted and [U-^{14}C]glucose (10 mM) was used as substrate. The conditions of incubation with [1-^{14}C]C_{10} were the same as those described earlier (3). After incubation, total lipids were extracted from the slices and the lipid classes were isolated by preparative thin layer chromatography (TLC) (3).

Enzymatic hydrolysis of TG with pancreatic lipase was carried out as described by Lands et al. (4). Porcine pancreatic lipase was obtained from Calbiochem (La Jolla, CA). ^{14}C-Labeled TG produced by mammary gland slices (ca. 10,000 cpm) and carrier TG (1 mg), obtained from mouse milk, both dissolved in ether, were coated on the sides at the bottom of a 15 ml centrifuge tube by evaporation under a gentle stream of nitrogen. Tris buffer pH 8.05 (1.0 M; 0.1 ml), NaCl (1.0 M; 0.1 ml) and lipase (0.2 units in 0.1 ml) were added and the tube and its contents (0.3 ml) were shaken vigorously on a vortex mixer for 2 min at room temperature. The reaction was stopped by the addition of 0.2 ml of N HCl. The total lipids were extracted and the classes were separated by TLC and isolated as given

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previously (3). The TG, 2-monoacylglycerol (MG) and diacylglycerol (DG) were individually saponified and the fatty acids (FA) were isolated. Fatty acid methyl esters were prepared with diazomethane and were separated by gas liquid chromatography (GLC) programmed at 10 C/min over the range of 80-180 C (3). A Varian Aerograph (model 2740) provided with a flame ionization detector, a stream splitter and stainless steel column (6 ft. $\times 1/8$ in.) packed with 15% diethylene glycol succinate on H/P chromosorb G was used for GLC. Individual fatty acid methyl ester fractions were collected and assayed for radioactivity (3).

RESULTS AND DISCUSSION

As observed earlier (2,3), the major lipid (> 90%) produced from either [1-^{14}C]acetate, [U-^{14}C]glucose or [1-^{14}C]C_{10} by slices of mammary glands of lactating mice was TG (Table 1). Furthermore, in both DG and TG synthesized from acetate or glucose, C_{10} comprised ca. 20% of the labeled fatty acids. The radioactivity in C_{10} found in the FA and the phospholipid (PL) fractions was considerably less. The level of C_{12} was also greater in TG and DG than in FA and PL fractions (Table 1). As observed previously, when mammary gland slices were incubated with [1-^{14}C]C_{10} and glucose, ca. 93% of the radioactivity in TG was still found in C_{10} (3), indicating that when presented to the tissue as such, this fatty acid was not elongated but was incorporated into complex lipids (TG and DG) as an intact unit.

Among the fatty acids synthesized from labeled acetate or glucose by mouse mammary gland slices, significant levels of monounsaturated fatty acids (16:1 and 18:1) were also observed (Table 1). Earlier we showed that Δ9 desaturase in the microsomes from lactating mouse mammary glands uses stearate rather than palmitate or myristate as the preferred substrate (5). The distribution of radioactivity in the fatty acids synthesized from [1-^{14}C]acetate or [U-^{14}C]glucose (Table 1) suggests that this is also the situation in the intact cell, since the proportion of monounsaturated to saturated (16:1/16:0 and 18:1/18:0) was greater in the case of 18 carbon FA than with 16 carbon FA.

The distribution of labeled fatty acids in PL was different from that in TG (Table 1). Phospholipids contained a relatively high level of long chain FA and only traces of C_{10}. These observations with mice are similar to those with rats and show a distinct difference in the fatty acid composition of PL and TG in milk fat and tissue lipids (6). Although DG are generally considered to be common intermediates in the biosynthesis of PL and TG, the data (Table 1) indicate that only selected species of DG such as those with short or medium chain FA and those with C-2 saturated FA are converted to TG. This selective use of DG for glycerolipid biosynthesis may be due either to enzyme specificity (7) or to a separation of the DG pools (8).

To determine the positional distribution of the C_{10}, TG produced from either [1-^{14}C]acetate, [U-^{14}C]glucose or [1-^{14}C]decanoate were separately

TABLE 1

[^{14}C] Fatty Acid Distribution in Lipids Synthesized by Slices of Mammary Gland from Lactating Mice

Lipid class	Percent [^{14}C] in total lipid	Labeled percursor	[^{14}C] Fatty acid (mole %) C_8	C_{10}	C_{12}	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$
Triacylglycerol	92.8	Acetate	2.9	24.0	25.3	22.7	15.6	2.8	3.9	2.5
	90.5	Glucose	1.9	24.0	27.3	22.1	16.2	1.9	3.9	2.6
	94.5	Decanoate	1.6	92.5	1.4	1.2	1.6	T	1.0	T
1,2-Diacylglycerol	3.6	Acetate	1.3	19.5	26.2	26.8	22.1	1.3	2.0	0.6
	5.1	Glucose	2.6	17.8	28.6	26.4	19.7	0.6	3.2	1.1
	4.4	Decanoate	1.5	91.1	2.4	1.6	1.3	T	1.0	T
Fatty acid	1.5	Acetate	T	1.5	16.4	25.4	40.3	2.2	11.9	2.2
	2.6	Glucose	T	0.8	13.7	28.3	45.6	3.1	6.8	1.3
Phospholipid	2.1	Acetate	T	3.0	11.4	22.9	42.8	3.0	13.7	3.0
	1.8	Glucose	0.5	1.8	14.0	21.6	44.2	5.7	10.8	1.4
	1.0	Decanoate	-	-	-	-	-	-	-	-

Mammary gland slices (100 mg wet wt) from mice lactating for 17 days were incubated for 2 hr at 37 C in 2 ml Krebs-Henseleit bicarbonate buffer containing [1-^{14}C]acetate (2.5 mM) and glucose (10 mM) or [U-^{14}C]glucose (10 mM) or [1-^{14}C]decanoate (1.25 mM) and glucose (10 mM) with 95% O_2 and 5% CO_2 as gas phase. After isolation of the total lipids from the slices, lipid classes were separated by preparative TLC. Following total saponification and preparation of methyl esters, fatty acids were analyzed by radio GLC. Values less than 0.5% are given as T. Analysis of radioactive fatty acids in the phospholipids obtained from C_{10} was not carried out due to the small amounts of ^{14}C activity in this fraction. The values are averages of closely agreeing duplicate determinations (± 5%) with slices from 2 lactating mice.

hydrolyzed by pancreatic lipase. The products of lipolysis, FA, MG, and DG, as well as the residual TG were isolated and the distribution of radioactive fatty acids in each fraction was analyzed (Table 2). In experiments with radiolabeled acetate and glucose, the fatty acid composition of labeled TG obtained after partial hydrolysis was similar to that of the original TG, a finding which suggests that pancreatic lipase shows no selectivity for a particular TG species. On the other hand, the liberated fatty acids contained a greater proportion of C_{10} (38%) than any other fatty acid (Table 2) and, as a consequence, the level of C_{10} in the MG was less (14%) than that in the original TG (24%). Hence, the liberated C_{10} must have been located predominantly in the C-1 and C-3 of TG. It is likely that the proportion of C_{10} acylated at C-1 was not appreciably greater than at C-2, since the level of this acid in the DG produced by hydrolysis was only 17%. Thus, our results show that, in TG synthesized from acetate or glucose by mammary gland slices in vitro, C_{10} is most likely acylated preferentially at the C-3 position. Furthermore, since the levels of C_{14} and C_{16} in MG are greater than in TG (Table 2), the data also suggest that these acids were esterified at C-2 relatively more than at C-1 or C-3.

On the other hand, the situation is quite different when the mammary gland slices were presented with a preformed medium chain length fatty acid. In the case of TG produced from [^{14}C]decanoate, we could show that for each 1000 cpm of the substrate hydrolyzed by the lipases, MG, DG and FA, were found to have 205, 218 and 577 cpm, respectively. If we assume that C_{10} was esterified in equal portions on the 3 carbons of glycerol, then after hydrolysis of TG, the [^{14}C] activity in the liberated FA would be expected to contain 519 cpm ($2\times205+218/2$). Since the value we obtained experimentally (577 cpm) was so close to that predicted, it would appear that when the free acid was presented to the tissue slices, the acyl transferases did not exhibit positional specificity for TG synthesis and almost all of the [^{14}C] activity was found in C_{10}.

During de novo synthesis, free C_{10} generated by fatty acid synthetase (FAS) does not accumulate to any appreciable extent in lactating mammary gland cells. Therefore, the acyl transferases are usually not exposed to high levels of C_{10} as the blood of mice does not contain appreciable amounts of this acid. We, therefore, suggest that the difference in the position on the glycerol moiety of the TG is due to the specificity of acyl transferases towards the free acid and that produced by FAS and is probably related to the concentration of the decanoate in the cell during the period when esterification takes place.

Our experiments with slices of mammary glands from lactating mice show that this preparation is capable of producing TG which contain a degree of acyl specificity analogous to that found in milk TG produced in vivo by the gland.

TABLE 2

[^{14}C] Fatty Acid Distribution of the Products of Pancreatic Lipase Hydrolysis of Triacylglycerol Produced by Mammary Gland Slices

Lipid class	[^{14}C] Fatty acid (mole %)							
	C_8	C_{10}	C_{12}	C_{14}	C_{16}	$C_{16:1}$	C_{18}	$C_{18:1}$
Triacylglycerol (original)[a]	2.9	24.0	25.3	22.7	15.6	2.8	3.9	2.5
Triacylglycerol (residual)	0.6	20.9	29.3	26.1	19.6	0.9	1.7	0.9
Diacylglycerol	0.9	15.9	29.2	29.9	17.3	3.0	1.8	2.0
Monoacylglycerol	0.5	12.9	27.9	34.1	17.7	2.7	1.4	2.7
Fatty acid	1.2	38.2	29.1	15.8	12.7	0.9	1.3	0.8
Triacylglycerol (original)[b]	1.9	24.0	27.3	22.1	16.2	1.9	3.9	2.6
Triacylglycerol (residual)	0.5	21.4	29.2	25.3	18.8	1.8	1.3	1.2
Diacylglycerol	0.9	16.5	30.5	28.5	17.9	1.9	2.0	1.9
Monoacylglycerol	1.0	14.0	26.9	33.3	18.7	2.7	1.5	2.0
Fatty acid	0.6	38.2	28.3	16.0	13.5	0.9	1.2	1.2

[a]Values given were obtained when mammary gland slices were incubated with [1-^{14}C]acetate and glucose and are averages of closely agreeing (±5%) duplicate determinations.

[b]Results from experiments with [U-^{14}C]glucose are given as averages of closely agreeing (±5%) duplicate determinations.

About 50-60% of TG were hydrolyzed by lipase in these experiments. In some cases, lipase reaction was stopped after 1 min incubation, in which case only 20% of TG were hydrolyzed. The distribution of radioactivity in FA of various chain lengths in MG, DG and FA in these instances was similar to that given in Table 1.

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Altered Microsomal Phospholipid Composition in the Streptozotocin Diabetic Rat

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ABSTRACT

Streptozotocin diabetes in the rat alters liver microsomal membrane fatty acid composition. The present study was undertaken to determine if such changes in fatty acid composition were due to changes in the amount of individual phosphoglycerides or to disproportionate changes in fatty acid composition in any of the individual phosphoglycerides. The diabetic animals showed a small increase in total microsomal phospholipid, which is due to a selective increase in the phosphatidylethanolamine fraction. The changes in fatty acid composition in the total lipid extract (decreased palmitoleic, oleic and arachidonic acids and increased linoleic and docosahexaenoic acids) from the diabetic animals were present in both the major phosphoglycerides, phosphatidylcholine and phosphatidylethanolamine, with very little change in fatty acid composition in the phosphatidylserine and inositol fraction. Further studies are necessary to delineate the cause of the abnormal membrane phospholipid composition in the diabetic animal.

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INTRODUCTION

Alterations in fatty acid composition of liver microsomal phospholipid have been reported in the streptozotocin diabetic rat (1). These include decreased proportions of palmitoleic (16:1ω9), oleic (18:1ω9) and arachidonic (20:4ω6) acids and an increased proportion of linoleic (18:2ω6) and docosahexaenoic (22:6ω3) acids. Decreases in liver microsomal phospholipid without an alteration in the proportion of individual phospholipids have been reported in the alloxan diabetic rat (2), but a slight increase in liver microsomal phospholipid per mg protein occurred in the streptozotocin diabetic rat (1). An increase in total platelet phospholipids has been described in poorly controlled diabetic patients with the largest increase being in the phosphatidylethanolamine fraction and a smaller increase in the phosphatidylserine fraction (3). The methyl transferase enzymes that convert phosphatidylethanolamine to phosphatidylcholine showed decreased activity in the severely alloxan diabetic rat (4). It was speculated that this decrease in the methylating pathway in the diabetic rats might change the composition of phospholipids and properties of cellular membranes since this pathway provides phosphatidylcholines with more polyunsaturated fatty acids than the CDP-choline pathway (4,5).

The present study was undertaken to determine if streptozotocin diabetes caused either changes in the amount of individual liver microsomal phosphoglycerides or disproportionate changes in fatty acid composition in any of the individual phosphoglycerides.

MATERIALS AND METHODS

Animals and Their Treatment

White, male, Sprague-Dawley rats from Charles Rivers Laboratories were maintained on a Purina Laboratory Chow #5001 diet ad libitum unless indicated otherwise. A sample analysis from one batch of the diet contained 5.0% total lipid with a fatty acid composition of 1.7% 14:0, 22.9% 16:0, 1.9% 16:1ω9, 7.8% 18:0, 32.4% 18:1ω9, 29.8% 18:2ω6 and 3.1% 18:3ω3. Experimental diabetes was produced by the intravenous injection of streptozotocin in a dose of 75 mg/kg body weight. After several days, blood glucose was estimated using Dextrostix reagent strips and an Ames Reflectance Meter. Only those rats with blood glucoses greater than 300 mg/dl were considered diabetic. Animals treated with insulin were given 8 U protamine zinc insulin subcutaneously daily for 2 days prior to death. Control and untreated diabetic animals were injected with saline. Before injection of insulin or saline and immediately before death, blood was taken for glucose determination by a commercial glucose oxidase method. Rats were killed 14-21 days after streptozotocin injection in the fed state.

Microsomal Lipid Analysis

Washed liver microsomes for phospholipid and fatty acid analyses were prepared and total lipids extracted as described previously (6). A portion of the total lipid extract from each sample was taken for total phosphorus determination. Another portion was taken for phospholipid separation by thin

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Abbreviations: The abbreviated fatty acid nomenclature refers to the number of carbon atoms in the chain, the number of unsaturated bonds, and the position of the first unsaturated bond counting from the terminal methyl group; thus arachidonic acid, 5,8,11,14-eicosatetraenoic acid, is 20:4ω6.

layer chromatography on Silica Gel H by a modification of the method of Skipski et al (7).

2',7'-Dichlorofluorescein was used to locate the lipid spots. Phosphatidylserine and phosphatidylinositol travelled together in this system. The spots were identified by comparison with authentic standards. The spots were scraped and eluted with chloroform/methanol/7M ammonium hydroxide (13:7:1, v/v/v). One portion of the eluate was used for lipid phosphorus determination to calculate the proportion of each major phospholipid. Mean recovery of lipid phosphorus was 94% of the amount applied to the chromatogram. The remainder of the eluate was used for preparation of fatty acid methyl esters, which were separated by gas liquid chromatography as described previously (6).

Analytical Procedures

Phospholipid phosphorus and protein determinations were carried out as described previously (8,9).

Statistical Analysis

Significant differences between groups were determined by using the one-way analysis of variance.

RESULTS

Metabolic State of Diabetic Animals

The untreated diabetic animals had overt polydipsia and polyuria and overtly elevated plasma glucose levels (404 ± 16 mg/dl, mean ± SE) compared to control rats (103 ± 2 mg/dl). The diabetic rats treated with 8 U protamine zinc insulin daily for 2 days had less polyuria and significantly lower glucose levels (321 ± 23 mg/dl) than the untreated diabetic animals at the time of sacrifice ($p < 0.01$).

Microsomal Phospholipid Composition in Diabetic Animals

Figure 1 shows that total microsomal phospholipid phosphorus was increased 11% in the diabetic animals (482 ± 3 nmol/mg protein) compared to controls (434 ± 8 nmol/mg protein). This increase was prevented by insulin treatment of the diabetic animals (421 ± 8 nmol/mg protein). The phosphoglycerides were separated into their major individual fractions, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine plus inositol (the latter two migrating together in the thin layer chromatography system used). Control microsomal phospholipid contained 54% phosphatidylcholine, 27% phosphatidylethanolamine, and 11% phosphatidylserine plus inositol. There were no significant changes in the amount of phosphatidylcholine or phosphatidylserine plus inositol in the diabetic animals. However, there was a 26% increase in the phosphatidylethanolamine content of the microsomal phospholipid from diabetic animals (149 ± 3 nmol/mg protein) compared to controls (118 ± 2 nmol/mg protein). This increase accounted for the increase in total phospholipid in the diabetic animals and was likewise prevented by insulin treatment of the diabetic animals (109 ± 3 nmol/mg protein).

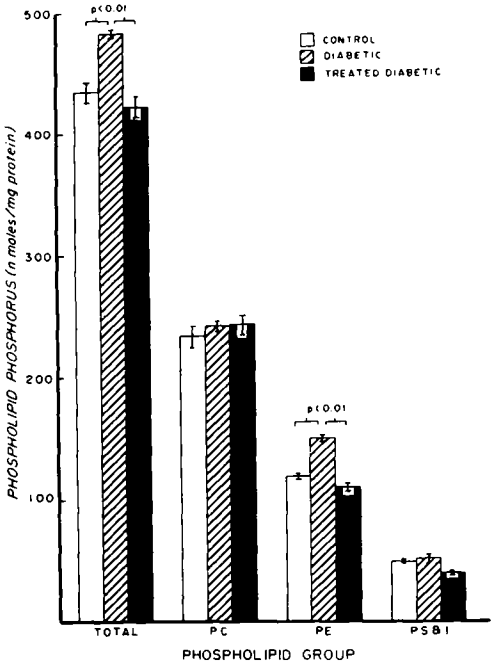

FIG. 1. Phospholipid composition of liver microsomes in diabetes. The individual phosphoglycerides from liver microsomes were separated by TLC and lipid phosphorus measured as described in the Methods section. Nondiabetic □, untreated diabetic ▨, and insulin-treated diabetic ■ rats (5 animals/group) were studied. Results are shown as mean ± SEM. Statistical differences between groups are shown above the bars.

Fatty Acid Composition of Individual Phosphoglycerides in Diabetic Animals

Figure 2 shows the fatty acid composition of the total lipid extract and of the individual phospholipid fractions from the liver microsomes of the control, diabetic and insulin-treated diabetic animals. Changes in fatty acid composition in the total lipid extract from the diabetic animals were similar to those described previously (1), namely, decreased proportions of 16:1ω9, 18:1ω9 and 20:4ω6 and increased proportions of 18:2ω6 and 22:6ω3. As noted before, all of these changes except for the decreased 20:4ω6 were prevented by insulin therapy

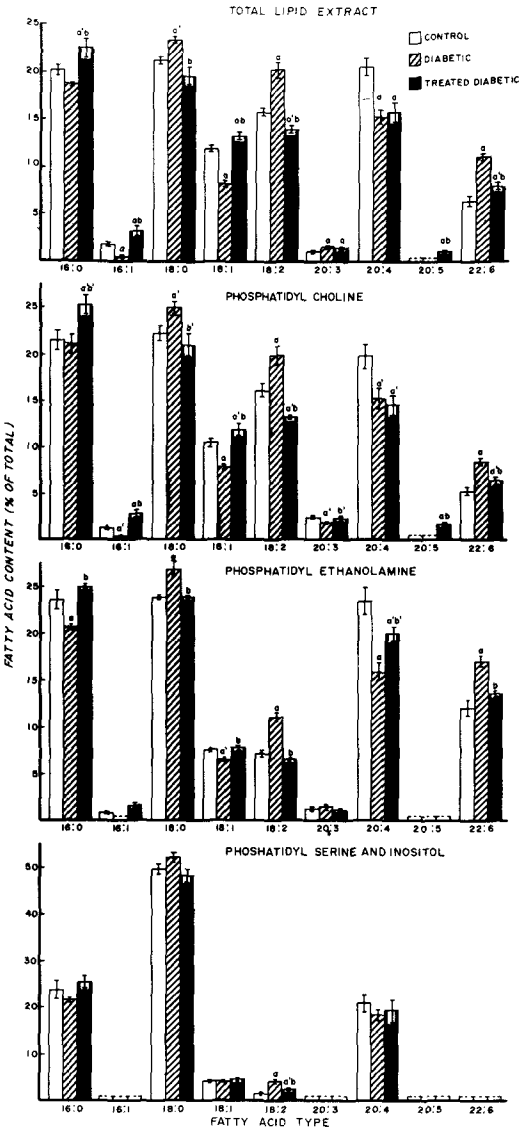

FIG. 2. Fatty acid composition of individual phosphoglycerides in experimental diabetes. The individual phosphoglycerides of the liver microsomal extracts were separated and fatty acid analysis of each fraction was carried out as described in the Methods section. Nondiabetic □, untreated diabetic ▨, and insulin-treated diabetic ■ rats (5 animals/group) were studied. Results are shown as mean ± SEM (a) $p<.01$, (a') $p<.05$, experimental vs control; (b) $p<.01$, (b') $p<.05$, treated vs untreated diabetic.

(1). In addition, there were two changes not noted before. These are a slight increase in the proportion of 20:3ω6 in the diabetic lipid extract (1.6 ± 0.1%) as compared to controls (1.1 ± 0.1%) which was prevented with insulin therapy and an apparent increase in 20:5ω3 in the insulin-treated diabetic animals (1.2 ± 0.1%) with unmeasurable levels (< 1%) in control and diabetic animals. There appeared to likewise be a small increase in 22:5ω3 in the insulin-treated diabetics, but the levels were too small to measure accurately.

To determine if these changes were fairly evenly distributed in all the major phospholipid fractions or occurred predominantly in one particular fraction, the fatty acid composition of the phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine plus inositol fractions from the control, diabetic and insulin-treated diabetic liver microsomes was determined. These data are also shown in Figure 2. It can be seen that the decreased proportions of 16:1ω9, 18:1ω9, and 20:4ω6 and the increased proportions of 18:2ω6 and 22:6ω3 occurred in both of the dominant phospholipid components, phosphatidylcholine and phosphatidylethanolamine. Again, these were prevented by insulin therapy, except for the diminished arachidonate levels. 20:3ω6 was paradoxically slightly diminished in phosphatidylcholine and unchanged in phosphatidylethanolamine. 20:5ω3 was increased in the insulin-treated diabetics in the phosphatidylcholine fraction as in the total lipid extract, and was unmeasurable in the phosphatidylethanolamine fraction.

The control fatty acid composition of the phosphatidylserine plus inositol fraction was markedly different from that seen in the other phospholipid fractions with a higher percentage of saturated fatty acids, arachidonic acid being the only unsaturated fatty acid present in substantial amounts. The only change in fatty acid composition in the phosphatidylserine plus inositol fraction in the diabetic animals was a significant increase in 18:2ω6, although this fatty acid is only a minor component in this fraction.

There also is a trend toward a decreased 16:0 with an associated increased 18:0 in the diabetic animals which is prevented by insulin but these changes only reached statistical significance in some fractions. The same trend was noted in previous experiments (1).

DISCUSSION

The streptozotocin diabetic rats showed a selective increase in liver microsomal phosphatidylethanolamine content which accounted for a slight increase in total microsomal phospholipid. This is consistent with similar findings in human diabetic platelet phospholipids (3). The increased levels of phosphatidylethanolamine could be due to the reported decreased methyl transferase enzyme activity in the alloxan diabetic rat (4). This enzyme converts phosphatidylethanolamine to phospha-

tidylcholine.

Whereas diabetic liver microsomes show increased linoleate and diminished arachidonate levels, human diabetic platelets showed decreased linoleate and increased arachidonate levels, although in both instances increased phosphatidylethanolamine levels occurred. This suggests that the changes in fatty acid composition are due to different mechanisms in these two instances. In addition to the fatty acid changes noted previously in the diabetic microsomes (1) (decreased 16:1ω9, 18:1ω9, and 20:4ω6 and increased 18:2ω6 and 22:6ω3), the present studies showed increased 20:5ω3 in the insulin-treated animals, suggesting a decrease in the untreated diabetic animals, although the levels were too low to measure accurately. This is consistent with a report of decreased 20:5ω3 in the fatty liver of diabetic subjects (10). The implications of this in relation to prostaglandin formation and platelet aggregation remain to be determined.

The changes in fatty acid composition in the total liver microsomal lipid from the diabetic animals occurred in both the major phosphoglycerides, phosphatidylcholine and phosphatidylethanolamine. The phosphatidylserine and inositol fraction showed only a small increase in 18:2ω6. Therefore, the changes in fatty acid composition of the total lipid extract are not due solely to the accumulation of phosphatidylethanolamine with an unusual fatty acid composition. They, likewise, are probably not due to a shift in the source of phosphatidylcholine from the methyl transferase pathway to the CDP choline pathway as postulated by Hoffman et al. (4), but are probably due to diminished Δ^6 and Δ^9 fatty acid desaturase activities as described previously (1). Further work is necessary to delineate the cause and consequences of the abnormal membrane phospholipid composition in the diabetic animal.

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Inhibition of Cholesterol and Fatty Acid Biosynthesis in Liver Enzymes and Chicken Hepatocytes by Polar Fractions of Garlic[1,2]

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ABSTRACT

Different concentrations of polar fractions, methanol-soluble (MESF), or water-soluble (WASF), of 1-8% equivalent to fresh garlic paste were added to yellow corn-soybean based diets and fed to 5-week-old male broiler chickens for 3 weeks to measure the inhibition of hepatic β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase, cholesterol 7α-hydroxylase (7α-hydroxy) and fatty acid synthetase (FAS). Dose-related decreases in the activities of these enzymes were obtained. Decreases in serum total cholesterol and in low density lipoprotein (LDL) levels were also observed. There was no effect on the level of cholesterol in high density lipoprotein (HDL). The most effective dose for these decreases was found 0.54% (MESF) and 1.2% (WASF) equivalent to 6% of the fresh garlic. The inhibition of HMG-CoA reductase and FAS by 25-300 μg of MESF or WASF for 15 min was tested in vitro, in male and female chicken hepatocytes. Inhibitions of activity were dose-dependent and the degree of inhibition increased with duration of incubation (150 μg of MESF or WASF 5 to 60 min). Dietary supplementation of odorless WASF of garlic was found to be very effective in lowering the total and LDL cholesterol levels compared to control chickens.

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INTRODUCTION

Although the rate of mortality has been considerably reduced, heart disease still remains as the leading cause of death in America. Most of the studies in this area are preventive in nature and focused on lowering plasma cholesterol. The role of nutritional factors such as the type of carbohydrate and dietary fiber in changing plasma cholesterol concentrations has been reported by a number of investigators (1-9).

The hypocholesterolemic, hypoglycemic and antibacterial properties of garlic oil and nonfibrous substances present in garlic bulb have been reported (10-15). Most studies reported in the literature have described the effects of garlic or its fractions on lowering the total cholesterol and lipids in serum and liver only after feeding cholesterol or fat to the experimental animals (11, 12). We have recently found that diets supplemented with different garlic fractions, particularly with polar solubles, decreased not only cholesterol and fatty acid biosynthesis, and serum total cholesterol but also lowered the cholesterol levels in low density lipoproteins (LDL) without affecting the high density lipoproteins (HDL) in chickens (16).

The inhibition of lipid metabolism and significant lowering of serum cholesterol and chol-LDL levels in chickens by polar fractions of garlic prompted us to determine the effective level of these fractions for the inhibition of lipid metabolism in male broiler chickens. We also report the inhibition of cholesterogenesis and lipogenesis by these fractions of garlic using isolated hepatocytes of male and female chickens.

MATERIALS AND METHODS

Experimental materials were purchased from the following sources: acetyl-CoA, malonyl-CoA, RS-mevalonic acid, glucose-6-phosphate, dithiothreitol, $NADP^+$, NADPH, glucose-6-phosphate dehydrogenase, cysteamine, Tween-80, triethanolamine hydrochloride, sodium malate, coenzyme A, malate dehydrogenase, nicotinamide, and DL-3-hydryoxy-3-methylglutaryl-CoA (Sigma Chemical Co., St. Louis, MO); cholesterol (Aldrich Chemical Co., Milwaukee, WI) was recrystallized twice in glacial acetic acid; 7α-hydroxycholesterol (5-cholesten-3β,7α-diol) and 7α-ketocholesterol (5-cholesten-3β-ol-7-one) (Steraloids, Inc., Wilton, NH); EDTA (Fisher Scientific Co., Itasca, IL); bovine serum albumin (Nutritional Biochemicals Corporation, Cleveland, OH); and DL-3-hydroxy-3-

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[1]Cooperative investigation between the Agricultural Research Service, US Department of Agriculture, and College of Agriculture and Life Sciences, University of Wisconsin, Madison. A preliminary report of this work was presented at the 66th Annual Meeting of Federation of American Societies for Experimental Biology, New Orleans, LA, April 1982, Abs. 41:554 (1982).

[2]Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the US Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

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methyl-[3-^{14}C] glutaryl-CoA (sp act 26.3 mCi/mmol), [4-^{14}C] cholesterol (sp act 50-60 mCi/mmol) and Aquasol (scintillation solution) (New England Nuclear, Boston, MA). Fresh garlic (*Allium sativum*) and the other diet components were obtained in Madison, WI. All other chemicals were of analytical grade.

Animals and Diets

Five-week-old broiler male or female chickens, weighing ca. 800-900 g were obtained from a commercial source (Madison, WI). The birds were maintained on a corn-soybean based diet as a control and the addition of methanol- (MESF) or water-soluble fractions (WASF) of garlic in the control diet served as experimental diets.

Procedure for the Extraction of MESF and WASF of Garlic

The clean garlic cloves (600 g) were homogenized into a paste using a Waring blender. The paste was extracted with petroleum ether (600 ml) 3 times to remove fatty acids and terpenes. The residue was freeze-dried and lyophilized. The resulting powder (315 g) was extracted successively with methanol and water 3 times each, using 600 ml of solvent each time. The methanol-soluble extracts were combined and concentrated under vacuum at 60 C, yielding 55.3 g of semisolid material. The water-soluble combine extracts were lyophilized, resulted in 171.5 g of powder. These materials were stored at 4 C. The required amount of each fraction for each diet (equivalent to 1, 2, 4, 6 and 8% of the fresh garlic paste) was taken up either in a minimum volume of the methanol solvent (20 ml for 10 kg feed) or WASF powder was mixed thoroughly with the corn-soybean based diet (Table 1). The MESF-based diets were allowed to evaporate overnight in a pan (air-dried under the hood). These diets were fed to different groups of chickens. The WASF and MESF were also tested in vitro in chicken hepatocytes.

Experiment I: Effect of Different Concentrations of MESF or WASF of Garlic on Hepatic Enzyme Activities of Cholesterol and Serum Lipids in 8-week-old Male Broiler Chickens

Forty eight 5-week-old male broiler chickens were divided at random into 6 groups, housed in wire bottom cages. Eight chickens fed corn-soybean diet served as the control group; other groups of 8 were fed different levels of MESF or WASF of garlic, corresponding to 1, 2, 4, 6 and 8% of fresh garlic as shown in Table 1. The diets (Table 1) and water were provided ad libitum for 3 weeks with the photoperiod of 12 hr. At the end of the feeding period, the birds were killed and samples of blood and liver were removed, washed, held on ice, weighed and then prepared for the analysis as described previously (16).

Experiment II: Effect of Different Concentrations of MESF and WASF of Garlic on the Enzymic Activities of HMG-CoA Reductase and FAS in Isolated Hepatocytes of Chickens

Eight-week-old male or female chickens were fed corn-soybean diet. They were fasted (48 hr) and refed (72 hr) so that the enzymic activities were measured at the high points of activity, prior to the preparation of liver perfusion. During fasting and refeeding period, 12 hr of photoperiod was employed.

Preparation of Isolated Hepatocytes of Chicken for *in vitro* Assays

The recirculating perfusion buffer system was similar to that described by Zahlten and Stratman (17), which gave good yields of viable cells (2-4$\times$ 10^8 cells per liver) in the present experiment; the cell viability was determined by the dye exclusion method (0.004% erythrosin B) which showed 72-78% viable cells.

Calcium-free perfusate buffer. Krebs improved Ringer I (K-RI) buffer was prepared from the following solutions: 80 ml 0.154 M NaCl, 4 ml 0.154 M KCl, 3 ml H_2O, 1 ml 0.154 M KH_2PO_4, 1 ml 0.154 M $MgSO_4 7H_2O$, 21 ml 1.3% $NaHCO_3$, 4 ml 0.16 M Na pyruvate, 7 ml 0.1 M Na fumarate, 4 ml 0.16 M Na L-glutamate, and 5 ml 0.3 M glucose.

Calcium-free incubation buffer. Krebs-Heneleit (KH) buffer had the following composition: 100 ml 0.154 M NaCl, 4 ml 0.154 M KCl, 3 ml H_2O, 1 ml 0.154 M KH_2PO_4, 1 ml 0.154 M $MgSO_4 7H_2O$, 21 ml 1.3% $NaHCO_3$ and 1.5% gelatin.

Experimental Procedure

In the preparation of birds for liver perfusion, bile duct canulation was omitted, and perfusion of the liver was made through the portal vein. The bird was anesthesized with 50 mg/kg sodium pentobarbital injected intravenously in the wing vein, placed on its back on a support rack and secured in place with tape across each limb with the head slanted down. The abdominal skin was incised lengthwise using scissors and the skin was peeled from the muscle to each side. A midline incision was then made through the muscular layer up to the point where the diaphragm begins. The exposed muscles and organs were swabbed with saline solution. The intestine was displaced to the right. During the rest of the procedure, the liver was bathed with perfusion buffer and kept at 42 C.

The bird was then heparinized and a loose tie was placed around the inferior vena cava above where the artery branches off to the kidney. The splenic vein was tightened with a knot, the thoracic cavity

TABLE 1

Percent Composition of Chicken's Diets and Body Weight of 8-Week-Old Male Broiler Chickens

Ingredients (corn-soybean base)	Diets[a]		Body weight (g)			Gain in weight (%)	Feed consumption (kg)
	Corn (%)	MESF[b] (%)	WASF[b] (%)	Initial[c]	Final[d]		
Corn (9.5% control)	70.00	-	-	810 ± 102[e]	1710 ± 169[e]	111	12.96
Corn + MESF or WASF[b]	70.00	0.09 (1.0%)[f]	0.20 (1.0%)[f]	888 ± 117	1766 ± 141	99	12.90
Corn + MESF or WASF[b]	70.00	0.18 (2.0%)	0.40 (2.0%)	860 ± 112	1716 ± 161	100	12.94
Corn + MESF or WASF[b]	70.00	0.36 (4.0%)	0.80 (4.0%)	895 ± 121	1759 ± 142	96[g]	12.92
Corn + MESF or WASF[b]	70.00	0.54 (6.0%)	1.20 (6.0%)	892 ± 123	1739 ± 146	95[g]	12.91
Corn + MESF or WASF[b]	70.00	0.72 (8.0%)	1.60 (8.0%)	848 ± 111	1645 ± 132	94[g]	12.92

[a]Each diet also contains soybean meal-44% protein (23.0%); alfalfa meal-17% protein (2.0%); meat and bone meal (2.0%); dicalcium phosphate (1.0%); calcium carbonate (1.0%); iodized salt (0.5%); vitamin and mineral mixture (0.5%); vitamin and mineral mixture contains/kg feed: vitamin A 3000 IU, vitamin D_3 500 ICU, riboflavin 2.5 mg; calcium-pantothenate 3.0 mg, vitamin B_{12} 0.005 mg; zinc sulfate ($ZnSO_4$) 70 mg and manganese dioxide (MnO_2) 65 mg; grit (5.0%) was also incorporated at the expense of each diet.

[b]MESF and WASF = Methanol- and water-soluble fractions of garlic were added to the corn-soybean based diets. Out of the 380 g of clean garlic cloves; 1.4 g petroleum ether-soluble fraction, 34.2 g methanol-soluble fraction, 79.3 g water-soluble fraction, 12.1 g residue were obtained.

[c]Weight of five-week-old male chickens.

[d]Weight of eight-week-old male chickens. These weights were obtained with WASF of garlic. Similar gain in weights were obtained with MESF.

[e]Mean ± SD, N = 8 chickens per group.

[f]Percentages of respective amounts equivalent to fresh garlic paste are in parentheses.

[g]Significantly different from control at $P < 0.05$.

was opened to expose the superior vena cava and a loose tie was placed around it. The buffer pump was started slowly so that calcium-free K-RI buffer which was equilibrated to 42 C and gassed constantly by 95% O_2/5% CO_2 was just slightly dripping out of the syringe. The inferior vena cava was cut well below the first loose tie to allow blood to escape, a hole was made in the ventricle of the heart and a needle inserted through the heart into the superior vena cava and the second loose tie was tightened. The first loose tie was then tightened to make a closed circuit.

This perfusion was conducted for 10 min without added collagenase; then 40 mg collagenase (the best results were obtained by using the preparation made by Worthington; dissolved in 10 ml 0.154 M NaCl) was added and perfusion was continued for 7 min. After digestion, the liver was removed and transferred into a plastic beaker containing 50 ml perfusion buffer at room temperature. The liver was minced with scissors and the crude suspension gassed for 2 min with 95% O_2/5% CO_2 and filtered through one layer of cheesecloth into a second plastic beaker. The crude cell suspension containing hepatocytes and nonparenchymal cells was transferred to a centrifuge tube.

The cells were counted in a Neuhauer hemocytometer (total number of viable cells $37\text{-}40 \times 10^6$) and protein was estimated by the Biuret method (total protein 200-225 mg). The total volume was adjusted according to the number of viable cells ($5\text{-}6 \times 10^6$) or protein concentration (30 mg/0.9 ml) used per incubation. These cells were incubated with MESF or WASF of garlic (dissolved in saline solution) in a final volume of 1 ml at 42 C for 15 min. MESF was dissolved with 10 μg Tween 80 per assay. After incubation, the assay mixture was centrifuged at 5000 × g for 2 min at 4 C and the supernatant discarded. Homogenizing buffer (0.4 ml) was added, the cells were homogenized and processed to obtain the cytosolic and microsomal fractions.

Preparation of Tissues for Analyses

Homogenates of the liver and the sedimented hepatocytes from in vitro assays were prepared in 0.1 M potassium phosphate buffer, pH 7.4 containing 4 mM $MgCl_2$, 1 mM EDTA and 2 mM dithiothreitol. Livers were chopped and suspended in the buffer (1:2, w/v). Homogenization was done with a Polytron homogenizer. The 100,000 × g supernates (cytosolic fraction) and the microsomal fractions were stored at −20 C until they were assayed for enzymatic activities (18,19). Protein concentrations were estimated by a modification of the Biuret method using the bovine serum albumin as a standard (20).

Enzyme Assays and Estimation of Cholesterol in Serum

Assays for HMG-CoA reductase (EC 1.1.1.34), cholesterol 7α-hydroxylase (EC 1.14) and fatty acid synthetase were carried out as reported previously (18,19).

Serum cholesterol concentrations were estimated using Worthington "Cholesterol Reagent" set obtained from Worthington Diagnostics Division of Millipore Corporation, Freehold, NJ.

LDL and very low density lipoproteins (VLDL) were isolated from the serum (100 μl) by precipitation with a mixture of phosphotungstic acid 9.7 mM (10 μl) + $MgCl_2$ 0.4 M (10 μl). After standing for 5 min at room temperature, the mixtures were centrifuged at 2000 × g for 10 min, the supernatant was removed and was used to determine the level of cholesterol in HDL. The precipitate was dissolved in 0.1 M sodium citrate buffer (100 μl) and the level of cholesterol (LDL + VLDL) was determined using the above method.

Expression of Data and Statistical Methods

Enzyme data are presented as specific activities (units/mg cytosolic or microsomal protein/min). Statistical comparison of results was performed by a one-way analysis of variance (21).

RESULTS AND DISCUSSION

In those parts of the world where unrefined cereals and vegetable products form the major part of the diet, the incidence of cardiovascular disease is much lower than that found in the American population. Garlic has been credited since the days of ancient Rome with special health benefits (10-12), when used in trace amounts in food preparations. A number of investigators report that consumption of garlic reduces serum cholesterol levels in cholesterol- or lard-fed rats and rabbits (10-15,22). The significant decreases in the activities of HMG-CoA reductase, 7α-hydroxy, and FAS by feeding MESF or WASF of garlic at a 5% level in low cholesterol chicken diets (16), prompted us to determine the effective levels of these fractions in chickens. Male/female broiler chickens were used in the current studies due to their higher rate of feed efficiency conversion to muscle and lower rate of catabolism compared to those of White-Leghorn chickens (16).

Chickens were fed a normal corn-soybean diet consisting of corn (70.0%) and soybean (23.0%) as the major source of protein (Table 1). This diet was supplemented with MESF or WASF methanol or garlic equivalent to 1.0, 2.0, 4.0, 6.0 and 8.0% of fresh garlic paste. Weight gain and feed consumption are shown in Table 1. Weight gain was suppressed with increasing concentrations of the garlic fractions. Feed consumption by all experimental groups was slightly lower than that of the control group. The maximum suppression of weight gain (−8%) was found with 0.72% of MESF and 1.60% WASF equivalent to 8.0% of fresh garlic. Birds which were fed higher doses of these fractions (10, 15, 20%) did not show a further decrease in weight gain (unpublished results), and exhibited no visible evidence of any abnormal change in any organ upon sacrifice.

A dose-related decrease in activity was observed for each of the rate-limiting enzymes for the synthesis (HMG-CoA reductase) and the degradation (7α-hydroxy) of cholesterol and for fatty acid synthetase over the range of the concentrations of these fractions (Table 2). Values ranged from 14% to 42%, 11% to 36% and 3% to 54%, respectively, compared to the control. These results may reflect only in vivo response to the lowering of the substrate pool in liver effected by the inhibition of the biosynthetic activities of both cholesterogenesis and lipogenesis in liver.

These effects were accompanied by significant decreases in serum cholesterol levels (−18% to −25%), compared to control (Table 2). The levels of chol-HDL were not changed but significant decreases were found in chol-LDL levels with WASF of garlic (−32% to −37%) compared to the control. These values are also reflected in the ratios of total-cholesterol to chol-LDL 2.28 vs 2.99 (24% decrease) and chol-LDL to chol-HDL, 0.89 vs 1.39 (36% decrease), compared to control (Table 2). Similar inhibitions of these parameters were obtained with MESF of garlic. The most effective dose of MESF and WASF for these inhibitions was found to be equivalent to 6.0% fresh garlic paste.

The relationship between chol-HDL, chol-LDL levels and the risk of coronary heart disease is now well established, whereas it is not so between dietary practices and cholesterol levels in the serum lipoprotein fractions. The protective effect of HDL is suggested to lie in its role in the removal of cholesterol from arterial tissue (23,24). A decrease in the serum cholesterol level caused by antihypercholesterolemic agents is usually accompanied by the reduction of serum chol-LDL (25). The significant decrease in the chol-LDL affected by the addition of WASF of garlic to a corn-soybean based diet suggests that these materials might have a similar or closely related mode of action.

The effects of MESF and WASF of garlic on lipid metabolism were tested in isolated hepatocytes of female or male broiler chickens, which were incubated with 25-300 μg of each of the fractions for 15 min. Dose-related decreases in the activities of HMG-CoA reductase and FAS were obtained with increasing concentration of these fractions (Table 3) or time of incubation using 100 μg, 5-60 min, (Table 4) in these hepatocytes. The maximum inhibition of these enzymes occurred within 20 min, in incubation containing 100 μg of each of these fractions (Table 4). Slightly lower activities of these two enzymes were found when incubated with MESF compared to WASF (Tables 3 and 4), which is due to the presence of Tween-80 for dispersing MESF in the incubation. Moreover, the activities of these enzymes were also slightly higher in hepatocytes prepared from female broiler chickens (Table 3) than male chickens (Table 4).

TABLE 2

Effect of Different Concentrations of Methanol- and Water-Soluble Fractions of Garlic on Hepatic Enzyme Activities of Cholesterol and Serum Lipids in 8-Week-Old Male Broiler Chickens[a]

WASF of garlic[a]				Concentration (serum mg/100 ml)		
Concentration (%)	HMG-CoA reductase[b]	Cholesterol 7α-hydroxylase[c]	Fatty acid synthetase[d]	Total cholesterol	Chol-HDL	Chol-LDL
Corn (control)	332±24[f] (100)[e]	2.8±0.1[f] (100)[e]	126± 4[f] (100)[e]	165±14[f] (100)[e]	55.2±3.7[f] (100)[e]	76.8±7[f] (100)[f]
Corn+0.20	290±23[f] (86)	2.5±0.2[f] (89)	122± 4[f] (97)	136±10[g] (82)	53.6±4.1[f] (97)	52.5±5[g] (68)
Corn+0.40	280±25[f] (84)	2.4±0.1[f] (86)	118± 6[fg] (94)	131± 8[g] (79)	54.1±4.3[f] (98)	50.2±6[g] (65)
Corn+0.80	200±20[g] (60)	2.3±0.1[f] (82)	117±10[fg] (93)	130±10[g] (79)	53.2±4.2[f] (96)	48.3±5[g] (63)
Corn+1.20	200±20[g] (60)	1.9±0.1[g] (68)	112± 6[g] (89)	126± 8[g] (76)	53.5±4.5[f] (92)	47.6±4[g] (62)
Corn+1.60	190±20[g] (58)	1.8±0.2[g] (64)	96± 3[h] (76)	123± 6[g] (75)	53.9±5.0[f] (98)	48.1±6[g] (63)

[a]Feeding period was 3 weeks; time of killing was 0800; data expressed as mean±SD: N=8 chickens per group; HMG-CoA reductase=β-hydroxy-β-methylglutaryl-CoA reductase. WASF=water-soluble fractions of garlic. Amount is equivalent to 1, 2, 4, 6, 8% of fresh garlic paste. Similar data was obtained with MESF=methanol-soluble fractions of garlic.
[b]pmol of mevalonic acid synthesized/min/mg of microsomal fraction.
[c]pmol of [^{14}C]cholesterol into [^{14}C]7α-hydroxycholesterol/min/mg of microsomal fraction.
[d]nmol of NADPH oxidized/min/mg of cytosolic fraction.
[e]Percentage of respective control activity data are in parentheses.
[f-h]Values not sharing a common superscript letter are different at $p<0.05$.

The present data confirmed the in vivo studies by these fractions of garlic and the inhibition of lipid biosynthesis was independent of age and sex of the birds.

The present studies revealed the inhibition of cholesterol and fatty acid biosynthesis in vivo and in vitro by methanol- and water-soluble fractions of garlic, followed by the significant lowering of serum cholesterol and chol-LDL levels in chickens. Further studies in relation to the effects of these fractions of garlic, and their components on lipid metabolism are in progress.

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TABLE 3

Effect of Different Concentrations of Methanol- and Water-Soluble Fractions of Garlic on the Enzymic Activities of β-Hydroxy-β-Methylglutaryl-CoA Reductase and Fatty Acid Synthetase in Isolated Hepatocytes of Female Chickens[a]

Methanol- or water-soluble fractions of garlic	β-Hydroxy-β-methylglutaryl-CoA reductase[b]		Fatty acid synthetase[c]	
Concentration (μg)	Methanol-soluble fraction	Water-soluble fraction	Methanol-soluble fraction	Water-soluble fraction
0.0	22.5 (100)[d]	24.3 (100)[d]	69.9 (100)[d]	77.8 (100)[d]
25.0	15.3 (68)	14.5 (60)	68.8 (98)	75.2 (97)
50.0	14.7 (65)	14.0 (58)	65.2 (93)	71.3 (92)
75.0	11.2 (50)	10.2 (42)	59.7 (85)	65.4 (84)
100.0	10.4 (46)	9.5 (39)	53.8 (77)	61.2 (79)
200.0	9.7 (43)	9.0 (37)	46.7 (67)	54.3 (70)
300.0	8.6 (38)	9.1 (37)	44.4 (64)	52.2 (67)

[a]Eight-week-old female chickens were fed standard corn-soybean diets. They were fasted for 48 hr and refed 72 hr prior to the preparation of liver perfusion. Incubation period was 15 min. Values represent means of replicate within incubation set.
[b]pmol of mevalonic acid synthesized/min/mg of microsomal fraction.
[c]nmol of NADPH oxidized/min/mg of cytosolic fraction.
[d]Percentage of respective control activity data are in parentheses.

TABLE 4

Effect of Length of Incubation with Methanol- and Water-Soluble Fractions of Garlic on the Enzyme Activities of β-Hydroxy-β-Methylglutaryl-CoA Reductase and Fatty Acid Synthetase in Isolated Hepatocytes of Male Chickens[a]

Incubation time (min)	β-Hydroxy-β-methylglutaryl-CoA reductase[b]		Fatty acid synthetase[c]	
	Methanol-soluble fraction	Water-soluble fraction	Methanol-soluble fraction	Water-soluble fraction
0	15.5 (100)[d]	18.5 (100)[d]	52.0 (100)[d]	58.5 (100)[d]
5	13.0 (84)	14.5 (78)	46.0 (88)	52.5 (90)
10	11.5 (74)	11.0 (59)	44.0 (85)	46.0 (79)
15	9.5 (61)	10.0 (54)	40.5 (78)	43.5 (74)
20	8.5 (55)	9.5 (51)	38.4 (73)	41.0 (70)
40	8.0 (52)	9.0 (49)	37.0 (71)	41.0 (70)
60	8.0 (52)	9.0 (49)	35.5 (68)	37.0 (63)

[a]Eight-week-old male chickens were fed standard corn-soybean diets. They were fasted for 48 hr and refed 72 hr prior to the preparation of liver perfusion. Each incubation contains 100 μg of methanol- or water-soluble fractions of garlic; value represents means of replicate within incubation set.

[b]pmol of mevalonic acid synthesized/min/mg of microsomal fraction.

[c]nmol of NADPH oxidized/min/mg of cytosolic fraction.

[d]Percentage of respective control activity data are in parentheses.

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Fatty Acid Metabolism and Cell Proliferation. III. Effect of Prostaglandin Biosynthesis either from Exogenous Fatty Acid or Endogenous Fatty Acid Release with Hydralazine

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ABSTRACT

Primary cultures of smooth muscle cells were established from the medial layer of guinea pig aorta. Cells were seeded at from 40 to 80 cells per cm^2 and cloned for 8 days. Media were analyzed for PGI_2 (6-keto-$PGF_{1\alpha}$) using radioimmunoassay. Prostanoids were synthesized when cells were grown in media alone. Arachidonic acid stimulated prostanoid synthesis and promoted cell proliferation. Indomethacin blocked prostanoid synthesis and abolished the stimulatory effect of arachidonic acid on cell proliferation. Hydralazine stimulated fatty acid release and prostanoid synthesis in confluent cells. Hydralazine also stimulated prostanoid synthesis and promoted proliferation in growing cells. Indomethacin blocked prostanoid synthesis and abolished the stimulatory effect of hydralazine on cell proliferation.
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INTRODUCTION

Several studies have shown that low concentrations of arachidonic acid [20:4(n-6)] promote the proliferation of a number of cell lines in tissue culture. These cell lines include HeLa cells (1), XS 63.5 cells (2), 7,12-dimethylbenz(a)anthracene tumor cells (3,4) and smooth muscle cells (5). Arachidonic acid is the precursor of prostaglandins and prostacyclin (PGI_2). A number of studies have shown that prostaglandins either promote (5-8) or inhibit (5-7,9-12) cell proliferation depending on the concentration of prostaglandin added to, or generated by, the cells in tissue culture. Other studies have suggested that PGI_2 either promotes (13) or inhibits (14,15) cell proliferation. In the present investigation, we have examined the effects of exogenous 20:4(n-6) and endogenous 20:4(n-6) release on the cellular biosynthesis of prostaglandins and on the proliferation of aorta smooth muscle cells.

MATERIALS AND METHODS

Materials

Arachidonic acid was purchased from NuChek (Elysian, MN) and was shown to be peroxide-free by thin layer chromatography (5). Hydralazine HCl and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). Reference prostanoids were kindly supplied by Dr. J. Pike. Antiserum for the radioimmunoassay (RIA) of 6-keto-$PGF_{1\alpha}$ was kindly supplied by Dr. L. Levine. Antimyosin (Chicken gizzard) was kindly supplied by Dr. U.G. Stewart.

Tissue Culture

Primary cultures of smooth muscle cells were established from the dissected medial layer of guinea pig aorta from prepubertal males (5,16). Smooth muscle cells were identified by their reactivity to antibodies prepared from smooth gizzard muscle (17). The medium used for growing cells to confluency (growth medium) was prepared from 1X Eagle's minimum essential medium containing Hank's salts and 25 mM HEPES buffer (GIBCO, Grand Island, NY) supplemented with 50 μg per ml gentamycin sulfate (Schering, Kenilworth, NJ), 2 mM glutamine, 1X nonessential amino acids (Microbiological Associates, Walkersville, MD), 1 mM pyruvate, and 1.3 mg per ml of sodium bicarbonate. This medium was supplemented with 10% fetal bovine serum (FBS) (Sterile Systems, Logan, UT: Hyclone, lots 100331 and 100348). The medium in cell proliferation and prostanoid experiments (experimental medium) consisted of growth medium supplemented with either 10% or 20% FBS, 1X essential amino acids, and essential vitamins.

Arachidonic acid was dissolved in 95% ethanol and diluted 1:500 with experimental medium. Hydralazine HCl and indomethacin were dissolved in 95% ethanol and diluted 1:2500 with the medium. Control cultures were treated with experimental medium containing the same amount of ethanol.

Cell Proliferation

Smooth muscle cells, passage number 4 and 3-5 days postconfluent, were seeded at low densities (from 40 to 80 cells per cm^2) in Falcon single-well plates (60 × 15 mm) or Costar tissue culture dishes (60 × 15 mm). Cells were allowed to attach to the plastic petri plates for one day before initial treatments. Cells were retreated with a media change at day 5 of the incubation period. After an 8-day incubation period, cells were fixed in 2.5% phosphate-buffered glutaraldehyde or 3.7% phos-

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phate-buffered formalin and stained with filtered Giemsa.

Total cell area was measured by image analysis using the Optomax Visual Analysis System. A relative cell count was obtained from the total cell area on the plate. The relationship between cell area and cell number was validated both with a microscope (18) and with a Coulter counter (19). When cultures were grown in medium alone, typical Coulter counter data gave 137,000 ± 6,600 cells per plate. The cell number depended on the primary culture and the source of FBS. Cells from the same primary cultures and batch of growth medium were compared in each treatment series.

Prostaglandin and Prostacyclin Biosynthesis

Media were obtained from confluent cultures (Corning T-25 flasks) or cloning cultures (Costar tissue culture dishes). Media aliquots from eight Costar dishes were pooled for analysis in cloning experiments. PGI_2 was measured as the 6-keto-$PGF_{1\alpha}$ metabolite by a standard RIA procedure (20). The cross-reactivity of the 6-keto-$PGF_{1\alpha}$ antibody was: PGE_2, 0.15%; PGD_2, 0.02%; $PGF_{2\alpha}$, 0.10%; arachidonic acid, 0.005%. Data for 6-keto-$PGF_{1\alpha}$ are reported both as nmol/culture and relative concentration in percent.

RESULTS AND DISCUSSION

Effect of Fatty Acids on Cell Proliferation

Cells were cloned in media alone or media containing 10 μM 18:2(n-6), 20:3(n-6) or 20:4(n-6). These fatty acids were not interconverted because smooth muscle cells in culture have no desaturase activity (21,22). Only 20:4(n-6) enhanced cell proliferation (Fig. 1). Previous studies from our laboratory show that prostanoids, synthesized from 20:4(n-6) but not 20:3(n-6), enhance smooth muscle cell proliferation (5,23). The data in Figure 1 suggest but do not prove that 20:4(n-6) stimulates cell proliferation through prostanoid synthesis rather than a generalized fatty acid effect that would be found with other related polyunsaturated fatty acids.

Prostanoid Biosynthesis in Cell Cultures

Previous studies have shown that hydralazine enhances prostanoid biosynthesis in tissues and cell cultures (24,25). We find that hydralazine enhances prostanoid biosynthesis (6-keto-$PGF_{1\alpha}$) when it is added in media alone to confluent smooth muscle cells (Fig. 2). Hydralazine does not stimulate prostanoid synthesis when it is added in media together with excess 20:4(n-6) (Fig. 2). Similar results are obtained with 6-keto-$PGF_{1\alpha}$ and PGE_2 (data not shown). These data show that hydralazine

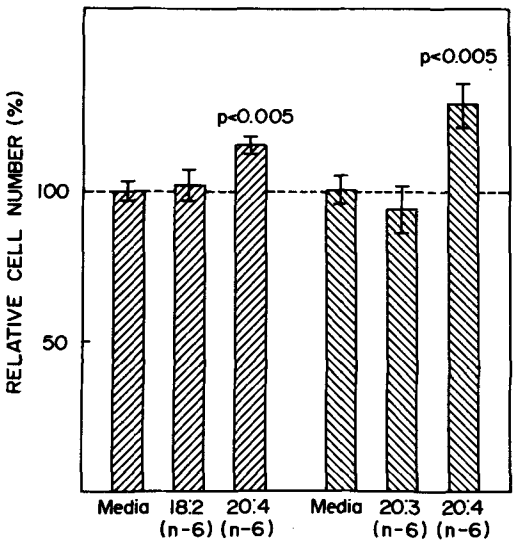

FIG. 1. Effect of 18:2(n-6) and 20:3(n-6) and 20:4(n-6) on cell proliferation. Cells were cloned for 8 days in Costar tissue culture dishes containing media alone or media supplemented with 10 μM fatty acid. Each group contained 8 plates. Data are expressed as relative cell number compared to control (media alone). Vertical lines show SEM. Treatment groups that differed significantly (Student t-test) from the control group are noted in the figure.

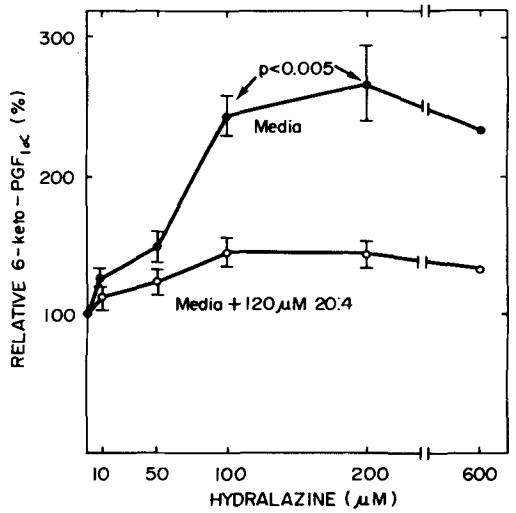

FIG. 2. Effect of hydralazine concentration on PGI_2 (6-keto-$PGF_{1\alpha}$) biosynthesis in confluent cultures of smooth muscle cells incubated for 24 hr in media alone or media containing 120 μM 20:4(n-6). Data are expressed as relative 6-keto-$PGF_{1\alpha}$ amount compared to control (media without hydralazine). Control cultures contained 285 ± 51 pmol/culture with media alone and 2190 ± 280 pmol/culture with 120 μM 20:4(n-6). Data represent mean ± SEM for 8 different primary cultures. Vertical lines show SEM. Significant differences (Student t-test) are noted in the figure.

stimulates fatty acid release rather than fatty acid conversion to prostanoids.

Exogenous 20:4(n-6) and endogenous fatty acid release through hydralazine both enhance 6-keto-$PGF_{1}\alpha$ levels in growing cultures (Fig. 3). Indomethacin blocks these effects. Similar results are obtained for 6-keto-$PGF_{1}\alpha$ and PGE_2 (data not shown).

Relationships between Prostanoid Biosynthesis and Cell Proliferation

Exogenous 20:4(n-6) (Figs. 1 and 4) and hydralazine (Fig. 5) both stimulate cell proliferation. Indomethacin blocks the stimulatory effects of 20:4(n-6) and hydralazine on cell proliferation (Figs. 4 and 5) just as it blocks enhanced prostanoid synthesis in these cultures (Fig. 3).

It is difficult to establish a causal relationship leading from an effector through prostaglandin biosynthesis to cell proliferation. Studies with exogenous 20:4(n-6) and endogenous fatty acid release through hydralazine indicate a causal relationship and other studies in the recent literature support this hypothesis. For example, vasopressin enhances prostaglandin synthesis in cell cultures (26) and this agent promotes the proliferation of 3T3 fibroblasts (27).

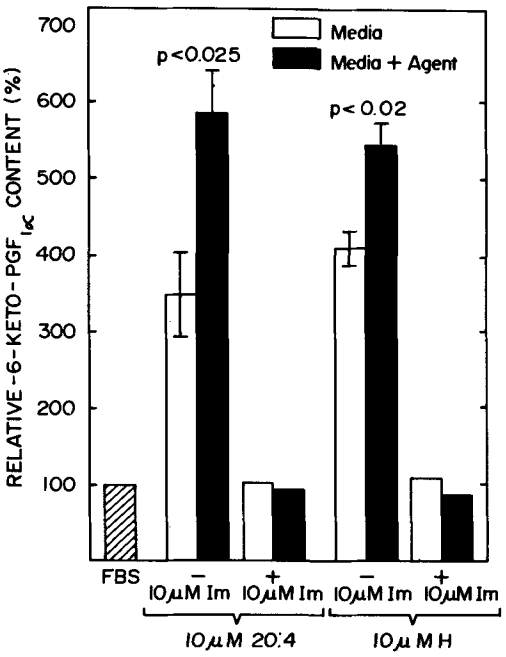

FIG. 3. Effect of either 20:4(n-6) or hydralazine (H) with or without indomethacin on PGI_2 biosynthesis in growing cultures. Cells were cloned either in 10% or 20% FBS. Data are expressed as relative 6-keto-$PGF_{1}\alpha$ content compared to FBS. Media with 20% FBS contained 3.59 ± 0.14 pmol/culture (mean $\pm$ SEM for 24 samples). Vertical lines show SEM for data from 3 different primary cultures. Significant differences are noted in the figure.

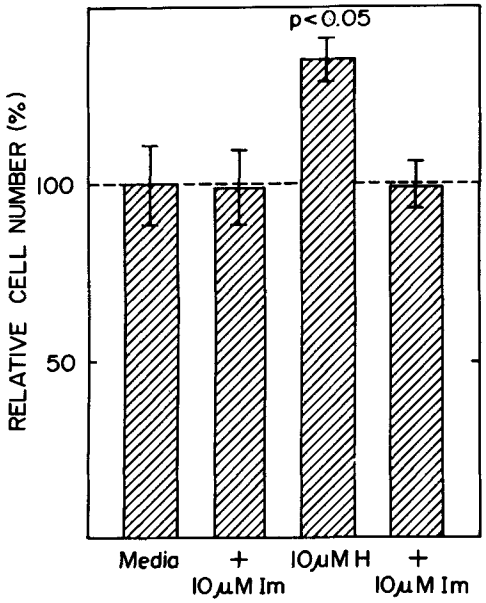

FIG. 4. Effect of 20:4(n-6) and indomethacin (Im) on cell proliferation. Cells were cloned for 8 days in Costar tissue culture dishes containing media alone or media supplemented with 20:4(n-6) and/or indomethacin. Each group contained 8 plates. Data are expressed as relative cell number compared to control (media alone). Vertical lines show SEM. Significant differences are noted in the figure.

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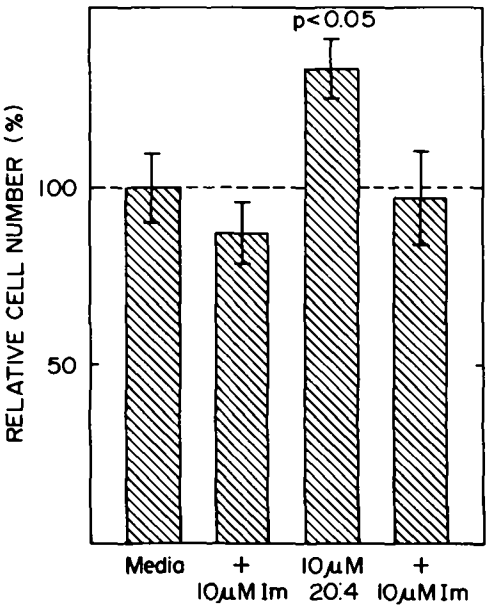

FIG. 5. Effect of hydralazine (H) and indomethacin (Im) on cell proliferation. Cells were cloned for 8 days in Costar tissue culture dishes containing media alone or media supplemented with hydralazine and/or indomethacin. Each group contained 8 plates. Data are expressed as relative cell number compared to control (media alone). Vertical lines show SEM. Significant differences are noted in the figure.

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Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: VIII. Volatile Thermal Decomposition Products of Hydroperoxy Cyclic Peroxides

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ABSTRACT

Secondary oxidation products are important sources of volatiles because of their susceptibility to further decomposition. Volatiles from the thermal decomposition of hydroperoxy cyclic peroxides have been identified by capillary gas chromatography followed by mass spectrometry (GC-MS). By using a saturated hydroperoxy cyclic peroxide as a synthetic model, the thermal decomposition pathways have been elucidated. Main cleavage occurs between the peroxide ring and the carbon-bearing hydroperoxide group. Volatiles produced were generally similar to those from corresponding monohydroperoxides. New volatiles identified included methyl furan octanoate, methyl ketones, and conjugated diunsaturated aldehyde esters. The general fragmentation observed between the peroxide ring and the hydroperoxide-bearing carbon is sufficiently predictable that it can be used as a tool for the structural characterization of hydroperoxy cyclic peroxides. Hydroperoxy cyclic peroxides from autoxidized and photosensitized oxidized methyl linolenate are suggested as important precursors of volatiles that may affect flavor quality of lipid-containing foods. *Lipids* 18:353-357, 1983.

Monohydroperoxides formed as primary products of autoxidation and photosensitized oxidation of unsaturated lipids are generally recognized as the principal precursors of volatile products causing a decrease in flavor quality and safety of foods (1-4). Studies on the decomposition of pure hydroperoxides showed that a major part of the volatiles can be accounted for by the classical scheme involving carbon-carbon scission on either side of alkoxy radical intermediates (5,6). However, recent evidence showed that this scheme can become complicated by further reactions or interactions of the hydroperoxides (4,6). The fundamental mechanisms of flavor deterioration in oxidized fats are still poorly understood (4,7,8). A better understanding is needed of the mode of decomposition of lipid oxidation products to provide the basis for predicting the course of the reactions contributing to flavor problems.

In the previous paper of this series (6), the hydroperoxy cyclic peroxides (epidioxides) from methyl linoleate (photosensitized oxidation) and methyl linolenate (both autoxidation and photosensitized oxidation) were suggested as important precursors of volatiles. Direct evidence for the contribution of hydroperoxy epidioxides to volatile lipid oxidation products was reported in studies of the thermal decomposition of these secondary products in photosensitized oxidized methyl linoleate (9). This paper presents an extension of these studies to clarify further the decomposition pathways and the role of secondary oxidation products on flavor deterioration of lipids. Secondary oxidation products investigated include the hydroperoxy epidioxides synthesized from methyl ricinoleate (10), those isolated from autoxidized and photosensitized oxidized methyl linolenate (11,12), and the hydroperoxy *bis*-epidioxides from photosensitized oxidized methyl linolenate (12,13).

EXPERIMENTAL

The synthesis of saturated hydroperoxy epidioxides from methyl ricinoleate was described previously (10). The allylic unsaturated hydroperoxy epidioxides were isolated and purified from oxidized methyl linolenate. The secondary oxidation products were first separated by silicic acid chromatography and combining the polar fractions eluting after monohydroperoxides. The hydroperoxy epidioxides were then purified by high pressure liquid chromatography (HPLC) (11,12) into positional isomers. These cyclic peroxides were shown to be functionally pure by thin layer chromatography (11). Any diastereomers separated were combined prior to decomposition.

The fractions of hydroperoxy epidioxides were decomposed in the injector port of a capillary gas chromatograph-mass spectrometer system at 200 C by interrupting the carrier flow for 30 sec. The gas chromatograph was a Perkin-Elmer Model Sigma 3 (Norwalk, CT). The capillary column, 0.32 mm × 15 m, was of fused silica with a 1.0 μm film of polymethyl and phenyl siloxane Durabond DB-5 bonded phase (J&W Scientific, Rancho Cordova, CA), programmed from 25 to 250 at 5 C/min with an initial hold of 5 min. The mass spectrometer was a Kratos MS 30 with glass separator (Manchester, England), taking one scan every 4 sec, 70eV EI

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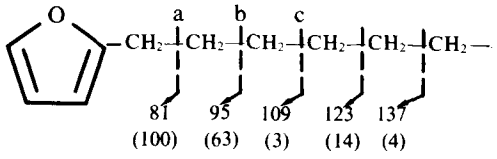
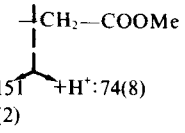
[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture over other firms or similar products not mentioned.

spectra. The volatile decomposition products were identified by computer matching mass spectra with those of reference compounds and confirming by GC retention data (14).

RESULTS AND DISCUSSION

The saturated hydroperoxy epidioxide (**I**) prepared from methyl ricinoleate (10) forms a good model to elucidate the type of cleavage caused by thermal decomposition. The fragmentation scheme shown in Figure 1 accounts for 80% of the total volatile products identified by capillary GC-MS after decomposition of saturated cyclic peroxide **I** (Table 1). Cleavage A between the peroxy ring and the alkoxy radical (derived from the hydroperoxide), and cleavage B between the peroxy ring and the alkyl radical, are the most important. Methylfuran octanoate [methyl 8-(2-furyl)-octanoate] expected by cleavage B is identified by formation of characteristic ions, m/z (relative %): M^+, 224 (14.7); M-31, 193 (8.1), and fragmentation according to the following scheme:





(2)

The importance of cleavages a, b and c above has also been reported for disubstituted C-18 furan esters and monosubstituted furans (15).

Less favorable cleavages C and D (Fig. 1) involving the peroxy ring produce heptanal on one hand, and 2-octanone on the other hand, that may rearrange into octanal by oxygen migration from carbon-2 to carbon-1 through an epoxy intermediate, R-CH(O)-CH2, or a ketene intermediate, R-CH=C=O (16,17). Ring opening rearrangement of oxiranyl to α-keto radicals has also been reported (18).

The formation of heptane may be explained by cleavage E involving the addition of two hydrogen radicals. This cleavage is apparently unique to saturated cyclic peroxides. Cleavage A may produce 2-nonenal by loss of a hydroxyl radical (Fig. 1). The importance of cleavage A agrees with previous observations made with the unsaturated hydroperoxy cyclic peroxides from linoleate (9). However, cleavage B was less important because allylic unsaturation was present between the peroxy ring and the alkyl chain.

Fragmentations of conjugated dienoic hydroperoxy epidioxides **II** and **III** are more complex than for our Model **I**. Fragmentation schemes in Figure 2 account for 87% (peroxide **II**) and 76% (peroxide **III**) of the total volatiles identified (Table 1). Cleavages between peroxy ring and alkoxy radical (A in **II** and B in **III**) are, as expected, most important. Cleavage on the other side of the peroxy ring becomes much less important because of the

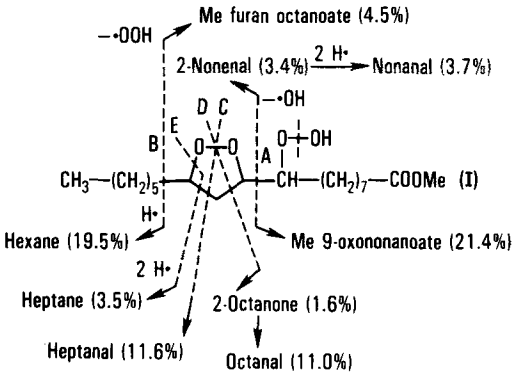
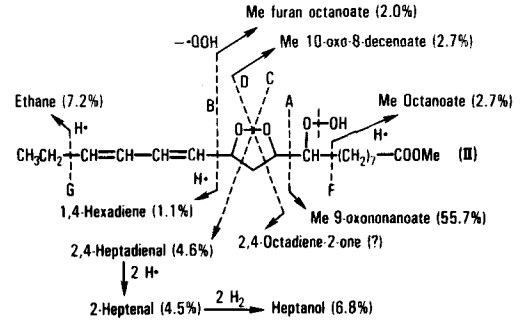


Fig. 1. Thermal decomposition cleavage of methyl 9-hydroperoxy-10,12-epidioxy-octadecanoate (**I**).



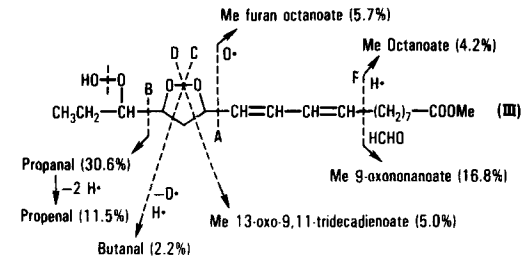
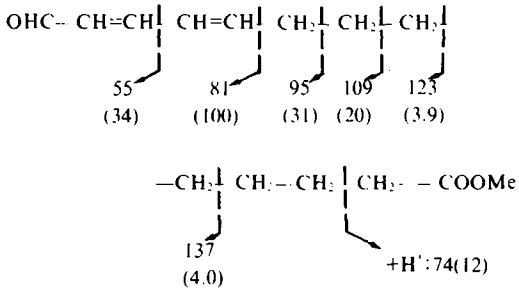


Fig. 2. Thermal decomposition cleavage of methyl 9-hydroperoxy-10,12-epidioxy-*trans*-13,*cis*-15-octadecadienoate (**II**) and methyl 16-hydroperoxy-13,15-epidioxy-*cis*-9,*trans*-11-octadecadienoate (**III**).

presence of the allylic diene system. The formation of methyl furan octanoate in **II** can be rationalized by cleavage B as in epidioxide **I**. However, the formation of the same furan ester from epidioxide **III** may be explained by a different mechanism involving hydroperoxidation of the conjugated diene radical formed by cleavage A, followed by cyclization of an alkoxy radical intermediate (4). Peroxide ring cleavage C is more significant in **II** than in **III**, and the converse is true for cleavage D. However, the diunsaturated ketone expected by cleavage D from epidioxide **II** was not identified. The conjugated diene C-13 aldehyde ester expected from **III** by cleavage D is identified by the characteristic ions, m/z (relative %); M^+, 238 (7.2); M-31, 207 (3.8); M-32, 206 (4.6), and the following fragmentation:



The corresponding C-12 conjugated diene aldehyde ester expected from cleavage A (Table 1) has the same MS fragmentation.

Cleavage F explains the formation of methyl octanoate in **II** and **III**. To explain the formation of significant amounts of methyl 9-oxononanoate from **III**, a reaction between the octyl ester radical is postulated with formaldehyde (Fig. 2). Formaldehyde is indeed a commonly observed degradation product of unsaturated lipids (4).

Fragmentations of the *bis*-epidioxides **IV** and **V** follow the same pattern as the corresponding monocyclic peroxides. The schemes suggested in Figure 3 account for 80% of the volatiles identified from **IV** and 84% of the volatiles from **V** (Table 1). As with monocyclic peroxides, cleavage A is most important in *bis*-epidioxide **IV** and cleavage B in **V**. The allylic unsaturation in **V** makes cleavage D' as important as cleavage A. Other cleavages C and D involving the peroxy ring are similar to those observed with the monoepidioxides. Cleavage G between the two peroxide rings apparently results in the formation of small amounts of methyl 12-oxo-10-dodecenoate and methyl furan octanoate. The formation of this furan ester from **IV** can be postulated in the same way as the monoepidioxides **I** and **II** (Figs. 1 and 2). To explain the formation of methyl furan octanoate from **V**, a different mechanism is apparently necessary which involves the loss

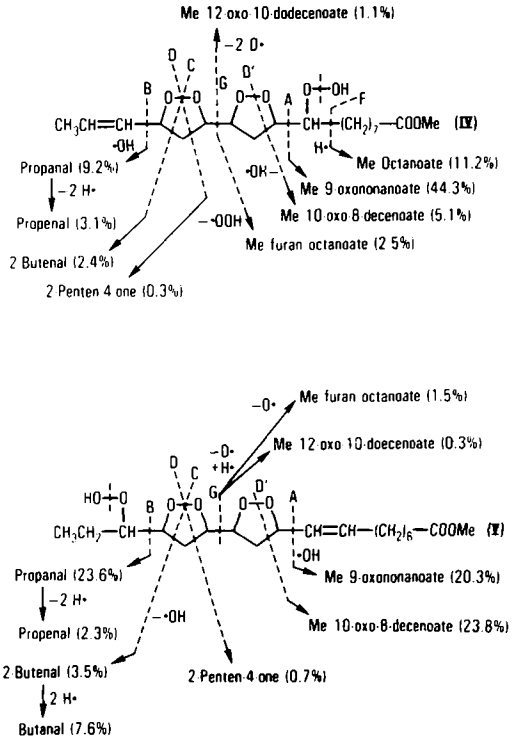


Fig. 3. Thermal decomposition cleavage of methyl 9-hydroperoxy-10,12,13,15-*bis*-epidioxy-*trans*-16-octadecenoate (**IV**) and methyl 16-hydroperoxy-10,12,13,15-*bis*-epidioxy-*trans*-8-octadecenoate (**V**).

of an oxygen radical. Cleavage G appears to be surprisingly unimportant by our experimental approach. However, other fragmentation products may be formed by cleavage G that were either not identified by our methodology or too unstable under our conditions. Nondestructive methods based on HPLC and NMR are now being investigated for this purpose.

To generalize the present work, hydroperoxy cyclic peroxides undergo thermal cleavage mainly between the peroxide ring and the hydroperoxide-bearing carbon. This fragmentation is sufficiently predictable that it can be used as a tool for the structural characterization of these types of cyclic compounds. This paper confirms our previous results (9) in providing direct evidence that hydroperoxy cyclic peroxides are important precursors of volatile oxidation products that may affect the flavor of foods containing polyunsaturated lipids.

ACKNOWLEDGMENT

We are indebted to R. D. Plattner and R. E. England for GC-MS analyses, and to C. R. Smith, Jr., for useful discussions.

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TABLE 1

GC-MS Analysis[a] of Volatiles from Thermally Decomposed Hydroperoxy Epidioxides (I-V, Figs. 1-3)

Volatile compounds	Relative retention	Relative percent (cleavage type)[b]				
		I	II	III	IV	V
Acetaldehyde	0.11	1.0	0.2	0.4	0.9	0.1
Ethane	0.12		7.2(G)	1.1		0.4
Acetone	0.12	0.5	0.6	0.4	3.4	0.6
Propanal	0.13			30.6(B)	9.2(B)	23.6(B)
Propenal	0.13		0.1	11.5(B)	3.1(B)	2.3(B)
Hexane	0.15	19.5(B)				
Butanal	0.16		0.8	2.2(C)	1.1	7.6(C)
2-Butenal	0.17		0.2		2.4(C)	3.5(C)
2-Pentanone	0.17				0.9	
Heptane	0.23	3.5(E)				
2-Penten-4-one	0.23				0.3(D)	0.7(D)
1,4-Hexadiene	0.24		1.1(B)	2.2		
Et Furan	0.24					1.3
2-Pentenal	0.24			1.2	0.1	1.7
2-Hexanone	0.32				0.1	1.4
Octane	0.37	1.6				
Hexanal	0.52			1.1		
2,4-Hexadienal[c]	0.58-0.61				0.5	
Heptanal	0.62	11.6(C)				
Me Hexanoate	0.64				0.2	0.9
2-Heptenal	0.74		4.5(C)		1.5	0.2
Heptanol	0.79	0.1	6.8			
2,4-Heptandienal[c]	0.80-0.81		4.6(C)		0.6	
Me Heptenoate	0.82				0.3	0.2
Me Heptanoate	0.83			0.1	0.4	0.2
2-Octanone	0.83	1.6(D)				
Octanal	0.85	11.0(D)				
Nonanal	0.95	3.7(A)				
Me Octenoate	0.99				0.4	0.2
Me Octanoate	1.00	0.8	2.7(F)	4.2(F)	11.2(F)	2.7
2-Nonenal	1.07	3.4(A)				
4-Heptanone	1.11	0.3				
2-Decenal	1.18	0.7				
Decanol	1.25	4.2				
Me 8-oxooctanoate	1.35			1.3	0.3	0.4
Me Decanoate	1.46	0.2				
Me 9-oxononanoate	1.53	21.4(A)	55.7(A)	16.8(F)	44.3(A)	20.3(A)
Me 10-oxodecanoate	1.63		0.2	0.9	0.6	0.3
Me 10-oxo-8-decenoate	1.71		2.7(D)	0.3	5.1(D')	23.8(D')
Me Furan octanoate[c]	1.73-1.74	4.5(B)	2.0(B)	5.7(A)	2.5(B)	1.5(G)
Me 11-oxo-9-undecenoate	1.84			4.3	1.1	0.9
Me 12-oxo-10-dodecenoate	1.90		1.2		1.1	
Me 12-oxo-8,10-dodecadienoate[c,d]	1.94-1.96			0.8(A)		0.2
Me 13-oxo-9,11-tridecadienoate[c,d]	2.15-2.19			5.0(D)		
Unidentified	-	10.1	8.7	6.6	9.3	4.2

[a]Quantitation based on flame ionization detection.
[b]Based on schemes in Figures 1-3.
[c]Separated *cis,trans* and *trans,trans* isomers are combined.
[d]Tentative identification.

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Effect of 6-Substituted Sterols on Sterol-induced Reproduction in *Phytophthora cactorum*

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ABSTRACT

6-Methylcholesterol and 6-ketocholestanol do not promote oospore development in *Phytophthora cactorum* when added as sole sterol supplement of media, and they inhibit development induced by cholesterol. 6-Fluorocholesterol promotes oospore formation; 6-chlorocholesterol does not, and neither inhibits cholesterol-induced development. A strain of *P. cactorum* has been found which is exceptional among Phytophthoras or Pythiums examined hitherto in forming numerous oospores with cholestanol. In this strain, 6-ketocholestanol inhibits cholestanol-induced oospore formation. These results indicate the importance of C-6 as a point of interaction with cellular sites controlling oospore formation.
Lipids 18:358-362, 1983.

INTRODUCTION

Sexual reproduction in the fungus *Phytophthora cactorum* requires the presence of sterols in the medium. In the absence of sterol, the fungus remains vegetative, but addition of sterols induces the development of oospores (1,2). The added sterol is taken up and metabolized by the fungus (3). A number of Δ^5- and Δ^7-sterols promote oospore development (4,5). The double bond at C-5 appears to be of particular importance. Thus, Δ^7-sterols are converted to Δ^5-sterols by the fungus (6), and inhibition of this conversion by the compound AY 9944 reduces oospore formation when Δ^7-ergostenol is the sterol added to the medium (7). Of perhaps greater significance, Δ^5-sterols are much more active than their saturated counterparts, e.g., cholesterol than cholestanol (8,9) and sitosterol than stigmastanol (5). It is suggested (7,10) that sterols are metabolized to hormones which are the active agents controlling reproduction. The hormone might require a double bond at C-5, or the double bond of the sterol could facilitate the introduction of other groups, e.g., a hydroxyl or a keto group at C-7. (Antheridiol and oogoniol, hormones of the fungus *Achyla,* are derived from fucosterol, and both are 7-oxo steroids (10).) The effects of several 6-substituted sterols on reproduction in *P. cactorum* has therefore been examined, on the supposition that substituents at this position would interfere with binding to a site important in controlling reproduction.

MATERIALS AND METHODS

Fungus Strains

Two strains of *P. cactorum* were used, one derived from IMI 21168 (from the Commonwealth Mycological Institute, Kew, Surrey, England) (= ATCC 46908); and IMI 270425 (originally from Dr. G.W.F. Sewell, East Malling Research Station, Maidstone, Kent).

Medium

The medium was that described by Elliott and Knights (3). Sterols were added in ether or acetone solution after the medium had been autoclaved.

Sterols

Cholestanol and 6-ketocholestanol were from Sigma. 6-Fluorocholesterol was the gift of Dr. T. C. McMorris. 6-Chlorocholesterol was synthesized from cholesteryl benzoate through 5α, 6α-dichlorocholestan-3β-yl benzoate according to the method of Barton and Miller (11). It formed colorless needles when recrystallized, after thin layer chromatography (TLC) purification, from chloroform/ethyl acetate, and melted at 150-151 C. It contained 0.2% cholesterol as an impurity. 6-Methylcholesterol was synthesized according to the method of Ushakov and Madaeva (12) by the reaction of methyl magnesium iodide on cholesterol-α-oxide (13), followed by the acid catalyzed *cis*-dehydration of the resulting 6β-methylcholestan-3β,5α-diol (14). It formed colorless needles when recrystallized from methanol and melted at 140-141 C.

Under the gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) conditions (on a 25 m fused silica capillary column coated with methyl silicone, and on a 12 m s/s capillary column coated with Dexil-300, and operated isothermally at 265 C and 250 C respectively) which resolved authentic mixtures of cholestanol and cholesterol TMS ethers, TMS ether of cholestanol used in these experiments (Sigma, Lot No. D1228-96) gave a single peak which was identified from its mass spectrum as cholestanol TMS. GC-MS instrument sensitivity in selected and multiple ion monitoring mode was such that 2-10 ng quantities of sterol TMS ethers could be detected without difficulty. Ultraviolet (UV) spectra of

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cholestanol and of its Liebermann-Burchard reaction mixture (8,15), and comparison of these with corresponding cholesterol standards, indicated that the cholestanol contained neither cholesterol nor other impurities, as have been reported to be present by other workers (16).

Assay of Oospore Production

The fungus was grown in plastic petri dishes, 50 mm diameter, containing 5 ml medium (solidified with 1% agar), inoculated centrally and incubated at 23 C. The numbers of oospores were counted in radial transects 1.05 mm wide, viewing a single plane of focus through the bottom of the dish.

Study of Metabolism of Sterols

The fungus was grown without shaking in 100 ml conical flasks containing 15 or 20 ml medium and inoculated either with a disc, 4 mm diameter, cut from an agar culture, or with a suspension of zoospores. The mycelium was freeze-dried, and extracted with chloroform/methanol (2:1, v/v). The extract was evaporated under vacuum, taken up in diethyl ether and chromatographed on silica gel plates. The free sterol zone was eluted, the trimethylsilyl derivatives prepared by reaction with N,O-*bis*-trimethylsilyl acetamide, and analyzed on a Kratos MS-30 mass spectrometer coupled to a Pye 104 gas chromatograph via a single-stage glass jet separator. In addition to the columns specified above, the main analyses were performed with a 30 m SP2250 SCOT column at 280 C. Helium was used both as a carrier gas and make-up gas giving a total flow of 25 ml/min. The source was operated at 1000 resolution, 70 eV and 280 C. The separator was maintained at 250 C. Spectra were scanned at 10 sec/decade. Sterol trimethylsilyl ethers were identified by their characteristic fragment ions and relative retention times. Selected and multiple ion monitoring using appropriate ions (at m/z 129, 255, 257, 327, 329, 343, 367, 368, 370, 384, 386, 396, 402, 458, 460, 472, 476, 492) were carried out and the responses compared with those of the standards.

RESULTS

Results of an experiment with strain IMI 21168 are given in Table 1. This shows that: (a) 6-methylcholesterol, added to media with either 2 or 8 mg/l cholesterol, inhibits oospore production; (b) 6-chlorocholesterol is not inhibitory with low concentrations of cholesterol and only slightly inhibitory with 8 mg/l cholesterol; (c) 6-ketocholestanol is inhibitory, but a considerably greater effect is evident with cholesterol at 2 mg/l than at 8 mg/l. (d) Cholestanol, included for comparison, showed, in agreement with earlier results (8), an inhibitory effect with 8 mg/l cholesterol, but at 2 mg/l cholesterol the addition of a small amount of cholestanol stimulates oospore production; larger amounts are inhibitory.

The effect of these sterols as sole supplement of the medium was as follows: with 6-methylcholesterol and 6-ketocholestanol, few normal oogonia (but a number of small abnormal ones) and no oospores were produced. Nes et al. (5) also found 6-ketocholestanol did not induce oospore formation. With 6-chlorocholesterol, many normal-looking oogonia were produced, but they all degenerated and no oospores were seen. With cholestanol, normal oogonia were produced, but most of them degenerated; an average of 1.1 oospores per transect was observed.

Experiments were also carried out with strain IMI 270425. With this strain, it was found that 6-fluorocholesterol stimulated oospore production as sole sterol supplement in a manner quantitively similar to cholesterol (Fig. 1). It did not inhibit oospore formation when added with cholesterol; rather, the number of spores increased (Fig. 2). A few oospores were formed with 6-chlorocholesterol as sole supplement (ca. one-fifth of the number formed with cholesterol). It had no effect on oospore production when added with cholesterol. 6-Methylcholesterol was strongly inhibitory when added to media with cholesterol (Fig. 2), and no oospores were seen when it was sole sterol supplement. 6-Ketocholestanol was inhibitory to oospore production when added to low concentrations of cholesterol (2 or 2.5 mg/l) but, at higher concentrations (8 or 10 mg/l), it appeared to be stimulatory (Fig. 3).

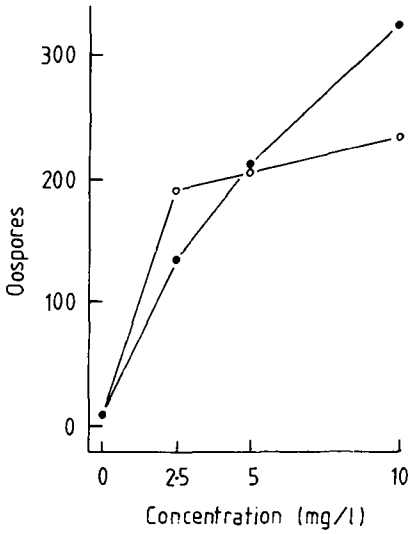

FIG. 1. Oospore production by *P. cactorum* (IMI 270425) with cholesterol (●–●) or 6-fluorocholesterol (o–o) as the sole sterol supplement of the medium.

TABLE 1

Oospore Production by *Phytophthora cactorum* (IMI 21168): Tests of Inhibitory Effects of 4 Sterols on Cholesterol-induced Oospore Formation[a]

Inhibiting sterol	Concentration of cholesterol (mg/l)	Concentration of inhibiting sterol (mg/l)				
		0	2	4	8	16
6-Methylcholesterol	2	41.5	31.3	10.0	0.1	0.7
	8	107.6	80.3	41.0	3.3	0.5
6-Chlorocholesterol[b]	2	41.5	34.7	43.3	32.4	32.9
	8	107.6	95.8	79.3	74.7	81.7
6-Ketocholestanol	2	41.5	26.0	2.6	0.5	0
	8	107.6	107.5	85.8	48.9	32.5
Cholestanol[c]	2	41.5	51.4	30.0	11.1	3.0
	8	107.6	83.7	69.5	41.2	35.9

[a]Values are means of 15 counts (generally 3 in each of 5 dishes).

[b]For 2 mg/l cholesterol, the differences in oospore count between concentrations of 6-chlorocholesterol are not significant compared to variation between replicate petri dishes. For 8 mg/l cholesterol, there are significant differences between concentrations but the linear component of the oospore number/concentration relationship is not significant.

[c]Differences between concentrations of cholestanol highly significant compared to differences between replicate dishes; linear and nonlinear regression components both significant for both 2 and 8 mg/l cholesterol. For 2 mg/l cholesterol, the oospore count at 2 mg/l cholestanol is significantly different ($P = .04$) from the count at 0 mg/l cholestanol.

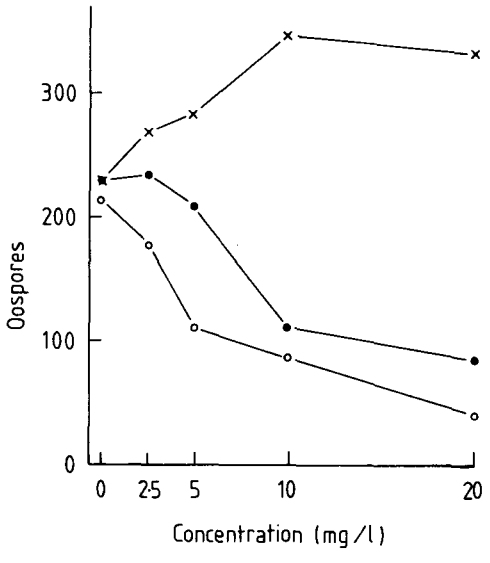

FIG. 2. Oospore production by *P. cactorum* (IMI 270425) with varying concentration of 6-fluorocholesterol added with 5 mg/l cholesterol (x–x) and with varying concentrations of 6-methylcholesterol added with 5 mg/l (●–●) or 2.5 mg/l cholesterol (o–o). For all 3 curves, the variation in numbers of oospores between concentrations is highly significant compared to variation between replicate petri dishes. Points are means of 20 counts.

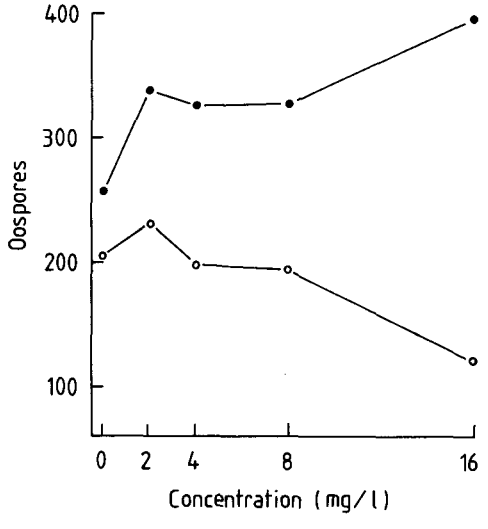

FIG. 3. Oospore production by *P. cactorum* (IMI 270425) with varying concentration of 6-ketocholestanol added with 2 mg/l (o–o) or 8 mg/l (●–●) cholesterol. For both curves, the linear component of the oospore number/concentration relationship is significant, and the nonlinear components nonsignificant. Points are the means of 12 counts, except for the two 0 mg/l 6-ketocholestanol points where there were 24 counts each, and for the 16 mg/l 6-ketocholestanol, 8 mg/l cholesterol point, where there were only 5 counts.

The notable difference between IMI 270425 and IMI 21168, however, was that the former produced numerous oospores with cholestanol, about as many as with cholesterol, and few abortive oogonia were seen. With IMI 270425, 6-ketocholestanol inhibited oospore production induced by cholestanol (Table 2). Cultures supplied with mixtures of cholestanol and 6-ketocholestanol had some oospores but many abortive oogonia. Plots of the data in Table 2 suggest that 6-ketocholestanol competes competitively with cholestanol for some cellular site controlling oospore development; thus, plotting the reciprocal of the number of oospores against the reciprocal of the cholestanol concentration (8) gives, for 6-ketocholestanol concentrations 3.75, 7.5, and 30 mg/l, lines whose intercepts on the abscissa do not differ significantly. Experiments of similar design, also using strain IMI 270425, showed that 6-methylcholesterol competes competitively with cholesterol for oospore-determining sites.

The free sterols in extracts of mycelium grown with 6-chlorocholesterol, 6-methylcholesterol, 6-ketocholestanol and cholestanol were examined. No sterol other than that added was found. The 6-chlorocholesterol contained a trace (0.2%) of cholesterol, but cholesterol was not present in the extract in greater amount than in the material added. No cholesterol was found in the extracts of mycelium of either IMI 270425 or IMI 21168 grown on cholestanol; there was a small peak in the scan for m/z 458 (cholestanol TMS ether - 2), but this had the retention time of cholestanol, not of cholesterol. Nes et al. (17) also found no conversion of cholestanol to cholesterol by their strain of *P. cactorum*.

DISCUSSION

Our hypothesis was that the activity of sterols in inducing oospore development depends either on a molecular configuration requiring the presence of a double bond at C-5, or that this double bond facilitates, or is required for, a reaction such as oxidation or hydroxylation at C-7. The hypothesis is supported by the ineffectiveness of sterols with bulky groups at C-6 (6-methylcholesterol and 6-ketocholestanol) in promoting oospore formation by themselves, and their inhibition of reproduction when mixed with the active sterol, cholesterol. An analogous and well known effect of a methyl substituent in mammalian systems is the inhibition by 2α-methylcortisol of the reduction of the C-4 double bond of cortisol (18-20). 6-Fluorocholesterol as sole supplement is not inhibitory to oospore development; it has an activity similar to cholesterol. 9-Fluorination of cortisol and related steroids does not reduce, and may even enhance, their physiological activity (21-23). Also, 2-fluoroadenosine is a better substrate than adenosine for adenosine kinase, whereas 2-methyladenosine is a poor substrate (24). The fluorine effect is attributed to the strongly electronegative character of the C-F bond, which is believed to enhance attachment of an enzyme to the substrate at the critical point near the substituted fluorine atom (21-23). 6-Chlorocholesterol has only weakly inhibitory effects on cholesterol-induced oospore development. The difference here between the effect of chlorine and fluorine substitution is similar to the differences observed elsewhere (21,24).

Strain IMI 270425 of *P. cactorum* is the first Phytophthora or Pythium found to produce abundant oospores with cholestanol. The usual effect with cholestanol is development of oogonia which mostly (> 95%) degenerate. There are evidently differences between strains in their steric requirements in ring B. One strain requires a double bond at C-5; in another, it is not obligatory. IMI 270425 does not convert cholestanol to cholesterol; at least, if it does, the cholesterol must be rapidly metabolized as it is not found in the free sterol fraction. It would now seem unlikely that IMI 21168 requires the double bond at C-5 to facilitate the insertion of groups at C-7, since the presence or absence of the double bond is a matter of indifference to IMI 270425. The observation that in IMI 270425 (but not in IMI 21168) 6-ketocholestanol appears sometimes to have a synergistic activity

TABLE 2

Oospore Production by *P. cactorum* (IMI 270425): Inhibitory Effect of 6-Ketocholestanol on Cholestanol-induced Oospore Formation

Concentration of cholestanol (mg/l)	Concentration of 6-ketocholestanol (mg/l)				
	0	3.75	7.5	15	30
2.5	69.9	19.9	15.9	0.6	4.5
5	177.3	29.0	21.5	13.6	5.4
10	–	41.9	40.5	17.9	19.4
15	143.1	72.0	45.3	22.0	20.9

Values are means of 8 counts.

with cholesterol is difficult to explain. However, 6-ketocholestanol inhibits cholestanol-induced oospore development. All the evidence thus indicates that this corner of the sterol molecule is still vitally concerned with attachment to some cellular site controlling oospore development.

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Variations of Fatty Acid Composition of Erythrocyte and Plasma Lipids in the Rat During the First Period of Life

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ABSTRACT

The changes occurring in the fatty acid composition of the erythrocyte lipids during the first weeks of life were studied in the rat. The major changes consisted of a progressive decrease in oleic acid and a progressive increase in linoleic acid. A lower but significant increase in arachidonic acid was also observed. These changes are not related to variations in erythrocyte age; rather, they appear to be related to the age of the animal. Since somewhat similar changes were observed in the fatty acid composition of the major lipid classes of plasma during the first weeks of life, the possibility that these variations could account for the changes in the fatty acid composition of erythrocyte lipids was considered. Some support to this possibility was found in the results of experiments in which erythrocytes taken from 15-day-old rats were incubated with plasma taken from newborn rats. The changes in the fatty acid composition of erythrocytes and plasma lipids do not appear to be dependent on dietary lipids, since they occur during the suckling period, i.e., before the rats begin to ingest the pelleted diet which presents a fatty acid pattern completely different to that of the dams' milk.

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INTRODUCTION

It is known that erythrocytes from newborn and adult subjects of various animal species and humans significantly differ in the fatty acid pattern of membrane lipids (1-4). However, little is known about the period of life in which such changes occur and about the metabolic mechanisms underlying these variations.

Early studies (2) indicated that differences in the composition of the ingested lipids account at least to a significant extent for the changes in the fatty acid pattern of red cell lipids. A significant variation in the ingested lipids, from both a quantitative and a qualitative point of view, occurs when infants undergo weaning after the suckling period (5). Differences have been also reported in the lipid composition (6-8) of dams' milk during suckling. It seemed, therefore, of interest to investigate the time-course variations of the fatty acid composition of both erythrocyte and plasma lipids in a laboratory animal, i.e., the rat, in which the dietary regime can be accurately controlled. The aim of the present study was to search any possible relationship between the erythrocyte and plasma lipids and the modifications of the dietary regime occurring during growth.

MATERIALS AND METHODS

Sprague-Dawley rats, maintained on a pelleted diet (Nossan, Correzzana, Milan, Italy) free of preservative compounds, were used. Male newborns of each litter were caged with the mother until weaning, at the 27th day of life. The young rats had free access to the pelleted diet since the 15th day of life. This experimental schedule was followed since it was observed previously that lack of access to the pelleted diet prior to weaning resulted in a significant decrease in weight gain of the young rats.

The blood was withdrawn from newborn rats, from 3, 5, 10, 15, 30 day old rats and from adult (220-250 g; 2 months old) rats, using heparin as anticoagulant. Blood samples were collected from the heart (in newborn and 3, 5 day old rats) or from the abdominal aorta (in 10, 15, 30 day old and in adult rats). Except for adult rats, each blood sample was derived from a number (3-6) of animals.

Rat erythrocytes were prepared as follows: blood samples were centrifuged at 1100 g for 5 min at room temperature. The plasma and the buffy coat was discarded. The packed red cells were washed 3 times by resuspension in 0.9% NaCl and resedimentation as above. The final sedimentation was performed at 1100 g for 15 min. Plasma samples were obtained by centrifuging blood samples at 1100 g for 15 min.

The livers were removed from newborn and from adult rats. Pools of livers from each group were used.

The lipids of the various samples were extracted according to Folch et al. (9). Different lipid fractions were separated by thin layer chromatography (TLC) on Silica Gel H (Merck, Darmstadt, Germany), using a solvent system of *n*-heptane/isopropyl ether/formic acid (60:40:2, v/v/v). The various fractions, identified with the aid of known standards, run at the same time and separately exposed to iodine vapor, were scraped off.

Fatty acid methyl esters were prepared by refluxing total lipids or different lipid fractions with a mixture of methanol/benzene/H_2SO_4 (43:5:2, v/v/v) for 2 hr at 85 C.

After the addition of 1.5 vol of H_2O, the methyl

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esters were extracted twice with *n*-hexane. The hexane extract was purified by washing with aqueous $NaHCO_3$ (1% w/v) and dehydrated with anhydrous sodium sulfate. Gas liquid chromatography (GLC) was performed with a Fractovap apparatus Mod. GI (C. Erba, Milan, Italy), equipped with a flame ionization detector. Glass columns (2.5×3 mm id) packed with 10% diethylene glycol succinate on Chromosorb W (C. Erba) were used. Columns oven and flash heater temperatures were 190 C and 225 C, respectively. Carrier gas was nitrogen (20 ml/min). The peak areas were calculated by multiplying the peak height by the width at half height. Each area was corrected by a factor obtained from the detector yield of known amounts of standards.

Separation of different phospholipid fractions of erythrocyte was carried out by one-dimensional TLC, as described by Skipski and Barclay (10).

Total lipids were determined colorimetrically according to Chiang et al. (11). Phospholipid phosphorus was determined by the method of Shin (12). Triglycerides and total cholesterol were determined by the methods of Van Handel and Zilversmit (13) and of Bowman and Wolf (14), respectively. Because of the high content in triglycerides, milk samples were saponified in 33% ethanolic potassium hydroxide and extracted with petroleum ether (bp 40-70) (15) before being processed for colorimetric assay according to Bowman and Wolf (14). Free fatty acids were determined by the method of Duncombe (16).

Reticulocytosis was produced in adult rats by subcutaneous injections of 0.25 ml/kg body wt of 2.5% (w/v) phenylhydrazine solution according to Allen and Schweet (17). The phenylhydrazine solution was prepared everyday and neutralized to pH 7.0 with NaOH. It was injected daily for 4 days and the rats were sacrificed on the 7th day when the reticulocytosis reached values of more than 80%.

Rat milk was obtained from nursing mothers at 1, 5, 15 days postpartum. The animals were injected with 2 UI of oxytocin (Syntocinon, Sandoz) and 5 min later the milk was collected from all nipples by aspiration.

RESULTS

The fatty acid pattern of erythrocyte total lipids in rats at various ages of life is reported in Table 1. As can be seen, the percentages of the unsaturated fatty acids markedly change during the first days of life. The content in oleic and palmitoleic acids sharply decreases within the first 10-15 days of life when it reaches the value observed in the erythrocytes of adult rats. The content in linoleic acid, on the contrary, gradually increases during the same period. The increase is already significant at 3-5 days of life. The arachidonic acid content increases significantly within the first 3 days; a second significant increase occurs within 15 and 30 days of life. The content in docosahexaenoic acid remains constant for 15 days and then decreases. The relative percentages of the saturated fatty acids (16:0 and 18:0) remained fairly constant.

Similar differences between newborn and adult rats are present in the fatty acid pattern of the erythrocyte phospholipids (Table 2). The changes are not accounted for by differences in the percentages of the major phospholipid fractions of the red cell membrane, since no variations between newborn and adult rats were seen in the percentages of phosphatidylcholine (34.4±2.0 and 34.1±3.1, for newborn and adult rats, respectively) and phosphatidylethanolamine (21.6±1.8 and 22.5±0.4, for newborn and adult rats, respectively). Also

TABLE 1

Fatty Acid Composition of Erythrocyte Total Lipids in Rats at Various Ages of Life

Age	Fatty acids						
	16:0	16:1	18:0	18:1	18:2ω6	20:4ω6	22:6ω3
Newborn (5)	33.5±0.6	3.6±0.3	11.2±0.3	18.5±0.5	5.9±0.09	22.9±0.5	4.4±0.5
3 days (3)	34.1±2.5	2.7±0.2	9.9±0.3	16.7±1.2	6.4±0.1	26.0±0.9[b]	4.3±0.9
5 days (3)	34.5±0.7	2.0±0.2	10.8±0.7	14.9±0.6	7.3±0.1[a]	25.5±0.6	4.9±1.5
10 days (3)	38.4±2.1	1.8±0.0	10.4±0.9	9.9±0.5[b]	8.7±0.2[a]	25.4±1.31	5.4±0.6
15 days (4)	33.9±1.5	1.9±0.4	11.1±0.1	9.3±0.3	10.7±0.3[a]	27.0±1.4	6.2±1.4
30 days (6)	29.6±0.7[a]	1.0±0.07[a]	13.5±0.3[b]	10.5±0.2	10.8±0.2	31.1±0.6[a]	3.5±0.09[a]
Adult (9)	30.7±1.1	1.3±0.1	14.5±0.5	10.8±0.4	11.3±0.2	29.5±1.2	2.1±0.2[b]
P	<0.01	≪0.01	≪0.01	≪0.01	≪0.01	<0.01	<0.01

Values, expressed as mean±SEM, are given as the percentage of total fatty acids. Number of samples is reported in brackets.

P was calculated by the analysis of variance (F test). Superscripts (a,b) mean P values (Tukey test) <0.05 and <0.01, respectively, with respect to the figure immediately above. The oleic/linoleic ratio in the newborn rats is 3.1 and in adult rats is 0.95.

TABLE 2

Fatty Acid Composition of Erythrocyte Phospholipids in Newborn and Adult Rats

Age	Fatty acids						
	16:0	16:1	18:0	18:1	18:2ω6	20:4ω6	22:6ω3
Newborn (3)	33.1±1.7	2.8±0.08	10.8±0.3	18.6±0.4	6.7±0.2	23.4±2.2	4.7±0.2
Adult (3)	31.0±1.1	trace	14.7±0.3[a]	10.8±0.6[b]	11.1±0.1[c]	31.0±0.3[d]	1.4±0.3[e]

Values, expressed as mean±SEM, are given as the percentage of total fatty acids. Number of samples is reported in brackets.

Statistical significance, P value for a: P<0.001; b: P<0.001; c: P<0.001; d: P<0.05; e: P<0.01.

the ratio between total lipids and phospholipids (TL/PL) or between phospholipids and total cholesterol (PL/CL) was the same in newborn (TL/PL = 1.31±0.06; PL/CL = 2.21±0.03) and adult rats (TL/PL = 1.19±0.01; PL/CL = 2.19±0.04).

In order to see whether the difference in the fatty acid pattern of erythrocyte lipids observed from birth to adult age could be due to differences in the age of the erythrocytes, we examined the fatty acid pattern of a population of erythrocytes rich in cells of young age, i.e., reticulocytes. To this end, a marked reticulocytosis was induced by treatment with phenylhydrazine. Since the fatty acid pattern (Table 3) of lipids of erythrocytes of rats treated with phenylhydrazine did not substantially differ from that of erythrocytes of control rats, it seems that the difference between newborn and adult rats is not due to changes in erythrocyte age. It appears, therefore, that such differences are dependent on the age of the animals; that is to say, during the first days of life the fatty acid pattern of erythrocyte lipids is attaining the stable feature of the adult. This possibility is supported by the finding of similar changes between newborn and adult age in the fatty acid composition of liver phospholipids studied as an example of membrane lipids (Table 4).

Since it has been shown (18) that plasma lipids largely exchange with lipids of erythrocyte membrane, it is reasonable to assume that the differences between newborn and adult rats in the fatty acid pattern of erythrocyte lipids are due to parallel variations of plasma lipids. Therefore, the fatty acid composition of total lipids and of the major lipid fractions (phospholipids, triglycerides and cholesteryl esters) of plasma was analyzed at the same times of life studied for erythrocyte lipids. As shown in Table 5, the percentage of oleic acid markedly decreases in each lipid fraction from birth to the adult age, as seen in erythrocyte lipids. The percentage of linoleic acid, on the other hand, increases in each lipid fraction, still in agreement with that observed in the erythrocytes. The increase is progressive in triglycerides, while in the other fractions a sharp peak increase occurs at 15 days and a decrease thereafter. The percentage of arachidonic acid shows marked variations during development in phospholipids and triglycerides. However, in cholesteryl esters, in which a high level of arachidonic acid is present, a marked increase in this fatty acid occurs from birth to the adult age, as observed in erythrocyte lipids.

The marked variations seen in the fatty acid pattern of plasma lipids within the first month of postnatal life mean that marked variations in lipid metabolism occur in this limited period of time. Therefore, the concentration of plasma lipids of the rats was evaluated at the same time of life. As can be seen in Figure 1, the concentration of plasma phospholipids, triglycerides and total cholesterol strikingly increases within the first 15 days and sharply decreases thereafter. The plasma content in

TABLE 3

Fatty Acid Composition of Erythrocyte Total Lipids in Phenylhydrazine Treated Adult Rats and in Respective Controls

	Fatty acids						
	16:0	16:1	18:0	18:1	18:2ω6	20:4ω6	22:6ω3
Treated rats (6)	31.2±0.4[a]	1.3±0.06	11.8±0.2[b]	13.2±0.5[c]	10.5±0.2	30.2±0.2	1.7±0.2
Control rats (6)	29.6±0.3	1.1±0.1	13.8±0.3	11.2±0.3	11.2±0.3	31.1±0.5	2.1±0.2

Values, expressed as mean±SEM, are given as the percentage of total fatty acids. Number of samples is reported in brackets.

Statistical significance, P value for a: P<0.01; b: P<0.001; c: P<0.01.

TABLE 4

Fatty Acid Composition of Liver Phospholipids in Newborn and Adult Rats

	Fatty acids						
Age	16:0	16:1	18:0	18:1	18:2ω6	20:4ω6	22:6ω3
Newborn	24.0	1.8	19.6	9.6	9.5	26.9	8.3
Adult	19.5	trace	25.8	6.7	12.9	30.8	4.3

Values are given as the percentage of total fatty acids.

TABLE 5

Fatty Acid Composition of the Total Lipids and of the Major Lipid Fractions of the Plasma in Rats at Various Ages of Life

		Fatty acids						
Plasma lipids	Age	16:0	16:1	18:0	18:1	18:2ω6	20:4ω6	22:6ω3
TL	Newborn (3)	23.5±0.7	4.6±0.6	8.0±0.6	20.3±2.2	17.3±1.9	23.5±3.3	2.9±0.5
	5 days (3)	23.5±0.9	1.4±0.05[a]	11.0±0.3[a]	10.6±0.8[a]	21.6±0.8	28.5±0.8	3.4±0.3
	15 days (3)	26.2±1.3	1.1±0.03	10.4±0.3	12.7±0.4	26.2±0.3[a]	21.2±0.6	2.3±0.3
	30 days (3)	21.4±0.7	1.9±0.2[a]	9.4±0.6	16.2±0.03[a]	21.6±0.1[a]	27.4±0.4	2.2±0.1
	Adult (3)	21.4±0.2	1.7±0.2	11.1±0.5	12.4±1.1[a]	23.4±1.0	28.1±2.2	1.9±0.4
	P	<0.05	<0.01	<0.01	<0.01	<0.01	NS	<0.05
PL	Newborn (6)	27.1±0.7	2.7±0.4	14.9±0.5	12.4±0.9	12.2±0.6	26.4±1.1	4.1±0.5
	5 days (3)	27.3±0.8	1.0±0.2[b]	17.7±0.2[a]	6.5±0.2[b]	20.3±0.6[b]	23.2±0.7	4.1±0.5
	15 days (3)	27.4±0.3	0.9±0.06	18.3±0.2	5.5±0.06	23.9±1.0	20.3±0.8	3.7±0.2
	30 days (6)	28.5±1.2	1.1±0.07	21.8±0.4[b]	7.4±0.2	14.4±0.4[b]	24.3±0.6[a]	2.4±0.3[a]
	Adult (6)	26.0±0.9	1.2±0.09	21.5±0.8	7.5±0.4	13.8±0.5	26.9±0.6	3.1±0.2
	P	NS	≪0.01	≪0.01	≪0.01	≪0.01	<0.01	<0.05
TG	Newborn (6)	25.3±1.2	4.8±0.3	4.8±0.4	25.0±0.7	14.8±0.7	21.6±2.9	-
	5 days (3)	30.9±1.3[a]	2.9±0.3[b]	4.4±0.2	24.4±0.8	18.6±1.1[a]	17.2±0.3	-
	15 days (3)	36.0±0.2[a]	2.2±0.07	4.9±0.03	24.3±0.4	22.4±0.5[a]	9.6±0.6[b]	-
	30 days (4)	23.6±0.7[b]	1.2±0.07[b]	5.1±0.2	19.9±0.3[b]	29.5±0.2[b]	20.7±0.3[b]	-
	Adult (6)	25.1±0.7	3.3±0.7	4.2±0.2	20.8±0.6	29.9±0.8	14.7±1.0[b]	-
	P	≪0.01	<0.01	NS	≪0.01	≪0.01	≪0.01	-
CE	Newborn (6)	20.4±0.7	10.7±1.4	4.1±0.3	22.0±0.8	10.9±0.5	32.0±1.8	-
	5 days (3)	17.5±1.7	3.0±0.06[b]	2.2±0.4[b]	9.1±1.6[b]	16.4±0.5[b]	51.3±3.8[b]	-
	15 days (3)	16.8±0.5	2.1±0.1[b]	1.8±0.2	10.9±0.6	23.9±0.4[b]	44.5±0.2	-
	30 days (6)	12.8±0.4[b]	2.1±0.2	2.8±0.2	12.3±0.7	17.9±0.5[b]	52.0±1.8	-
	Adult (6)	11.0±0.5[a]	3.0±0.4	2.2±0.3	11.8±0.7	16.7±0.7	55.5±1.6	-
	P	≪0.01	≪0.01	<0.01	≪0.01	≪0.01	≪0.01	-

TL = total lipids; PL = phospholipids; TG = triglycerides; CE = cholesteryl esters.

Values, expressed as mean±SEM, are given as the percentage of total fatty acids. Number of samples is reported in brackets.

P was calculated by the analysis of variance (F test). Superscripts (a, b) mean P values (Tukey test) <0.05 and <0.01, respectively, with respect to the immediately above figure. The oleic/linoleic ratio in plasma cholesteryl esters in the newborn rats is 2.0 and in adult rats is 0.7.

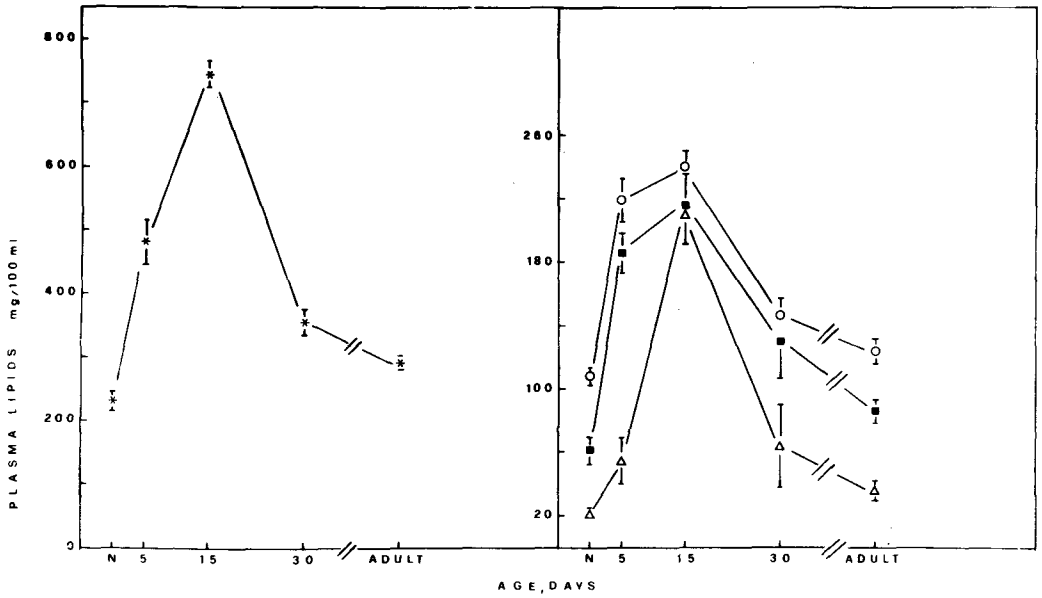

FIG. 1. Content of plasma lipids in rats at various ages of life. Vertical bars are the standard error of the mean. Six samples were examined for newborn and adult rats; three for 5, 15, 30 day-old rats. * — * total lipids; O — O phospholipids; ■ — ■ total cholesterol; Δ — Δ triglycerides.

total lipids, which shows similar variations, is significantly higher in the adult than in the newborn rats.

Since, according to some authors (2), the composition of plasma lipids reflects to some extent the composition of dietary lipids, the possibility must be considered that the changes in the fatty acid pattern of erythrocyte lipids observed within the first month of life are due to the changes in the dietary regime of the animals, as the rats ingested only dams' milk up to 15 days after birth and began to eat some pelleted food thereafter. The fatty acid composition of the milk at 1, 5 and 15 days after delivery and that of the pelleted diet used is reported in Table 6. As can be seen, the fatty acid composition of the pelleted diet is similar to soybean oil, whereas the milk lipids are rich in short-chain fatty acids and contain some arachidonic acid. No significant variations occur in the fatty acid pattern of the milk lipids within the first 15 days after delivery, with the only exception of arachidonic acid between 1 and 15 days, which corresponds to the time when changes in the fatty acid composition of erythrocyte lipids were observed. Therefore, the changes in plasma and erythrocytes could not be ascribed to difference in fatty acid composition of the milk. The lipid content of the milk and that of the diet is reported in Table 7, which shows a much higher level of triglycerides in the milk than in the diet, while the reverse is true for total cholesterol.

The possibility that the fatty acid composition of erythrocyte lipids is affected by that of plasma lipids was tested by incubating erythrocytes taken from 15-day-old rats with plasma taken from newborn rats (experimental details are given in legend to Table 8). After 2 hr of incubation, the fatty acid pattern of the erythrocyte lipids was changed compared to that of the lipids of the nonincubated cells. Oleic and palmitoleic acids were increased while arachidonic and docosahexaenoic acids were decreased. Thus, the fatty acid pattern of the incubated cell somewhat resembled that of the erythrocytes of newborn rats, although the content of linoleic acid was not changed. It must be noted that some hemolysis occurred during the incubation.

DISCUSSION

The present results demonstrate that the fatty acid pattern of the erythrocyte total lipids changes considerably during the first days of life. The most remarkable changes are represented by the decrease in oleic acid and by the increase in linoleic and arachidonic acids. The changes cannot be ascribed to variations in lipid fractions of cell membrane or to variations in erythrocyte age. The changes, on the contrary, seem to be related, in some way, to variations in the fatty acid pattern of the major lipid classes of the plasma during the first weeks of life. The experiment in which erythrocytes taken from 15-day-old rats were incubated with plasma taken from newborn rats gives some support to this

TABLE 6

Fatty Acid Composition of Rat Milk and of the Pelleted Diet

	Fatty acids										
	8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2ω6	20:0	20:4ω6
Milk Days postpartum											
1 (6)	7.1±3.2	21.0±4.4	5.6±1.3	3.5±0.7	20.3±1.5	2.7±0.2	3.2±0.5	20.2±2.9	20.3±3.1	-	3.8±0.9[a]
5 (5)	2.8±1.3	10.3±2.8	7.5±0.9	7.3±0.5	21.8±1.1	2.1±0.1	3.5±0.3	20.5±1.4	22.0±2.1	-	2.4±0.7
15 (4)	5.5±1.6	19.7±5.7	11.5±2.5	11.4±1.8	17.9±2.5	1.5±0.4	2.6±0.5	13.4±3.3	15.1±3.4	-	1.4±0.4[b]
Pelleted diet	-	-	-	0.8	19.2	0.9	5.6	27.9	42.9	2.6	-

Values, expressed as mean±SEM, are given as the percentage of total fatty acids. Number of milk samples is reported in brackets.
Statistical significance of the difference, P value for a-b: $P<0.05$.

possibility. It must be considered, however, that the dependence of erythrocyte lipids on plasma lipids is not demonstrated with certainty from the present data. A series of difficulties is in fact encountered when the values of Tables 1, 5 and 8 for individual fatty acids are compared. For example, the plasma level of oleic acid is down already at 5 days of age (Table 5), while the change in the erythrocytes proceeds more slowly (Table 1). Transferring the results of the in vitro study (Table 8) to an in vivo situation, the exchange should be almost complete within 2 hr. Similarly, the level of linoleic acid in the erythrocytes is much lower than that of the plasma throughout the rat life.

In a previous study from our laboratory (4), it was reported that changes in the fatty acid composition of erythrocyte membrane occur also in humans during the first weeks of life. In this case too, a progressive increase in linoleic acid was observed, but, contrary to what is seen in rat erythrocytes, the content of oleic acid increased and that of arachidonic acid decreased. As in the case of rat erythrocytes, the changes did not appear to be related to the erythrocyte age and seemed to be connected with similar variations of the fatty acid composition of plasma lipids. The previous and the present study therefore suggest that the fatty acid composition of the red cell membrane takes on the feature characteristic of the adult subject during the first period of life and that this arrangement could be at least in part dependent on the variations of plasma lipids.

Variations in fatty acid pattern of plasma lipids and lipids of various organs have been reported by other authors. Lopez-Santolino et al. (19) observed in newborn rats a typical "fetal" serum cholesteryl ester fatty acid (CEFA) pattern, characterized by a high oleate/linoleate (O/L) ratio (1.5:5.0), and in the mothers a typical "adult" CEFA pattern characterized by an O/L ratio of less than unity (0.2:0.6). Somewhat similar values in O/L ratio were found in the present study, not only for plasma cholesteryl esters (see legend to Table 5), but also for erythrocyte lipids (see legend to Table 1) of both newborn and adult rats. These characteristic "adult" and "fetal" CEFA patterns were found (19) to be maintained in spite of moderate alterations in the fatty acid composition of the prenatal diets, but could not be maintained in the face of exaggerated imbalances of diets supplying only single fatty acids. The authors (19) postulated that homeostatic mechanisms exist which function so as to maintain the characteristic adult and fetal CEFA patterns in the face of stresses consisting of variations in the dietary fatty acid composition.

Dobiasova et al. (20) demonstrated that marked changes occur in the fatty acid composition of the triglycerides and phospholipids of some rat organs (lungs, adipose tissue, intestine and liver) during

TABLE 7

Lipid Content of the Milk During Lactation and of the Pelleted Diet

	Total lipids	Triglycerides	Phospholipids	Total cholesterol
Milk Days of lactation				
1	15.5 ± 1.6 (6)	12.9 ± 1.4 (6)	0.127 ± 0.008 (6)	0.084 ± 0.012 (6)
5	17.0 ± 1.9 (4)	14.5 ± 1.8 (5)	0.163 ± 0.015 (5)	0.060 ± 0.011 (4)
15	19.5 ± 2.6 (4)	15.9 ± 1.0 (5)	0.180 ± 0.011 (5)	0.063 ± 0.006 (4)
Pelleted diet	4.1	1.2	0.321	0.228

Values of milk are expressed as mean ± SEM. Number of milk samples is reported in brackets. Data for milk and pelleted diet are presented as g/100 ml and as g/100 g, respectively. The pelleted diet in addition contains diglycerides and monoglycerides (0.28 g/100 g) and free fatty acids (1.4 g/100 g).

postnatal development. According to the above authors (20), however, most of these changes are not related to variations in the composition of the diet; only the triglyceride contents in myristic and lauric acids were found to vary with the dietary content of these fatty acids. Pronounced changes in the fatty acid composition of the liver phospholipids and triglycerides have been reported (21) to occur during the postnatal development of the rat; while in triglycerides the content of long-chain polyunsaturated fatty acids was mainly of dietary origin. A more complex mechanism involving metabolic routes of interconversion of fatty acids accounted, at least in part, for the fatty acid changes observed in phospholipids.

Our results do not suggest that the changes in the fatty acid composition of erythrocyte lipids during the first period of life are dependent on the ingested diet. In fact, the fatty acid composition of rat milk at various days postpartum did not show, in agreement with Kehrer and Autor (8), a significant variation in the content of oleic and linoleic acids; only a small variation in arachidonic acid was observed. The solid diet, that contains higher contents of oleic and linoleic acids when compared to milk, begins to be ingested by rats only 15-20 days after birth, when the fatty acid pattern of erythrocyte lipids has already taken the feature characteristic of the adult subject. That the changes in the fatty acid composition of erythrocyte lipids are not dependent on the ingested diet is further supported by the fact that a change in the dietary fatty acids, which occurred when the young rats started to eat the pelleted diet, resulted in no further change in fatty acid composition of erythrocyte lipids. Even if consistent changes occur in the fatty acid composition of plasma lipids from the 15th day to the adult age, such changes do not seem to be related to the variation in the dietary regime. It must also be noted that the plasma lipids do not assume the fatty acid composition of the mother's milk.

It seems, therefore, that the changes in fatty acid composition of the erythrocytes and plasma lipids are triggered off by something other than the dietary fatty acids. Neither is the increase in plasma lipids the cause for the change in fatty acid composition of the erythrocytes and plasma lipids, because the animal is simply responding to a high fat diet (mother's milk) and when the milk intake is

TABLE 8

Fatty Acid Composition of Total Lipids of Erythrocyte Taken from 15-Day-Old Rats Before and After Incubation with Plasma Taken from Newborn Rats

	Fatty acids						
Treatment	16:0	16:1	18:0	18:1	18:2ω6	20:4ω6	22:6ω3
Erythrocytes before incubation	34.9	1.5	11.3	8.2	10.9	28.3	4.9
Erythrocytes after incubation	34.6	3.8	12.5	15.0	11.2	19.1	3.8
Newborn rat plasma before incubation	22.1	6.7	9.3	22.5	20.5	17.5	1.4

0.5 ml of packed red cells from 15-day-old rats were incubated with 2.0 ml of plasma taken from newborn rats. The incubation was carried out aerobically at 37 C for 2 hr. At the end of the incubation, the erythrocytes were recovered by centrifugation, washed 3 times in 0.9% NaCl and extracted for lipids.

reduced, after the first two weeks of life, the plasma lipid content decreases. Increases in the concentration of plasma lipids have also been observed in humans during the first period of life (22). Furthermore, it has been reported (23) that serum cholesterol levels are much higher in suckling rats than in weaned rats. The low prenatal level increases rapidly after birth (24) and decreases again at the time of weaning (25). These changes seem to depend on the diet (26) and on the age-dependent endocrine factor, but the exact mechanism is not known.

In conclusion, the reason for the change in fatty acid composition of the plasma and erythrocyte lipids still remains unknown.

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Lipid Composition and de novo Lipid Biosynthesis of Human Palmar Fat in Dupuytren's Disease

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ABSTRACT

Seventy-two surgically obtained Dupuytren's disease palmar-fat (DDPF) specimens and 18 location-matched specimens from patients not suffering from this disease (controls) were studied for their total lipid composition and de novo lipogenic activity. Incubation of "DDPF" with 1-[^{14}C]acetate in oxygen produced [^{14}C]palmitate and [^{14}C]stearate in approximately equal yields as those obtained from "controls." No [^{14}C]octanate was formed in any of the palmar-fat preparations. The lipids and fatty acid analysis revealed differences: (a) DDPF specimens were richer in free fatty acids, methyl esters of fatty acids and free-cholesterol than specimens of controls. (b) DDPF specimens contained less phospholipids. (c) DDPF specimens showed a significantly higher content of octanoate and other short-chain fatty acids than specimens of controls. The above findings are not incompatible with the results expected if some mild hypoxia occurred in DDPF; this has been suggested in the statistical correlations observed for this disease and alcoholism with liver involvement.
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INTRODUCTION

Dupuytren's disease (DD) is an affliction characterized by a degeneration of elastic fibers (1) and a progressive irreversible contraction of one or more fingers (2). Several articles and reviews have recently been published (1,3,4) but no total lipid assays or lipogenic activity have been reported for the palmar-fat tissues.

Published reports (1,3,5) have shown statistically significant evidence linking chronic liver disease (from alcoholism) and the incidence of DD). Several Alcoholic Research Centers have demonstrated the presence of volatile (short to medium chain) fatty acids in breath and in the sera of patients with liver cirrhosis (6-11). This fact has been explained by the suggestion that, in alcoholism, a liver hypoxia is responsible for the incomplete oxidation of some of the long-chain fatty acids, and thus shorter chain fatty acids (6) are found in larger than normal amounts (8); since these fatty acids are water-soluble and volatile, they will be found in all tissues and in breath.

The associations of alcoholism and local palmar hypoxia with DD prompted us to investigate whether higher than normal levels of volatile fatty acids may be present in the palmar fat of DD patients, and if this result may be because of a special biosynthesis.

MATERIALS AND METHODS

Specimens of palmar fat were obtained from surgically hospitalized patients. Seventy-two DD and 18 location-matched surgical specimens from palms of patients not suffering DD or other metabolic disease were studied (controls). The tissues were kept chilled (1 C) and immediately taken to the laboratory, padded dry with filter paper and weighed.

The individual tissue was chopped into small pieces with fine scissors and the tissue divided into various weighed portions. One of the weighed aliquots was mixed with a 30× vol of chloroform/methanol (2:1, v/v). Lipid extraction was performed at 5 C for 48 hr, with agitation (wrist-action shaker). The lipid extract was then used for chemical analysis.

Neutral Lipid Analysis

Neutral lipids were separated from phospholipids by silicic acid column chromatography using sequential chloroform and methanol elution. The individual neutral lipids were separated by thin layer chromatography (TLC) using Silica Gel G on glass plates. The solvent system was petroleum ether/ethyl ether/acetic acid (90:10:1, v/v/v). Neutral lipids were identified by staining with iodine vapor.

Phospholipid Analysis

Phospholipids were separated by two-dimensional TLC using Silica Gel H on glass plates. The solvents used for the first and second dimension separation were chloroform/methanol/acetic acid/water (200:120:25:15, v/v/v/v) and chloroform/methanol/acetic acid/water (100:20:40:20, v/v/

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v/v), respectively. The R_f values were determined by the use of known standards (Supelco, Bellefonte, PA). Lipid fractions were estimated by the charring procedure. All of these analytical procedures, extraction methods, and quantitations have been previously reported in great detail (12-13).

Fatty Acid Analysis

Potassium salts of the fatty acids were prepared by saponification of the palmar fat under nitrogen with 20% KOH in 90% ethanol for 3 hr at 100 C (fraction S). An aliquot of fraction S was acidified and then extracted with diethyl ether. The recovered fatty acids were esterified with 14% boron trifluoride in methanol. The methylated fatty acids obtained were then purified by TLC using the neutral lipids procedure. Gas liquid chromatography (GLC) was performed using a Packard Model 846 with a 6-ft glass column packed with 10% DEGS-PS (Supelco, Bellefonte, PA) and a nondestructive mass detector (argon ionization). Argon was used as a carrier gas. Temperature programming was carried out from 150 to 200 C at 1 C/min and then maintained at 200 C for an additional 45 min. The retention time was used for identification; quantitation was by comparison with known amounts of authentic fatty acid methyl esters (Applied Sciences, State College, PA).

Volatile Fatty Acids

Another aliquot of fraction S was assayed by steam distillation in a closed glass system and assayed for volatile free fatty acids in a special GLC system (8).

Lipogenetic Activity

From fresh surgical samples of DDPF, weighed aliquots were obtained. The weighed tissue aliquot was homogenized in 2 1/2 vol of buffer containing K_2HPO_4, 0.067 M; KH_2PO_4, 0.042 at pH 7.0 at 0 C. The mixture was centrifuged for 7 min at 500 × g to remove unbroken cells, nuclei, and cell debris. For each test, 5 mg of 500 × g supernatant protein was added to a flask containing 1 mg of 1-[^{14}C]acetate (1 mCi/mmol) in pH 7.2 buffer. Final volume of each reaction was 5 ml. The flasks were incubated at 37 C for 3 hr with gentle shaking; the gaseous phase was 100% oxygen. After incubation, 2 mg of a carrier mixture of fatty acids (16:0, 16:1, 18:0, 18:1 and 8:0) was added to each flask. Solid pellets of metaphosphoric acid were added to bring the mixture to pH 2. The material was extracted in a closed glass container continuously with ether for 24 hr. Aliquots of the ether extract were evaporated to dryness and placed in a desiccator over KOH. One ml of 10% acetic acid was added to each residue which was again dried in a desiccator over KOH. Each residue was dissolved in absolute ether in a 1 ml volumetric flask. Aliquots were methylated (12), then taken for GLC radioassay (6). Other aliquots were used to obtain the volatile fatty acids by steam distillation (8) and then quantitated by GLC radioassay (6).

Sodium 1-[^{14}C]acetate (sp act 15 mCi/mmol) was obtained from New England Nuclear Corporation, Boston, MA. A Packard Tri-Carb Spectrometer was used for determination of radioactivity. Radioactive assay of the GLC products was by oxidation and subsequent assay in a proportional radioactivity counter attached to the GLC (Packard 894). Counting was to ± SD. These techniques have been reported previously (14-18).

Protein

The biuret technique was utilized for protein assays of tissue aliquots. Bovine serum albumin (ICN-Nutritional Biochemicals, Cleveland, OH) was used as the standard (19,20).

RESULTS

In Table 1 are shown the lipid assays obtained from DD and control palmar-fat specimens. The studies indicate that the differences between DD and controls are not statistically significant for most classes of lipids. Although some minor differences could be shown, DD samples yielded higher values compared to controls for only some of the neutral lipid components: free fatty acids, methyl esters and cholesterol, yet these were not outstanding. Lower values for all of the phospholipids were also seen in the DD specimens; but the levels found were so small that statistics are hard-pressed to indicate significance. The observed patterns for both types of specimens are not that much different than those observed for many fat-rich areas or lipid pads of the body (14).

In Table 2, we have presented the results of fatty acid analysis by GLC after saponification and esterification of aliquots of the samples. Results of the GLC assays indicated a similar pattern of fatty acid distribution for both the control and DD specimens in most of the medium- and long-chain fatty acids. But, in the assay of volatile fatty acids obtained by closed-vessel steam distillation and then GLC, the short-chain fatty acids (octanoate and smaller sizes) showed a statistically significantly higher value for the DD specimens than for the controls.

In Table 3, the results are presented for the 3-hr incubations of palmar-fat tissue homogenates under a gaseous phase of 100% oxygen. The incorporation of 1-[^{14}C]acetate into the fatty acids was small and in the range reported for similar tissues (14). No differences were observed in the lipo-

TABLE 1

Percentage of Lipid Composition of Human Palmar-Fat Surgical Specimens

	72 Specimens Dupuytren disease		18 Specimens (location-matched) controls	
		±SEM		±SEM
Total lipids (% net weight)	98.3	±2.1[a]	99.0	±1.7
Neutral lipids	96.5	±1.4	95.3	±1.2
Polar lipids	2.5	±0.6	3.5	±0.7
Undetermined lipids	1.0	-	1.2	-
Neutral lipids:				
Mono- and diglycerides	1.7	±0.4	0.9	±0.2
Cholesterol	1.9	±0.7	0.3	±0.1
Free fatty acids	4.4	±0.9	2.1	±0.7
Triglycerides	78.7	±2.4	86.6	±3.1
Methyl esters	3.8	±0.9	0.9	±0.1
Cholesteryl esters	3.9	±0.3	3.7	±0.8
Undetermined	2.1	-	0.8	-
Phospholipids:				
Phosphatidylethanolamine	0.4	±0.1	0.5	±0.1
Phosphatidylinositol	0.1	±0.1	0.2	±0.1
Phosphatidylserine	0.1	±0.1	0.2	±0.1
Phosphatidylcholine	0.2	±0.2	0.4	±0.2
Lysophosphatidylcholine	0.1	±0.1	0.3	±0.1
Sphingomyelin	0.2	±0.1	0.4	±0.1
Phosphatidic acid	0.7	±0.3	0.8	±0.2
Cardiolipin	0.3	±0.2	0.4	±0.1
Undetermined	0.4	-	0.3	-

[a]Standard error of the mean.

genetic activity of DD samples from that found in controls based on protein content.

When incubations were carried out using hypoxic conditions (a gaseous phase of 100% nitrogen), no incorporations of 1-[^{14}C]acetate were observed in any of the recovered fatty acids. These observations were repeated several times (four) for both DD and control homogenates.

DISCUSSION

Daris (3) and others (1,5) have made a case for the statistical relationship between the incidences of Dupuytren's disease and alcoholism. A hypothesis in Dupuytren's disease is that a mild hypoxic state may develop inside the tissues of the lipid pad of the palm of the hand. This hypoxic state may be caused by poor circulation to the area and the abnormal connective tissue (4,21) distribution and local accumulation in the area may further contribute to the local formation of an hypoxic state, especially in a traumatized palm.

In alcoholism, it has been shown (22) that the octanoate serum concentrations of fasting patients are much higher than in those who did not have hepatic involvement or in nondrinkers (7,8). The origin of the octanoate was attributed to incomplete oxidation of long-chain fatty acids in the liver (6). The biosynthetic ability under oxygenated conditions of tissue homogenates of palmar fat was shown in this study to be about the same for both DD and controls; yet, the analysis of fatty acids revealed a larger content of short-chain fatty acids in the DD specimens. Since serum octanoate levels were shown (8,23) to be directly proportional to the extent of liver impairment (in alcoholic cirrhosis), the great increase (3-4 times over the control values) for octanoate and other short-chain fatty acids in the total lipids of palmar fat of DD specimens is not inconsistent with the concept of a locally present mild hypoxic state exacerbated or induced by alcoholism or trauma in the palms of such patients.

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TABLE 2

Percentage Fatty Acid Composition of Human Palmar Fat

	Dupuytren	Control
Less than 8[a] and 8[a]	2.3±0.4[b]	0.5±0.2
10[a]	1.4±0.2	0.8±0.2
12[c]	2.7±0.4	2.9±0.3
14	2.6±0.6	2.7±0.2
14:1	0.4±0.3	-
16	29.2±2.4	27.2±1.1
16:1	1.7±0.3	6.3±0.7
18:0	8.7±0.5	7.2±0.9
18:1	34.1±4.7	36.7±3.9
18:2	5.4±2.1	7.2±2.0
20	1.2±0.3	1.4±0.9
18:3	3.0±0.7	2.0±1.1
20:2	1.9±0.4	1.7±0.6
20:3	1.3±0.5	7.4±0.5
Over 20	1.8±0.2	1.0±0.3
Undetermined	2.3	1.0

[a]Determined by closed-vessel steam distillation and then GLC (8).

[b]Standard error of the mean.

[c]Determined by GLC of esterified fatty acids (fatty acids of 12-20 carbons in chain).

TABLE 3

Recovered [^{14}C]Fatty Acids Obtained from 1-[^{14}C]Acetate by Homogenates of Palmar Fat (dpm/mg C$\times 10^{-3}$)/mg Protein (Average of 10 Assays; Protein Calculated from Wet Weight of Sample; Gaseous Phase 100% Oxygen)

Fatty acid chain length	DDPF	Controls
Less than 8 and 8:0	0	0
10:0	0	0
12:0	0	0
14:0	0	0
16:0	387±23[a]	365±31
10:1	trace	trace
18:0	185±37	231±43
18:1	trace	trace
Over 18	0	0

[a]Standard error of the mean.

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Effects of Dietary *Trans*-Fat on Biliary and Fecal Steroid Excretion and Serum Lipoproteins in Rats

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ABSTRACT

Rats were fed cholesterol-free or cholesterol-enriched diets containing olive oil or partially hydrogenated corn oil at the 10% level for ca. 30 days (*c*-18:1, 77.0% in the former diet and *c*-18:1, 24.7% and *t*-18:1, 42.5% in the latter). The linoleic acid content of these fat diets was made equivalent (1.7 energy %). After feeding cholesterol-free diets, *trans* fat compared to *cis* fat showed (a) no untoward effects on growth parameters, (b) a reduction of serum cholesterol levels without influencing concentrations of serum apolipoproteins A-I, B and E, (c) no effects on the bile flow and the concentration of biliary cholesterol and bile acids, (d) an increasing trend of fecal excretion of neutral and acidic steroids, both in terms of mg/day and mg/g feces, and (e) rather equivocal change in the composition of fecal, but not biliary steroids. Similar response patterns were also observed when cholesterol-enriched diets were fed except for a decrease in serum apo B and an ineffectiveness to increase fecal acidic steroids. Together with the results obtained from experiments simultaneously performed with safflower oil and completely hydrogenated corn oil, it seems that the steroid metabolism can be specifically modified by the geometry of dietary fats.
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INTRODUCTION

Currently, consumption of *trans* fatty acids, mainly as *trans* octadecenoic acid (*t*-18:1), as a constituent of hydrogenated fat products is increasing. Though there is still a controversy, *trans* unsaturated fatty acid of this type may not be specifically hypercholesterolemic and hence atherogenic (1,2). It is generally believed that many of the physiological effects of *trans* monoenes resemble those of saturated fatty acids with comparable chain length (1,2). Of additional interest with regard to the dietary *trans* fatty acids are the interrelationships with carcinogenesis, since dietary fat appears to promote the incidence of several types of cancer (3,4).

Although our preceding rat study with unhydrogenated and partially hydrogenated soybean oil indicated a probable modification by *trans* fat of steroid metabolism (5), it was difficult to ascribe the observed results solely to the difference in the geometry of constituent fatty acids, since the effects of *trans* fatty acids depend on the essential fatty acid status (6,7).

In the present study, care was taken to design the diets for the comparative purpose. To ensure the effects of *trans* fatty acids on sterol dynamics, dietary fats with a comparable level of octadecenoic acid but differing in the geometric configuration were chosen. The essential fatty acid level of these fat diets was equalized. Furthermore, polyunsaturated fat and completely saturated fat were simultaneously examined for reference purposes. Under these conditions, the effects of *t*-18:1 on serum cholesterol levels and biliary and fecal steroid excretion were studied in rats fed diets free of or containing cholesterol.

MATERIALS AND METHODS

Animals and Diets

Specific pathogen-free male Wistar rats obtained from a local breeder (Kyudo Co., Kumamoto) were used after several days of acclimation in an air-conditioned room (20-22 C, lights on 0800-2000 hours). The animals weighing an average of 86 g or 101 g for experiments with diets free of or containing cholesterol, respectively, were freely assessed experimental diets and water. The compositions of diets are shown in Table 1. Four different fats served as a fat source: olive oil (OL), partially hydrogenated corn oil (PHC) (5), completely hydrogenated corn oil (CHC) and safflower oil (SA). To make the linoleic acid content equivalent to that of the OL diet, a small portion of hydrogenated fats was replaced with safflower oil. Thus, OL, PHC and CHC diets all supplied ca. 1.7% of calorie as linoleic acid. Total octadecenoic acid contents of OL and PHC diets were similar. Food intake and body weight were measured every 2 days. After feeding these diets for 28 or 29 days, a minute volume of blood was withdrawn from the tail vein for analyses of cholesterol and apolipoproteins. The bile duct was cannulated under light ether anesthesia at 1000-1100 hours during the days 30-36. Rats were kept in restraining cages and the bile was collected for 2 hr by monitoring the flow rate at

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TABLE 1

Composition of Diets (%)

Ingredients	Groups			
	Cis fat diet (OL)	*Trans* fat diet (PHC)	Saturated fat diet (CHC)	Poly-unsaturated fat diet (SA)
Casein	20	20	20	20
Olive oil	10	-	-	-
Partially hydrogenated corn oil	-	9.03	-	-
Completely hydrogenated corn oil	-	-	9.03	-
Safflower oil	-	0.97	0.97	10
Mineral mixture[a]	4	4	4	4
Vitamin mixture				
Water soluble[a]	1	1	1	1
Fat soluble[b]	0.1	0.1	0.1	0.1
Choline chloride	0.15	0.15	0.15	0.15
Cellulose powder	4	4	4	4
Sucrose[c]	60.75	60.75	60.75	60.75
Fatty acid composition (%)				
16:0	11.0	13.1	10.7	6.4
c-16:1	0.7	-	-	-
18:0	2.7	8.5	79.2	2.2
t-18:1	-	42.5	-	-
c-18:1	77.0	27.4	1.5	13.1
cc-18:2	8.1	8.1	8.1	78.0

[a]Harper mixture (5) purchased from Oriental Yeast Co., Tokyo.
[b]Fat-soluble vitamins (retinyl palmitate 400 IU, cholecalciferol 200 IU and DL-α-tocopheryl acetate 10 mg per 100 g diet) dissolved in safflower oil.
[c]Cholesterol, 0.5%, was added at the expense of sucrose.

30-min intervals and kept frozen until analyzed. The bile drained at the constant rate was used for steroid analysis. After the bile drainage, rats were killed by decapitation, blood was collected and the liver and epididymal adipose tissue excised. Prior to blood withdrawal from the tail vein, feces were collected for 2 days and lyophilized.

Lipid and Steroid Analyses

Blood sera obtained prior to bile duct cannulation were analyzed for cholesterol enzymatically (Cholesterol C-Test, Wako Pure Chemicals Inc., Osaka) and for apolipoproteins A-I, B and E by immunoelectrophoresis (8). Cholesterol, triglyceride and phospholipid in post-drainage samples were analyzed as reported elsewhere (9). The procedures for analyzing biliary and fecal steroids and fatty acid compositions of tissue lipids by gas liquid chromatography were the same as reported previously (5). Fecal fat content was determined by the method of Kamer et al. (10).

Statistical Analysis

To evaluate the statistical significance with the probability level of 0.05, Scheffé's analysis of variance was adapted (11).

RESULTS

Growth Parameters and Liver Weight

The average weight gain and food intake per day were 6.3 g and 17.6 g for cholesterol-free diets and 6.0 g and 18.0 g for cholesterol-enriched diets, respectively. Feeding different types of fat diets caused no significant changes in these parameters in both trials. The liver weight was also essentially comparable in rats fed cholesterol-free diets (average 4.1-4.3 g/100 g body weight). Cholesterol feeding resulted in the enlargement of the liver (average 4.5-4.9 g/100 g body weight) except for the CHC group (average 4.1 g/100 g body weight), but again no significant dietary fat-dependent differences were observed.

Serum and Liver Lipids

As shown in Table 2, in rats fed diets free of cholesterol, the concentration of serum cholesterol was the highest, although not significant, in the OL group, whereas that for other fat groups stayed in the same range. The difference in the cholesterol level became more marked on feeding cholesterol-containing diets; the OL group had significantly higher level of serum cholesterol compared to any

TABLE 2

Effects of Dietary Fats on Serum and Liver Lipids

Lipids	Groups[a] OL	PHC	CHC	SA
Cholesterol-free diets				
Serum				
Cholesterol (mg. dl)	152 ± 26[b]	78.4 ± 11.7	81.9 ± 7.9	95.1 ± 6.4
Apolipoproteins (mg/dl)				
A-I	106 ± 3	91.1 ± 6.0	88.0 ± 3.9	90.5 ± 3.4
B	4.7 ± 0.2[c]	4.2 ± 0.4[c]	4.3 ± 0.2[c]	7.1 ± 0.2[d]
E	32.0 ± 6.5	29.7 ± 2.6	34.6 ± 2.9	38.8 ± 1.7
Liver				
Cholesterol (mg. g)	2.8 ± 0.1[c]	2.2 ± 0.1[d]	2.2 ± 0.0[d]	2.4 ± 0.1[c,d]
Triglyceride (mg. g)	17.8 ± 1.9	16.3 ± 1.5	18.7 ± 1.8	18.1 ± 1.7
Cholesterol-containing diets				
Serum				
Cholesterol (mg dl)	297 ± 17[c]	176 ± 15[e]	97.0 ± 5.3[f]	141 ± 4[d,e]
Apolipoproteins (mg. dl)				
A-I	99.1 ± 5.9[c,d]	137 ± 16[c]	77.9 ± 2.5[c,d]	68.5 ± 3.0[e]
B	6.2 ± 0.5[c]	3.2 ± 0.2[d]	4.5 ± 0.3[e]	8.9 ± 0.4[c]
E	32.5 ± 1.6	46.6 ± 5.4	27.0 ± 1.7	32.1 ± 1.8
Liver				
Cholesterol (mg g)	29.9 ± 2.1[c]	22.5 ± 2.5[c]	3.2 ± 0.2[d]	27.2 ± 2.1[c]
Triglyceride (mg g)	35.0 ± 0.8[c,d]	29.4 ± 2.2[c]	15.8 ± 2.2[e]	63.6 ± 9.9[d]

[a]See footnote to Table 1.
[b]Mean ± SE of 8-9 rats per group.
[c-f]Values with different superscript letters were significantly different at $P<0.05$.

one of other 3 groups. Triglyceride and phospholipid levels of post-drainage sera were comparable among groups regardless of with or without dietary cholesterol, respectively (data not shown).

There were no dietary fat-dependent differences in concentrations of serum apo A-I and apo E in rats fed cholesterol-free diets. The concentration of apo B was significantly higher in the SA group than any other groups. When cholesterol was fed, the apo A-I level was the highest in the PHC group and the lowest in the SA group; the difference between these two groups was significant. The serum apo B level was again high in the SA group and it was significantly lower in the PHC than in the OL group. The apo E levels were comparable among groups.

In rats fed cholesterol-free diets, the concentration of liver cholesterol was significantly higher in the OL group than that of 2 hydrogenated fat groups, but not the SA group. Triglyceride levels were comparable to each other. Dietary cholesterol increased the liver cholesterol level to a similar extent in rats fed three unsaturated fat diets, whereas with the saturated fat diet it remained apparently unchanged. The response patterns similar to cholesterol were also observed on hepatic triglyceride and the increase was most prominent in rats fed the SA diet followed by OL and PHC diets in decreasing order. The concentration of hepatic phospholipid was not influenced at all by fat types or dietary cholesterol (data not shown).

Biliary Steroids

The bile flow rate was comparable among different groups, though it was somewhat higher in the CHC group (Table 3). The concentration of biliary cholesterol was significantly higher in the SA than in the CHC group in 2 sets of experiments, but it was indistinguishable between the OL and PHC groups. The concentration of biliary bile acids was unaltered by fat types, irrespective of the presence or absence of dietary cholesterol. Dietary fat types showed no demonstrable effects on biliary steroid compositions except for the saturated fat diet free of cholesterol, where the percentage of the major constituents was somewhat variable.

Fecal Steroids

As shown in Table 4, feeding saturated fat significantly increased fecal weight both in the presence and absence of dietary cholesterol. Due to the increase in excreta, daily fecal neutral steroid excretion was the highest in the CHC group, whereas on the unit weight basis it was the lowest. The apparent absorbability of CHC was calculated to be as low as 34.2%, compared to 93.0-97.5% for other fat groups. Dietary cholesterol did not affect

TABLE 3

Effects of Dietary Fats on Biliary Cholesterol and Bile Acids

Biliary steroids	Groups[a] OL	PHC	CHC	SA
Cholesterol-free diets				
Bile flow (ml/hr)	0.95±0.10[b]	0.82±0.16	1.17±0.09	0.86±0.08
Biliary cholesterol (mg/ml)	0.12±0.01[c]	0.12±0.01[c]	0.08±0.01[c]	0.17±0.01[f]
Biliary bile acids				
Concentration (mg/ml)[c]	10.7 ±0.7	11.8 ±1.5	12.6 ±0.5	11.7 ±1.0
Composition (%)[d]				
Lithocholic	0.7 ±0.3	0.3 ±0.1	0.2 ±0.0	0.4 ±0.1
Deoxycholic	1.3 ±0.3	1.4 ±0.3	0.5 ±0.1	1.3 ±0.3
Chenodeoxycholic	3.6 ±0.5	4.9 ±0.4	2.7 ±0.7	2.9 ±0.4
Cholic+α-muricholic	51.0 ±3.9	55.7 ±1.6	42.1 ±2.7	52.3 ±2.4
12-Ketolithocholic	11.8 ±1.3	10.8 ±0.9	18.3 ±1.2	12.5 ±1.2
β-Muricholic	18.9 ±1.3	16.5 ±1.7	18.2 ±2.6	19.4 ±0.4
ω-Muricholic	4.3 ±1.4	2.2 ±0.3	4.7 ±1.6	3.8 ±0.5
Cholesterol-containing diets				
Bile flow (ml/hr)	0.79±0.09	0.92±0.05	1.03±0.07	0.80±0.09
Biliary cholesterol (mg/ml)	0.15±0.07[cf]	0.16±0.09[cf]	0.11±0.09[f]	0.19±0.10[c]
Biliary bile acids				
Concentration (mg/ml)	13.9 ±1.0	11.5 ±0.9	13.2 ±0.5	13.1 ±1.4
Composition (%)				
Lithocholic	2.0 ±1.0	3.1 ±1.4	1.1 ±0.3	3.7 ±1.9
Deoxycholic	3.3 ±0.4	2.8 ±0.9	1.1 ±0.4	2.3 ±0.8
Chenodeoxycholic	9.8 ±1.0	8.6 ±1.3	4.5 ±1.4	5.5 ±0.5
Cholic+α-muricholic	47.8 ±3.5	39.7 ±2.9	40.3 ±2.2	45.6 ±4.0
12-Ketolithocholic	8.2 ±0.9	7.1 ±1.3	8.8 ±1.4	8.2 ±0.9
β-Muricholic	13.5 ±2.6	14.2 ±2.3	9.5 ±1.8	16.5 ±3.3
ω-Muricholic	6.9 ±1.4	10.1 ±1.3	13.2 ±1.8	7.4 ±1.4

[a]See footnote to Table 1.
[b]Mean±SE of 6-8 rats per group.
[c]As taurocholate.
[d]Excludes 2-3 unidentified steroids.
[c-f]Values with different superscript letters were significantly different at $P<0.05$.

on this parameter. Thus, steatorrhea was evident in rats fed CHC. Steatorrhea would markedly reduce the absorption of cholesterol and bile acids and thus account for the large increase in steroid excretion and the lack of the response to dietary cholesterol in hepatic cholesterol content (Table 2) in rats fed CHC.

When cholesterol-free diets were fed, the PHC group excreted more neutral steroids than the OL group both in terms of daily output and the concentration, the difference in the concentration was significant. A similar but somewhat moderate extent of the increase was also observed on feeding cholesterol-enriched diets. Rats fed polyunsaturated fat in relation to olive oil excreted essentially the same amount of neutral steroids regardless of whether or not there was dietary cholesterol. The extent of bacterial transformation of cholesterol to coprostanol was comparable between the PHC and SA groups both on feeding diets free of and containing cholesterol, and it was considerably lower than that of the OL group. Saturated fat almost completely interfered with the transformation.

In the absence of dietary cholesterol, rats fed the PHC diet excreted more bile acids than those fed the OL diet, the difference in the concentration being significant. Cholesterol feeding diminished the difference between these groups. Fecal excretion of bile acids was low in the SA group, particularly when cholesterol-enriched diets were fed. Though statistically not significant due to considerable fluctuations, there were detectable differences in compositions of fecal acidic steroids between the PHC and OL groups in diets both with and without cholesterol. The pattern observed on the PHC diet did not resemble those on the SA diet. Rats fed a saturated fat diet had the very specific profile on the cholesterol-free diet and the composition roughly approximated to that of the PHC group on the cholesterol-enriched diet.

Fatty Acid Compositions

In rats fed cholesterol-free diets, percentages of linoleic and arachidonic acid in total lipids from serum and liver were similar in the OL and PHC groups and they were slightly lower than the

TABLE 4

Effects of Dietary Fats on Fecal Excretion of Neutral and Acidic Steroids

Fecal steroids	Groups[a] OL	PHC	CHC	SA
Cholesterol-free diets				
Feces excreted (g/day)[b]	0.81 ± 0.08[cc]	0.86 ± 0.07[c]	2.40 ± 0.12[f]	0.54 ± 0.07[c]
Neutral steroids				
Daily excretion (mg/day)	4.3 ± 0.6[c]	7.6 ± 0.5[cf]	8.5 ± 0.8[f]	4.3 ± 0.6[c]
Concentration (mg/g feces)	5.3 ± 0.4[e]	9.0 ± 0.2[f]	3.5 ± 0.3[e]	7.6 ± 0.3[ef]
Composition (%)				
Cholesterol	15.0 ± 1.9[e]	34.1 ± 1.3[c]	92.0 ± 3.4[f]	29.5 ± 7.0[c]
Coprostanol	85.0 ± 1.9[e]	65.9 ± 1.3[c]	8.0 ± 3.4[f]	70.5 ± 7.0[c]
Acidic steroids				
Daily excretion (mg/day)	3.4 ± 0.5	8.3 ± 1.5	10.0 ± 3.9	2.3 ± 0.7
Concentration (mg/g feces)	4.2 ± 0.2	9.0 ± 1.6	4.1 ± 1.6	4.1 ± 1.4
Composition (%)[d]				
Lithocholic	16.0 ± 3.7	18.9 ± 5.4	16.1 ± 6.8	8.2 ± 2.4
Deoxycholic	13.0 ± 2.2	12.8 ± 3.8	30.3 ± 5.3	20.2 ± 3.4
Cholic + α-muricholic	22.1 ± 6.4	17.6 ± 7.4	0.2 ± 0.1	14.3 ± 3.9
12-Ketolithocholic	26.2 ± 6.4	37.3 ± 6.0	19.2 ± 4.0	23.7 ± 5.6
β-Muricholic	8.7 ± 1.1	8.5 ± 2.1	16.0 ± 3.3	18.3 ± 3.2
ω-Muricholic	12.0 ± 5.5	3.3 ± 1.3	15.2 ± 4.6	15.1 ± 7.4
Cholesterol-containing diets				
Feces excreted (g/day)	0.81 ± 0.07[c]	0.88 ± 0.07[c]	2.41 ± 0.07[f]	0.78 ± 0.04[c]
Neutral steroids				
Daily excretion (mg/day)	44.4 ± 2.0[e]	63.7 ± 6.6[e]	104 ± 6[f]	45.1 ± 1.9[e]
Concentration (mg/g feces)	56.2 ± 3.0[ef]	71.6 ± 3.1[e]	42.9 ± 1.6[f]	58.9 ± 3.6[ef]
Composition (%)				
Cholesterol	50.4 ± 3.3[e]	68.2 ± 3.2[c]	97.5 ± 2.0[f]	60.7 ± 5.5[e]
Coprostanol	49.6 ± 3.3[e]	31.8 ± 3.2[c]	2.5 ± 2.1[f]	39.3 ± 5.5[e]
Acidic steroids				
Daily excretion (mg/day)	23.4 ± 2.1	19.6 ± 2.3	42.2 ± 8.9	11.8 ± 2.5
Concentration (mg/g feces)	27.0 ± 2.5	20.9 ± 2.3	17.8 ± 3.9	15.0 ± 2.9
Composition (%)				
Lithocholic	15.1 ± 1.7	16.8 ± 1.1	24.0 ± 6.4	21.9 ± 4.0
Deoxycholic	13.3 ± 0.6	9.5 ± 1.8	1.6 ± 1.1	20.2 ± 5.8
Cholic + α-muricholic	36.9 ± 4.2	19.0 ± 4.6	19.7 ± 4.0	24.1 ± 7.2
12-Ketolithocholic	5.7 ± 1.5	2.2 ± 1.1	5.3 ± 1.3	7.8 ± 2.4
β-Muricholic	11.0 ± 1.3	15.3 ± 2.9	20.9 ± 6.5	9.6 ± 2.7
ω-Muricholic	18.1 ± 4.7	35.6 ± 7.1	25.8 ± 6.4	14.4 ± 4.1

[a]See footnote to Table 1.
[b]Lyophilized feces.
[c]Mean ± SE of 8-9 rats per group.
[d]Excludes 2-3 unidentified steroids.
[c,f]Values with different superscript letters were significantly different at $P < 0.05$.

corresponding values for the SA group. The linoleic acid content of the adipose tissue was also comparable in these 2 groups but was less than one-sixth that of the SA group. Essentially no eicosatrienoic acid (20:3n9) was detected in the OL and PHC groups. Serum, liver and adipose tissue lipids from the PHC group contained a comparable amount of octadecenoic acid, and ca. 25% of that in serum and liver lipids was replaced with the *trans* isomer in the PHC group; the corresponding value for the adipose tissue was ca. 30%. Only marginal amounts of other types of *trans* acids such as *t*-16:1 and *tc*-18:2 or *ct*-18:2 were detected.

Similar patterns of the responses were also observed when OL and PHC diets containing cholesterol were fed. *Trans* fatty acid in the octadecenoic acid fraction approximated to 20% for serum and liver lipids and 40% for the adipose tissue.

DISCUSSION

In the present study, designed to elucidate the effects of *trans* fatty acids on sterol metabolism, the dietary fats containing a comparable amount of octadecenoic acid but differing in the geometry were fed to rats under the condition where the linoleic acid content was made equivalent. The only cognitive difference found in olive oil and partially hydrogenated corn oil was the content of stearic acid, a minor component fatty acid in both

fats. From the fatty acid profile of tissue lipids, the effects of essential fatty acid deficiency could obviously be ignored.

Trans fat in relation to the corresponding *cis* fat did not modify the rate of bile flow nor the concentration of biliary cholesterol and bile acids in two sets of experiments, with or without dietary cholesterol. *Trans* fat also did not always exert statistically significant effects on fecal steroid excretion. However, when diets free of cholesterol were fed, there was a 1.5-fold increase in the excretion of fecal steroids when expressed in terms of mg/day or mg/g feces. The results suggest a net increase by *trans* fat in fecal output of steroids. When cholesterol-enriched diets were fed, the magnitude of the increase in fecal neutral steroids by *trans* fat in relation to *cis* fat was marginal and there was no difference in acidic steroid excretion. It seems likely that the effect of dietary cholesterol on steroid metabolism outweighs that of the difference in the geometric configuration of dietary octadecenoic acid. A similar trend in the increment in fecal steroid excretion was also observed between PHC and SA groups and confirmed the results obtained in the previous study with unhydrogenated and partially hydrogenated soybean oil (5).

The biochemical and physiological significance of the present observation is hard to evaluate at the moment. It is concluded that *trans* fat is no more hypercholesterolemic than *cis* fat when the same amount of linoleic acid is supplied simultaneously. One possible objection to this conclusion is that hypercholesterolemia may be induced by olive oil which contains detectable amounts of squalene, a precursor of cholesterol (our olive oil contained ca. 4 mg squalene/g, thus supplying 40 mg/100 g diet). The hypercholesterolemia observed on feeding olive oil in the cholesterol-free diet may be relevant to the presence of squalene. However, when excess cholesterol was included in the diet, olive oil still elevated the serum cholesterol. Recently, Richter and Schäfer (12) observed that replacement of dietary triglyceride by squalene resulted in a significant reduction of cholesterol absorption in rats. The lower serum cholesterol level associated with feeding *trans* fat would be in part the reflection of increased fecal neutral steroid excretion and hence decreased absorption of cholesterol. In addition, in rats fed a cholesterol-free diet, the increased fecal excretion of bile acids may also account for the reduced serum cholesterol. When diets containing insufficient essential fatty acids were fed to rats, *trans* fat elevated the serum cholesterol level compared to polyunsaturated fat or saturated fat (13).

Together with the changes in serum cholesterol levels, PHC compared to OL decreased serum apo B when cholesterol-containing diets were fed. Although the mechanism whereby dietary *trans* fat exerts its effect on the level of serum apo B is unclear, the difference in the geometry of dietary fats seems also to influence the metabolism of serum apolipoproteins (14,15). The difference in the response of serum apo A-I to dietary PHC and SA was consistent with that observed previously (5).

The increase in steroids in the lumen of the colon is thought to promote the incidence of colonic cancer (16,17), but at present it seems inappropriate to correlate the observed effects of *trans* fat on fecal steroid excretion to the carcinogenesis (18-20). Although there were considerable differences in compositions of neutral and acidic steroids between *trans* and *cis* fat groups, the evidence was not enough to sustain the effects of *trans* fat on the microbial flora. It is therefore unlikely that, so far as the present data indicate, *trans* monoene has a distinct promoting effect on colonic carcinogenesis (21). Of course, long-term feeding trials should be carried out before any definite conclusions should be drawn. Nevertheless, Alfin-Slater and Aftergood (22) were able to observe any untoward effects of *trans* fats on the well being of rats in the multigeneration studies.

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METHODS

The Synthesis and Biological Activity of Thiolcarnitine and Its Thiolesters

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ABSTRACT

Acetyl-D,L-thiolcarnitine was synthesized by the acid-catalyzed addition of thiolacetic acid to 4-trimethylammonio-2-butenoic acid. Acetyl-D,L-thiolcarnitine was the precursor of D,L-thiolcarnitine, which was prepared by base hydrolysis. Thiolcarnitine significantly enriched in the L-isomer was prepared from acetyl-D,L-thiolcarnitine using carnitine acetyltransferase as the resolving agent. The C_2, C_8 and C_{16} carnitine thiolesters were obtained by acylating thiolcarnitine with the corresponding N-hydroxysuccinimide esters. As a substrate for carnitine acetyltransferase, acetylthiolcarnitine gave the same kinetic constants as did acetylcarnitine; on the other hand, thiolcarnitine and carnitine gave the same K_m but the V_{max} with thiolcarnitine was less than 5% of the value obtained with carnitine. With thiolcarnitine and acetylthiolcarnitine as reactants, the measured K_{eq}, at 30 C and pH 7.0, for the reaction catalyzed by carnitine acetyltransferase (see below) was $4.6 \pm .1$.

acetylthiolcarnitine + CoA ⇌ acetyl-CoA + thiolcarnitine

Lipids 18: 382-386, 1983.

A published abstract (1), appearing in 1970, stated that a long-chain carnitine thiolester was biologically active as an analog of the corresponding carnitine ester. Thiolcarnitine (i.e., 4-trimethylammonio-3-mercaptobutanoate) attracted our attention because its availability would allow us to synthesize affinity labels and photoaffinity labels for carnitine and carnitine ester binding sites by derivatizing the thiol group, just as we have done with CoA (2-4). We therefore developed a synthesis for thiolcarnitine and its thiolesters and evaluated their biological activity rather extensively. As we were completing these experiments, Ferri et al. (5) published a note on the mitochondrial handling of D,L-thiolcarnitine and acetyl-D,L-thiolcarnitine, thereby establishing that thiolcarnitine is indeed biologically active as a substitute for carnitine.

This communication reports a synthetic route to thiolcarnitine and its thiolesters that is relatively simple, uses reagents and equipment that are commonly found in biochemical laboratories and yields products in amounts suitable for most biochemical studies. A procedure for enriching the L-isomer content of thiolcarnitine (to 77-86%) was also developed. A synthesis of thiolcarnitine has not been published previously. Preliminary reports of our experiments have appeared (6,7).

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EXPERIMENTAL

Materials

CoA-Li^+ salt, dithiothreitol, acetyl-L-carnitine, and L-carnitine were purchased from P-L Biochemicals (Milwaukee, WI). NAD^+, ATP, ADP, mersalyl acid, citrate synthase (EC 4.1.3.7) type II from pig heart, malic dehydrogenase (EC 1.1.1.37) from pig heart, carnitine acetyltransferase (EC 2.3.1.7) from pigeon breast, and *cis*-oxalacetic acid were purchased from Sigma (St. Louis, MO). Thiolacetic acid, 5,5'-dithiobis(2-nitrobenzoic acid) (abbreviation: DTNB), N-hydroxysuccinimide and D,L-carnitine were purchased from Aldrich (Milwaukee, WI). Thiolacetic acid was vacuum distilled prior to use and DTNB was recrystallized from glacial acetic acid.

Acetyl-CoA (8) and D,L-acetyl carnitine (9) were synthesized by published methods. ATP-citrate lyase (EC 4.1.3.8), purified from rat liver to a specific activity of 2.2 units/mg by the method of Linn and Srere (10), was a gift from David C. Hammond.

Assays

The purity of carnitine compounds was assessed by thin layer chromatography (TLC) at room temperature on silica gel plates (E. Merck, # 5775) using as solvent chloroform/methanol/water/formic acid (50:42:8:2). Carnitine compounds

were visualized with iodine vapors. The R_f were: carnitine, 0.16; thiolcarnitine, 0.10; 4-trimethylammonio-2-butenoate, 0.11; acetylcarnitine, 0.20; acetylthiolcarnitine, 0.18; octanoylthiolcarnitine, 0.38; palmitoylthiolcarnitine, 0.52; dithiothreitol, 0.8. The carnitine thiolesters and 4-trimethylammonio-2-butenoate quench UV irradiation and thus they were detected with a UVS-11 lamp. Thiolcarnitine and dithiothreitol were detected by spraying with 3 mM DTNB, 40 mM potassium phosphate, pH 7.2.

The concentrations of acetyl-L-carnitine and L-carnitine in solutions were determined as described (11). Acetyl-L-thiolcarnitine was determined by the method for acetyl-L-carnitine (11).

RESULTS AND DISCUSSION

Acetyl-D,L-thiolcarnitine

D,L-Carnitine was dehydrated to form 4-trimethylammonio-2-butenoic acid, as described in (12) except that we refluxed the system up to 4 hr. The product separated from the reaction system as an oil upon adding 4 vol of anhydrous ether. The oil was triturated to a solid, which was then dissolved in methanol and treated with small amounts of activated charcoal to remove colored contaminants. Several crystallizations from 2-propanol/methanol (4:1) were required for purification. In a typical synthesis of acetyl-D,L-thiolcarnitine, 11 mmol of the α,β-unsaturated acid were treated with 7 ml of thiolacetic acid and a drop of H_2SO_4. The heterogeneous mixture was stirred under N_2 and heated at 60-70 C for $1^1/_2$-2 hr, then cooled to room temperature and left, with stirring, for several hours. Care was taken to avoid overheating, which led to formation of an intractable orange oil. Progress of the addition reaction was monitored by TLC. The product was filtered and washed with small volumes of acetone and ether. After crystallization from a minimal volume of absolute ethanol, yields ranged from 55 to 80%; mp = 166 C. The L-isomer accounted for 46-49% of the total thiolester content. Confirmation of the structure of acetyl-D,L-thiolcarnitine was provided by high resolution ^1H and ^{13}C NMR analysis (Fig. 1). Proton resonances a, b and d at 3.2, 3.8, and 2.9 ppm agree well with analogous values reported for O-acetylcarnitine (13). The main difference between the spectra of O-acetylcarnitine and acetylthiolcarnitine is the methine proton resonance, which occurs at 5.64 ppm in the former compound and at 4.22 ppm in the latter. Assignment of ^{13}C resonance lines was aided by an experiment (not shown) allowing ^1H and ^{13}C coupling (14). Thus, carbonyl lines g and c were not split, methylene lines b and d gave triplets, methyl lines a and f gave quartets, while the methine carbon c gave a doublet. By analogy to published spectra of trimethyllysine metabolites (15), the trimethylammonio carbon resonance was assigned at 56.27 ppm and the methylene b resonance at 71.19 ppm.

The stability of neutral aqueous solutions of acetyl-D,L-thiolcarnitine toward hydrolysis is good. Thus, 11.5 mM acetylthiolcarnitine was kept at room temperature under N_2 in 50 mM potassium phosphate, pH 7.0, for 4 days, and only 4.3% of the acetylthiolcarnitine had hydrolyzed.

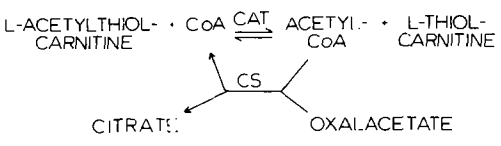
Thiolcarnitine

In a typical preparation of D,L-thiolcarnitine, 0.8 mmol of acetylthiolcarnitine was hydrolyzed with a several molar excess of KOH for 2 min at 0 C and then neutralized with HCl. D,L-Thiolcarnitine thus prepared was used in synthesizing carnitine thiolesters. In aqueous solution exposed to air, thiolcarnitine was relatively stable toward oxidation at pH $\leq$ 4, but oxidation to the disulfide was significant at neutral and basic pH values.

D,L-Thiolcarnitine solutions of known concentration were titrated with a standard NaOH solution to determine pKa values for the carboxyl and thiol groups. A Radiometer ETS 822 titrating system, kindly made available by Dr. R. Middaugh, was used for these studies. The pKa values were determined to be 3.4 and 8.1 for the carboxyl group and thiol groups, respectively. These values can be compared to published pKa values for the carboxyl group of carnitine, 3.88 (16), and the thiol group of cysteamine, 8.2 (17).

For the preparation of thiolcarnitine significantly enriched in the L-isomer, carnitine acetyltransferase was used to resolve the racemic acetylthiolcarnitine. The reaction system is shown in Scheme I (below) where CAT and CS are abbreviations for carnitine acetyltransferase and citrate synthase, respectively.

A reaction system contained the following reagents in 40 ml: acetyl-D,L-thiolcarnitine, 1.6 mmol; oxalacetate, 0.95 mmol; potassium phosphate, pH 7.0, 8 mmol; CoA, 22 μmol; citrate synthase, 1.2 mg; carnitine acetyltransferase, 2.0 mg. The system was maintained at 30 C under a N_2 stream. Aliquots were withdrawn periodically and tested for free thiol with DTNB; ca. 50 min were generally required for 90-95% of the theoretical yield of L-thiolcarnitine to be released. The isolation of L-thiolcarnitine was initiated by adding to the solu-



SCHEME I.

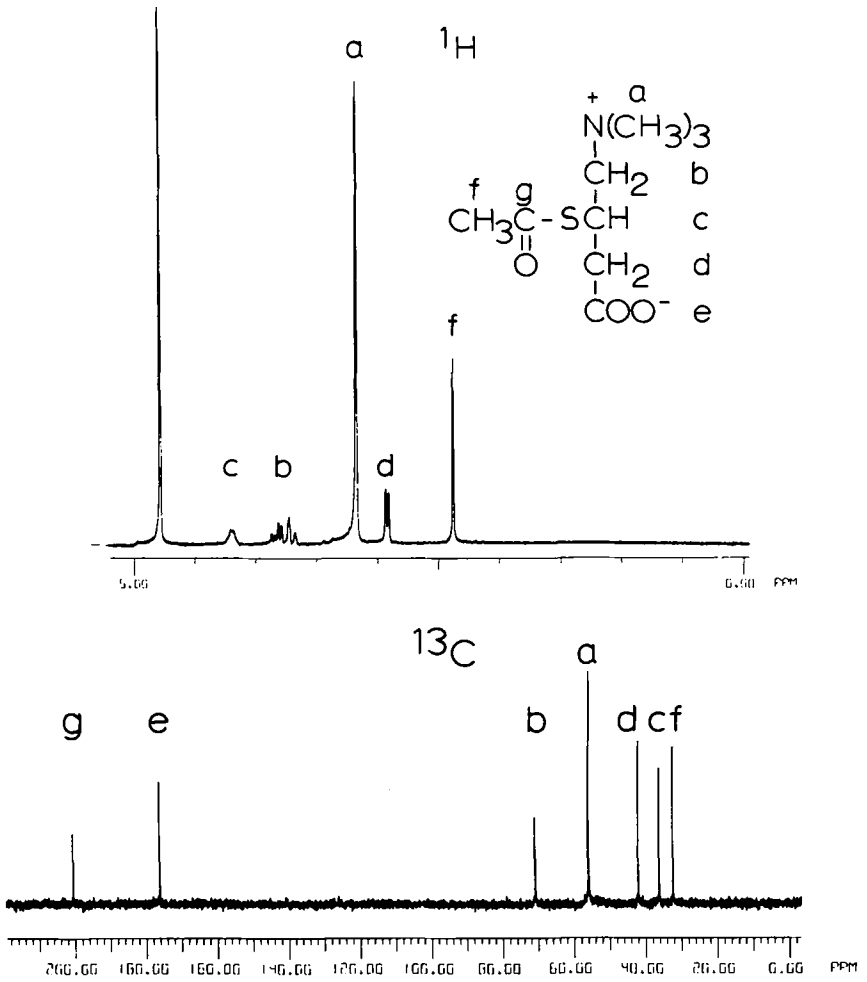


FIG. 1. ^{1}H (top) and ^{13}C (bottom) NMR spectra for acetyl-D,L-thiolcarnitine. Spectra were obtained with a JEOL FX 270 NMR spectrometer, using D_2O as a solvent. The external standard was 2,2-dimethyl-2-silapentane-5-sulfonate in D_2O. An HDO resonance was observed at 4.8 ppm in the ^{1}H spectrum.

tion an amount of mersalyl acid equimolar to the thiol content. The solution was stirred briefly and then chromatographed on a Bio Rad AG 1×8 anion exchange column (18×2 cm) in the Cl^- form. Due to its negative charge, the mercaptide of L-thiolcarnitine and mersalyl acid was retained on the column while other carnitine compounds washed through. After a water wash of ca. 3 column volumes, L-thiolcarnitine was eluted with 100 ml of 16 mM dithiothreitol followed by more water. Eluted fractions were carefully monitored for thiolcarnitine and dithiothreitol by TLC. Thiolcarnitine eluted prior to dithiothreitol. L-Thiolcarnitine fractions were collected and concentrated by rotary evaporation at 30 C; yields ranged from 30 to 65%. After conversion to acetyl-L-thiolcarnitine (see below), the stereoisomeric purity of several preparations ranged from 77 to 86% L-isomer.

Ferri et al. (5) reported that acetylthiolcarnitine is a potent acylating agent. We noted that acetylthiolcarnitine acetylates CoA at a slow but significant rate at neutral pH. Thus, in controls (i.e., without carnitine acetyltransferase) for the measurement of K_{eq} (see below), 20% of the available CoA was acetylated by nonenzymatic transesterification from acetyl-D,L-thiolcarnitine after 50 min of incubation. If 20% of the transesterification events in the reaction system for resolving acetyl-D,L-thiolcarnitine are nonenzymatic, then the maximum possible stereoisomeric purity is 90% L-isomer. We observed somewhat less than 90% stereoisomeric purity. We attribute the observed

lesser levels of stereoisomeric purity to the fact that, as the enzyme removes acetyl-L-thiolcarnitine, it becomes more probable that a nonenzymatic transesterification event will involve acetyl-D-thiolcarnitine rather than acetyl-L-thiolcarnitine.

Carnitine Thiolesters

The thiolesters were synthesized by treating an aqueous solution of thiolcarnitine with 1.5 equivalents of the acetyl, octanoyl, or hexadecanoyl ester of N-hydroxysuccinimide (18). A homogeneous system was achieved by adding 2-propanol, and then a 10-molar excess of $NaHCO_3$ was added. Progress of the reaction was monitored by testing for thiols with DTNB (19); generally, no thiol was detected after 20 min. Acetyl-L-thiolcarnitine was purified by TLC on cellulose plates (E. Merck, # 5503), using as solvent *n*-butanol/acetic acid/water (5:2:3). Octanoyl- and palmitoyl-thiolcarnitine were purified in the same way, except that the solvent was *n*-butanol/acetic acid/water (4:1:1). The product was located with a UVS-11 Mineralight lamp, and the band was scraped and extracted with methanol. Yields ranged from 25 to 40%; thiolcarnitine disulfide was the principal side product. Each of the carnitine thiolesters stimulated O_2 uptake by rat heart and beef heart mitochondria equally as well as the corresponding carnitine ester, supporting observations reported previously (1,5).

Comments on Biological Activity

Ferri et al. (5) showed that D,L-thiolcarnitine and acetyl-D,L-thiolcarnitine are substrates for carnitine acetyltransferase, but they did not determine kinetic constants. We determined whether carnitine and thiolcarnitine, and also acetylcarnitine and acetylthiolcarnitine, exhibited the same kinetic parameters with carnitine acetyltransferase (Table 1). The rate of catalysis with acetylcarnitine or acetylthiolcarnitine as the varied substrate was measured spectrophotometrically at 30 C using an assay solution that contained the following, in μmoles, in 1.0 ml: L-malate, 10; NAD^+, 0.5; EDTA, 1.0; CoA, 0.5; Tris-Cl pH 7.5, 100; and 5.1, 0.74, and 0.55 units of malic dehydrogenase, citrate synthase, and carnitine acetyltransferase, respectively. Concentrations tested ranged from 50 to 800 μM. When carnitine or thiolcarnitine was the varied substrate, the rate was measured spectrophotometrically at 30 C using an assay solution that contained the following, in μmoles, in 1.0 ml: citrate, 20; ATP, 5; $MgCl_2$, 10; NADH, 0.14; acetyl-CoA, 0.15; Tris-Cl, pH 8.0, 100; and 3.0, 1.3, and 0.34 units of malic dehydrogenase, ATP citrate lyase, and carnitine acetyltransferase, respectively. Carnitine and thiolcarnitine concentrations varied from 30 to 600 μM. In both assay systems, the reaction was initiated by adding carnitine acetyltransferase. Kinetic constants were calculated by fitting data to the HYPERO program written by Cleland (20).

TABLE 1

Kinetic Constants for the Carnitine/Thiolcarnitine and Acetylcarnitine/Acetylthiolcarnitine Pairs with Carnitine Acetyltransferase[a]

	K_m(mM)	V_{max}(ΔA_{340}/min)
L-Carnitine	0.21 ± .03	0.78 ± .06
L-Thiolcarnitine	0.19 ± .02	0.035 ± .002
D,L-Carnitine	0.33 ± .03	0.64 ± .02
D,L-Thiolcarnitine	0.17 ± .02	0.021 ± .001
L-Acetylcarnitine	0.069 ± .012	0.38 ± .02
L-Acetylthiocarnitine	0.10 ± .01	0.36 ± .01
D,L-Acetylcarnitine	0.10 ± .02	0.31 ± .02
D,L-Acetylthiolcarnitine	0.14 ± .02	0.31 ± .02

[a]Studies with carnitine and thiolcarnitine were done on the same day with the same assay solutions, and likewise for the acetylcarnitine, acetylthiolcarnitine studies.

The results show that essentially equivalent kinetic constants are obtained for the acetyl compounds when the substrate pair is CoA plus acetylcarnitine or acetylthiolcarnitine. However, when the substrate pair is acetyl-CoA plus carnitine or thiolcarnitine, the K_m values are still similar but the V_{max} for thiolcarnitine is less than 5% that of carnitine.

As reflected in the measured K_m values (Table 1), the binding of carnitine and thiolcarnitine, and their acetylesters, appears to be equivalent. Thus, considering the Haldane Relationship

$$\left(K_{eq} = \frac{V_f}{V_r} \cdot \frac{K_{MP}}{K_{MS}}\right) \quad (21),$$

the comparatively low value observed for V_{max} with thiolcarnitine should be reflected in the measured K_{eq}. That is, for the reaction

acetyl-thiolcarnitine + CoA ⇌ acetyl-CoA + L-thiolcarnitine

one predicts a significantly larger K_{eq} with acetylthiocarnitine and thiolcarnitine than with acetylcarnitine and carnitine. We determined the equilibrium constant at 30 C under N_2 for the reaction catalyzed by carnitine acetyltransferase. The initial system contained the following, in μmoles, in 3.0 ml: CoA, 1.5; acetyl-D,L-thiolcarnitine, 3.0; potassium phosphate, pH 7.0, 300; EDTA, 1.5; and 6 units of carnitine acetyltransferase. Duplicate reactions were stopped at 30, 45 and 60 min by immersion of the tube in water at 80 C for 1 min (22). Aliquots were then assayed for both acetyl-CoA and acetyl-L-thiolcarnitine. The value of K_{eq} was 4.6 ± .1, which differs significantly from the value (K_{eq} = 0.32) reported for essentially the same

system by Ferri et al. (5). The basis of the discrepancy is not known. When the experiment outlined above was repeated, but starting with CoA and acetyl-D,L-carnitine, the K_{eq} was $0.60 \pm .17$ which is the same value reported by Fritz et al. (22).

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Rapid Qualitative Analysis of Polar and Nonpolar Lipids in a Single Sample Using "Three-Way" Thin Layer Chromatography

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ABSTRACT

Plastic sheets, precoated with silica gel, are cut to give a square with a strip attached at the top right-hand corner. Lipid extract is applied to the square portion, just below the strip, and chromatographed in a solvent which moves nonpolar lipids on to the strip, leaving polar lipids at the origin. The strip is cut off and separation of the lipids on the strip and the remaining square portion of the plate is completed by conventional one- or two-dimensional chromatography, respectively. The application of this method and a multiple staining technique to the analysis of lipids in human bronchoalveolar lavage fluid is described. *Lipids* 18: 387-389, 1983.

Thin layer chromatography (TLC) is now well established as a standard technique for the analysis of lipid mixtures (1). Originally, chromatography was carried out using glass plates coated with silica gel in the laboratory, but now commercially available precoated plates are often used for convenience. The silica gel may be precoated on to plastic or aluminium sheets, instead of glass, so that plates can easily be cut to any desired shape before use. This paper describes two additional ways in which the special properties of plastic-backed plates may be exploited: (a) the plate may be cut between solvent runs, and (b) different lipid detection procedures may be applied consecutively to the same plate.

MATERIALS AND METHODS

Lipids, obtained from Sigma Chemical Co., St. Louis, MO, Calbiochem-Behring, La Jolla, CA, and Dr. K. Murray, were dissolved in chloroform or chloroform/methanol for use as standards. Other reagents were analytical grade, except for chloroform and ethanol, which were redistilled before use, and diisopropyl ether, tetrahydrofuran, hexane fraction and dimethoxymethane, which were laboratory reagent grade. Plastic-backed plates, 20 × 20 cm precoated with a 0.2-mm layer silica gel (Merck, Darmstadt No. 5748), were used for TLC.

Bronchoalveolar lavage fluid (BAL) was obtained during bronchoscopy of a normal volunteer, by washing three times with 50 ml NaCl (90 g/l), then centrifuged at 250 g for 10 min. A chloroform/methanol extract of the cell-free supernatant was prepared (2) and its total phosphorus content was determined (3).

'Three-way' TLC: General Procedure

Mark plates with a soft pencil and cut as indicated in Figure 1. Trim 1 mm silica gel coating from the three edges of each plate parallel to the arrow, using a scalpel blade and a straight edge. Prewash the plates, as indicated in Figure 1, if required. Apply lipid to the plate as a 4 mm streak, 10 mm below the dotted line (Fig. 2) and 8 mm from the right-hand edge of the plate. A 5 μl microdispenser (Drummond, Broomall, DE) is particularly suitable for this purpose.

Chromatograph in the direction of arrow 1 in a solvent which moves all or most nonpolar lipids on to the strip (Fig. 2). Remove the strip by cutting with scissors, as indicated by the dotted line (Fig. 2), and chromatograph in additional solvents in the direction of arrow 1 to complete the separation of nonpolar lipids, if required (Fig. 3C). During chromatography, it may be necessary to support the strip in a 2-cm wide stand made of Whatman 3 MM paper (Fig. 1B), so that the lower edge of the silica gel just touches the solvent (see Results and Discussion).

Trim 1 mm silica gel from the upper edge of the remaining square portion of the plate, then carry out conventional two-dimensional chromatography in the directions indicated by arrows 2 and 3 (Fig. 2) to separate polar lipids. During the first solvent run, support the plate in a 9-cm wide stand, as described above, if necessary.

Lipid Detection

The following reagents were used: reagent A, ninhydrin in water-saturated butanol (1); reagent B, stock molybdenum blue reagent (1), diluted with 1 vol each of distilled water and ethanol on the day of use; reagent C, 39 g cupric acetate monohydrate dissolved in 1 liter of 0.5 M H_2SO_4, then diluted with 0.25 vol ethanol; reagent D, 0.5 M H_2SO_4 diluted with 0.1 vol ethanol. Solvent-free plates were treated with reagent A, B or C for 1 min, either by vertical immersion or by placing face down on

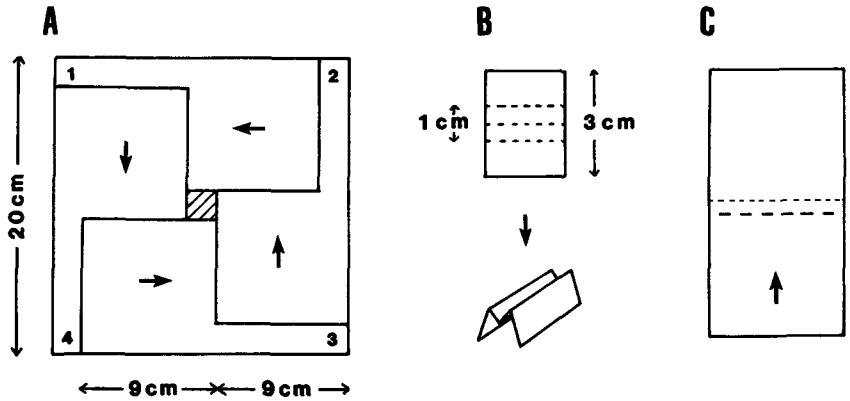


Fig. 1. Preparation for TLC. (A) Layout for cutting four plates from a single plastic-backed sheet of silica gel. For each plate, the arrow indicates the direction of solvent flow during prewashing and in the first solvent. (B) Stand for supporting the plate at the solvent surface during chromatography. (C) An alternative approach (see Results and Discussion section). Arrow, as for (A).

the surface of the reagent in a shallow dish, then drained and heated at 50 C for 5 min (A), dried at room temperature (B) or heated at 170-180 C for 10 min (C) to develop color. For multiple staining, the plate was treated consecutively with reagents A and B, washed by dipping for 5 min in reagent D, then treated with reagent C, with thorough drying between each reagent.

RESULTS AND DISCUSSION

An example of the application of the 'three-way' TLC method to the analysis of a standard lipid mixture and the lipids in bronchoalveolar lavage fluid is presented in Figures 2 and 3. Because, in the solvent system used, monoglyceride and cholesterol were located close to the cutting line after the first solvent run (Fig. 2), a stand was used to support the plate and the strip during subsequent chromatography to prevent these lipids being leached off by the solvent. The whole chromatographic procedure can be completed in less than 2 hr.

Figure 3B illustrates the use of a multiple staining procedure. Reagents A and B are specific for free amino groups and phospholipids, respectively; reagent C is a nonspecific charring reagent. This procedure takes advantage of the strong bonding of the silica gel to the backing material which enables the plate to withstand repeated dipping in staining reagents. It is possible that the unidentified minor components of BAL extract which stain with reagent C, but not with reagents A and B (Fig. 3B), are glycolipids of lung origin (4).

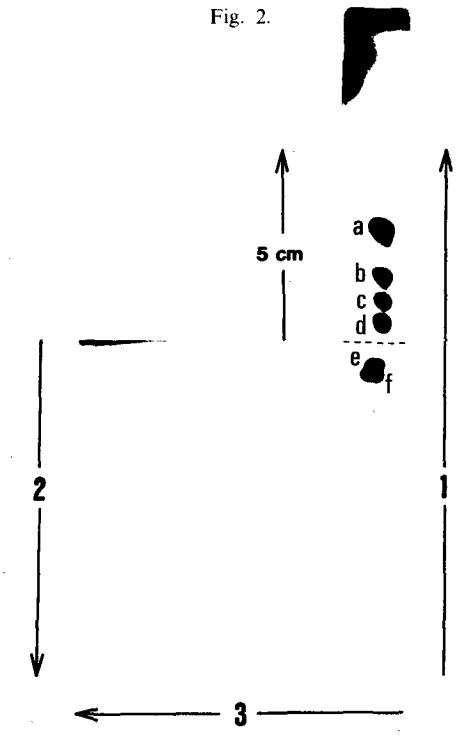
The example described in this paper (Fig. 3) illustrates only some of the possibilities inherent in the use of plastic-backed TLC plates. The principles of the method may be adapted in many ways to meet the specific needs of the user. For example, if only one-dimensional chromatography of both polar and nonpolar lipids is required, several different samples may be applied to a plate 18-cm long (Fig. 1C). The samples would be applied 10 cm from the top of the plate and, after running for 14 cm in the first solvent, the plate would be cut 9 cm from the top, as indicated by the broken line in Fig. 1C. Alternatively, if two-dimensional chromatography of both nonpolar and polar lipids is required, a single sample may be applied in the position closest to the right-hand edge of the plate (Fig. 1C). In addition, it may be possible to develop different combinations of compatible specific staining reagents.

This approach is especially suited for use as a rapid screening procedure, because it obviates the need to separate polar and nonpolar lipids by column chromatography prior to TLC. For example, it could be used in preliminary experiments when information about broad changes in lipid composition is required prior to designing more detailed studies or, alternatively, to monitor the composition of fractions obtained during lipid purification. When used in combination with a sensitive multiple staining procedure, such as that described in this paper, 'three-way' TLC also allows detailed qualitative analysis when only a small amount of lipid is available.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Health and Medical Research Council of Australia (C.D.-C.) and a fellowship from the Royal North Shore Hospital, St. Leonards, N.S.W. (G.J.B.); BAL fluid collected by Dr. D. Allen; technical assistance provided by Mrs. R. M. Price; highly purified cetyl palmitate provided by Dr. K. Murray, CSIRO Division of Food Research, North Ryde, N.S.W.

Fig. 2. The first stage of 'three-way' TLC of lipid standards. Plates were prewashed in chloroform/methanol (2:1, v/v) and 5 μg each lipid standard was applied. The plate was chromatographed in diisopropylether/methanol/acetic acid (50:0.6:0.8, v/v) as indicated by arrow 1, then stained with reagent C. Key: (a) cholesterol stearate, cetyl palmitate and tripalmitin; (b) palmitic acid; (c) 1,2-dipalmitin; (d) cholesterol; (e) monopalmitin; (f) mixture of phospholipids (as in Fig. 3A) at the origin. The dotted line indicates where the plate must be cut before proceeding with chromatography. The arrows indicate the three directions in which solvents travel in the complete procedure.



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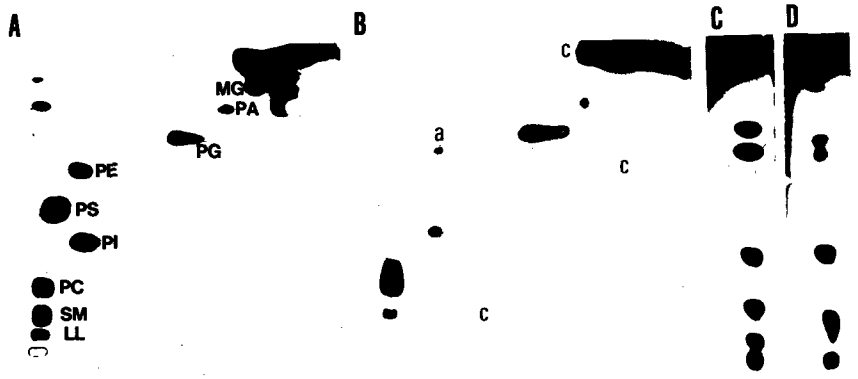

Fig. 3. 'Three-way' TLC of lipid standards and BAL extract. The plates, which contained 5 μg each lipid standard (A,C) or BAL extract (B,D) equivalent to 1.8 ml original BAL fluid (2 μg total phosphorus), were prewashed, chromatographed and cut as described in Fig. 2. The square portions were dried for 5 min in a fume hood, then for 5 min at 60 C and chromatographed in two dimensions using chloroform/methanol/acetic acid/water (25:8:8:1, v/v) followed by tetrahydrofuran/dimethoxymethane/methanol/water (10:6:4:1, v/v), with drying (as above) between solvents. Separation of nonpolar lipids on the strips was completed by chromatographing to the top of the strip in benzene/petroleum ether (40-60 C boiling fraction) (1:1, v/v), then hexane. The strips were dried for 10 min in a fume hood between solvents. Plates A and B were stained with reagent C or by the multiple staining procedure, respectively. The strips were stained with reagent C only. For A: MG, monopalmitin; PA, phosphatidic acid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; LL, lysophosphatidylcholine; unlabeled spots, unidentified impurities. For B: (a) spot which stained with reagent A; (c) spots which stained with reagent C but not with reagents A or B; all other spots reacted with both reagents B and C. For C: lipid standards were (from top to bottom) cholesterol stearate, cetyl palmitate, tripalmitin, palmitic acid, dipalmitin and cholesterol.

COMMUNICATIONS

Iodination of Arachidonic Acid by the Iron/H_2O_2/Iodide System

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ABSTRACT

The newly described iron/hydrogen peroxide (H_2O_2)/iodide antimicrobial system iodinates arachidonic acid to form the same products which are generated by peroxidase/H_2O_2/iodide systems. Arachidonic acid is multiply iodinated with the formation of *bis*-iodohydrins and monoiodinated products which were identified as iodolactones by their high performance liquid chromatography elution patterns and by gas chromatography-mass spectrometric analysis. Iodination of arachidonic acid by the iron/H_2O_2/iodide system appears to proceed via the formation of hydroxyl radicals as an intermediate species. Iodination of unsaturated lipids may contribute to the cytotoxicity of the iron/H_2O_2/iodide system.
Lipids 18: 390-392, 1983.

Arachidonic acid is iodinated when incubated with H_2O_2, iodide and either eosinophil peroxidase (EPO), myeloperoxidase (MPO), lactoperoxidase (LPO) or thyroid peroxidase (1-3). Peroxidase, H_2O_2 and a halide form a potent antimicrobial and cytotoxic system (for review see 4) and one of the mechanisms which may contribute to this toxicity is the halogenation of surface lipids. We have recently demonstrated that Fe^{2+} can replace peroxidase in a H_2O_2- and iodide-dependent antimicrobial system (5) and that this system also can iodinate tyrosine residues of protein (6). We report here that the $Fe^{2+}/H_2O_2/I^-$ system can iodinate arachidonic acid and that the products formed are the same as those generated by the peroxidase systems.

MATERIALS AND METHODS

Special Reagents

Arachidonic acid was obtained from NuChek Prep, Elysian, MN, and (5,6,8,9,11,12,14,15-$^3H_8$)-arachidonic acid from New England Nuclear, Boston, MA. (5,6,8,9,11,12,14,15-$^2H_8$)arachidonate was prepared from eicosatetraynoic acid and deuterium gas as previously described (7). Water was deionized to a resistance of greater than 1.8×10^7 ohms/cm and all salt solutions were passed twice over a Chelex 100 ion exchange resin (8) to remove trace metals. All organic solvents were obtained from Burdick and Jackson, Muskegon, MI, and all other reagents were of the highest commercial grade available.

Iodination of Arachidonic Acid

Arachidonic acid (10^{-4} M, 30 μCi $^3H_8$) was incubated for 30 min at 37 C in 15 ml of 0.02 M sodium acetate buffer, pH 5.5, containing 10^{-4} M ferrous sulfate, 10^{-4} M H_2O_2 and 10^{-4} M NaI. The reaction was terminated by the addition of 0.1 ml of 0.01 M sodium thiosulfate.

Product Analysis

As previously described (3), after addition of sodium thiosulfate, the products were extracted with CH_2Cl_2 and separated by reverse-phase high performance liquid chromatography (RP-HPLC) on a 3.9 mm$\times$30 cm μ Bondapak C_{18} column (Waters Assoc., Milford, MA) using methanol/water/acetic acid (80:20:0:0.1, v/v/v) or 100% methanol as the solvent and a flow rate of 1 ml/min. Radioactive peaks were extracted with CH_2Cl_2 and either rechromatographed or derivatized for gas chromatographic-mass spectrometric (GC-MS) analysis as previously described (3). C-values are expressed as the relative chain length of saturated fatty acid methyl esters.

RESULTS

^3H-arachidonic acid was incubated with Fe^{2+},

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H_2O_2 and iodide, the tritiated products separated by RP-HPLC and their elution profile compared to the profile resulting from incubation with the EPO/H_2O_2/iodide system (Fig. 1). The profiles were similar except for a relative preponderance of fraction C. Peaks A-E in the Fe^{2+}/H_2O_2/iodide system profile were collected, extracted and analyzed by GC-MS. Deuterated analogues of purified products of the LPO/H_2O_2/iodide system with the same elution profiles as those in peaks A, B, D and E were employed as internal standards, and octadeuterated arachidonic acid was used as an internal standard for the products in peak C. The presence of products previously isolated from the peroxidase systems (3) in the corresponding peaks formed by the Fe^{2+}/H_2O_2/iodide system was confirmed by coelution with the octadeuterated analogs with the appropriate chain length (C-value) during GC-MS analysis (Table 1). By these criteria, compound A was identified as a mixture of *bis*-iodohydrins arising from iodohydrin formation at the Δ^8 and Δ^{14} carbons, compound B as 6-iodo-5-hydroxy-eicosatrienoic acid, δ-lactone, compound C as unconsumed arachidonic acid and compounds D and E as a mixture of 14- and 15-iodinated macrolides. Compounds F and G were not amenable to vapor phase analysis but correspond by their retention volume on RP-HPLC to the mixture of nonpolar iodolactones generated by the EPO, MPO and LPO/H_2O_2/iodide systems. Fe^{3+} is unable to substitute for Fe^{2+} in the Fe^{2+}/H_2O_2/iodide microbicidal system (5) and iodinated products were not observed when Fe^{2+} was omitted from the Fe^{2+}/H_2O_2/iodide/arachidonate system.

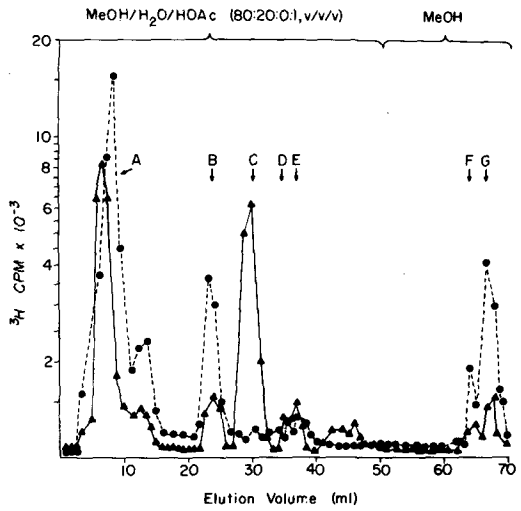

FIG. 1. Reverse-phase HPLC chrpmatogram of ^{3}H-arachidonic acid metabolites generated by the Fe^{2+}/H_2O_2/I^- system. The reaction mixture, incubation and product separation were performed as described in Methods. The products formed by the Fe^{2+}/H_2O_2/I^- system (▲—▲) were compared to those resulting from the incubation, as described in (3), of arachidonic acid with eosinophil peroxidase, H_2O_2 and iodide (●--●).

TABLE 1

GC-MS Analysis of Arachidonic Acid Metabolites Formed by the Fe^{2+}/H_2O_2/I^- System

Peak	C-Value	Ions monitored: Extracted compound m/z	Ions monitored: Internal standard m/z
A	26.0	313,383,437	387
B	23.9	303	311
C	19.3	318	326
D,E	21.8	303	311

DISCUSSION

The findings reported here indicate that a system consisting of Fe^{2+}, H_2O_2 and iodide can iodinate arachidonic acid to generate the same products formed by peroxidase/H_2O_2/iodide systems (3). Structural analysis of the products indicate that each of the 4 double bonds of arachidonic acid are susceptible to iodination. The EPO/H_2O_2/iodide system appeared to be more efficient than the iron-dependent system under our conditions since all the arachidonic acid (peak C) was consumed by the former and ca. 75% by the latter (Fig. 1).

The mechanism of iodination of arachidonic acid by the Fe^{2+}/H_2O_2/iodide system is presumably similar to that for the iodination of tyrosine residues of protein (6). H_2O_2 reacts with Fe^{2+} to generate hydroxyl radicals (OH·) as follows (9):

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH\cdot$$

The oxidation of iodide by the OH· formed would be expected, with the formation of one or more iodinating species (10). The following sequence of reactions,

$$\begin{array}{l} OH\cdot + I^- \rightarrow I\cdot + OH^- \\ I\cdot + H_2O_2 \rightarrow HOI + OH\cdot \\ \hline I^- + H_2O_2 \rightarrow HOI + OH^- \end{array}$$

for example, would generate both the iodine radical (I·) and hypoiodous acid (HOI). The hydroxyl radical does not appear to be an intermediate in the iodination reaction catalyzed by peroxidases (6). Halogenation of membrane arachidonic acid or other unsaturated lipids by toxic iodide oxidation products may contribute to the antimicrobial activity of the OH· generated by phagocytes.

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Bile Salt-Stimulated Lipase in Human Milk from 2 to 16 Weeks Postpartum

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ABSTRACT

Our objectives were to determine the change in bile salt-stimulated lipase (BSSL) activity in mature milk with time postpartum and to determine if BSSL activity was related to total lipid in the milk. Milk samples were collected from 12 mothers at 2, 6, 12 and 16 weeks postpartum. A significant ($P < .05$) decrease in BSSL activity with time was observed. The average values expressed as μmol free fatty acids released/ min/ ml milk were 5.3, 4.5, 4.1 and 3.6 at 2, 6, 12 and 16 weeks postpartum. Total lipid in the milk increased significantly ($P < .05$) from 4.1 g/ dl at 2 weeks to 5.4 g/ dl at 16 weeks postpartum but was not significantly correlated with BSSL activity.

Lipids 18: 393-396, 1983.

While breast milk is recognized as the best nutrient for infants, we are only beginning to appreciate some of its unique contributions in support of infant health. In this paper, we report on bile salt-stimulated lipase (BSSL), a lipase in breast milk which may be capable of contributing significantly to the digestion of milk lipids in the neonate. Since 50% of the calories consumed by the breast-fed infant are derived from lipids, their efficient digestion and absorption from the gastrointestinal tract is a major concern in neonatal nutrition. The physiological implications for the presence of BSSL in breast milk on infant nutrition have been reviewed (1-3).

In this study, we determined the activity of BSSL in milk collected from mothers 2 to 16 weeks postpartum. Our major objective was to determine if BSSL activity changed with length of lactation. In two previous studies, BSSL activity did not change significantly with time postpartum (4,5). However, in these reports, the same mothers were not repeatedly sampled and we believe that individual differences between mothers may have masked differences due to the length of lactation. Therefore, we felt that a study with healthy women repeatedly sampled under carefully controlled conditions might detect differences that were missed in the previous studies.

Bile salt-stimulated lipase activity also was compared to total lipid in the milk. Hall and Muller (5) observed a correlation between BSSL activity and lipid concentration within a feeding. A secondary objective of this study was to determine if the relationship between BSSL activity and lipid concentration could be extended to a broader spectrum of milk samples.

MATERIALS AND METHODS

Twelve mothers were recruited in the second and third trimester of pregnancy from the practices of cooperating obstetricians in North Central and Eastern Connecticut. Donors were given a full explanation of the project and written consent was obtained. Milk samples were collected between May 1980 and December 1980 from mothers whose milk was the total source of calories for their infants.

Milk samples were taken by trained investigators in a home situation using an electric breast pump (Egnell, Inc., Cary, IL). The total milk from one breast was taken at least 1 hr after a previous nursing in the morning (9.30-11.30 a.m.) and again in the afternoon (1.30-3.30 p.m.). The milk was immediately placed on dry ice and at the end of the day transported to the laboratory for storage at -70 C. Milk was collected at 2, 6, 12 and 16 weeks postpartum.

On the day of the analysis, milk was thawed and equal volumes of a.m. and p.m. milk were pooled for analysis. The total lipids were extracted by a modified Folch procedure and weighed as previously described (6).

The activity of BSSL in the milk was measured by the method of Hernell and Olivecrona (7). The substrate was prepared by the addition of ca. 0.9 μCi [carboxyl-^{14}C] triolein, 25 mg of carrier triolein, 1.25 ml 1 M Tris-HCl buffer (pH 9.0), and 1.0 ml of 10% gum arabic. The mixture was sonified at 2 × 15 sec (Branson Instruments, Stamford, CT). To this sonified mixture, we added 2.5 ml 1 M NaCl, 0.9 ml freshly prepared 10% sodium taurocholate and 2.5 ml of 18.6% bovine serum albumin. A second substrate was prepared without sodium taurocholate. The 180 μl of substrate was pipetted into a test tube and preincubated at 37 C for 15 min.

The enzyme source was milk diluted 1:150 with 5 mM veronal buffer, pH 7.4. Twenty μl of the diluted milk was added to the 180 μl of preincubated substrate and mixed. The final assay mixture was 3.1 mM triolein, 138 mM Tris-HCl buffer, 1.2% gum arabic, .28 M NaCl, 19 mM sodium taurocholate

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and 5.2% bovine serum albumin. The assay was incubated in a Metabolyte water bath (New Brunswick Scientific Co., New Brunswick, NJ) at 37 C, 60 cycles/min. For each milk sample analyzed, there were two blanks, one without enzyme and one without bile salts. Four percent of the samples exhibited some lipolytic activity in the absence of bile salts. However, this activity was minor, less than 0.2 μmol free fatty acids released/min/ml of milk.

The reaction was stopped by the addition of 3.25 ml of a mixture of methanol/chloroform/heptane (1.41:1.25:1.00), followed by 1.05 ml of 50 mM boric acid, potassium carbonate buffer, pH 10.0. To separate the free fatty acids from unhydrolyzed substrate, each sample was immediately vortexed and centrifuged for 15 min at 900 × g. One ml of the aqueous upper phase was drawn off and counted by liquid scintillation. Extraction of fatty acids was determined to be ca. 90%. Corrections for extraction and quench were made. Results were expressed in units defined as 1 μmole of free fatty acid released/min/ml of milk.

Milk from 3 mothers at least 2 weeks postpartum was used to determine the appropriate incubation time for the BSSL assay. The μmoles of free fatty acids released by BSSL after 0, 15, 30, 45, and 60 min incubation were determined. The linearity of this data was determined by least squares regression analysis.

Statistical analysis of our longitudinal study was based on analysis of variance of a complete block design with repeated measurements (8). The assumptions of equal variances and correlations between times needed for the analysis of variance were tested and confirmed. If a significant change with time was detected, orthogonal contrasts between times were performed to determine if the change was linear or quadratic. To determine if BSSL activity was related to total lipid in the milk, a regression analysis was performed which compared the within and between times relationship of BSSL and milk lipid. This analysis is described in Snedecor and Cochran (9).

RESULTS

Under our incubation conditions, the BSSL activity with time was found to be linear from 0 to 60 min. We chose 45 min in all subsequent BSSL assays.

The volume of milk collected during the study is reported in Table 1. The average volume collected was 57.4 ml with no significant difference between the various times postpartum. This volume represents two complete breast expressions and based on values in the literature represents slightly less than 10% of an average daily milk production (10). Although it would have been more desirable to obtain a 24-hr collection, in most cases this would have been impossible to obtain. Our collection procedure was carefully standardized, and we feel it was the most satisfactory procedure short of 24-hr collection.

TABLE 1

Milk Volume, Total Lipid, and Bile Salt-Stimulated Lipase Activity[a]

Milk analysis	Stage of lactation weeks of postpartum				
	2	4	12	16	CV[b]
Milk collected (ml)	51.5	59.0	60.4	58.7	35.0
Total lipid (g/dl)	4.1	4.4	4.9	5.4	12.9
Bile salt-stimulated lipase activity (units/ml milk)	5.3	4.5	4.1	3.6	22.5

[a]Twelve observations in each mean.
[b]Coefficient of variation $[(\text{error mean square})^{1/2}/\bar{x}] \times 100$.

In the longitudinal study, bile salt-stimulated lipase activity was observed in all the samples analyzed. The average activity was 4.4 units ± 1.0 SD. The range of values observed were from 2.6 to 10.1 units. There was a significant ($P < .05$) linear decrease in activity from 5.3 units at 2 weeks to 3.6 units at 16 weeks postpartum (Table 1).

There was a significant ($P < .05$) linear increase in total lipid from 4.1 g/dl at 2 weeks to 5.4 g/dl at 16 weeks postpartum. The mean lipid content of milk in this study was 4.7 g/dl ± 0.5 SD. The range was from 2.1 to 7.3 g/dl.

From the data in Table 1, it appeared that there might be a negative relationship between BSSL activity and total lipid in the milk. In Table 2, values for regression analysis are given. The overall regression between total lipid and BSSL activity was not significant. However, when the between times regression was broken out, a significant regression coefficient ($P < .01$, $B = -1.01$) was calculated between total lipid and BSSL activity. The between times relationship is clearly observed in Table 1 with concentration of lipid increasing with the time postpartum from 4.1 to 5.4 gm/dl and the BSSL activity decreasing from 5.3 to 3.6 units/ml milk. This relationship is not seen within times as there is no significant regression. From the regression analysis, we concluded that BSSL activity and lipid content in the milk are not related but are both influenced by time postpartum.

DISCUSSION

Two previous studies report no significant change in the activity of bile salt-stimulated lipase with time postpartum. The study by Hernell et al. (4) had several uncontrolled variables which might have masked any change in BSSL activity with

TABLE 2

Comparison of between and within Time Regression Analysis of BSSL Activity with Milk Lipid

Comparison	Regression coefficient	(Significance level)	Sum of squares		(df)
			Regression	Residual	
Between times	−1.01	(.01)	10.128	6.877	(10)
Within times	−0.06	(N.S.)	0.209	85.646	(35)
Total	−0.221	(N.S.)	2.934	99.926	(46)

time postpartum. Some of these uncontrolled variables were varying degrees of malnutrition, a lack of uniform sampling times postpartum and a large variation between mothers. For a study which was not closely controlled, they had relatively few observations which ranged from 9 at one time period to 29. It is not surprising that they observed no statistically significant change in BSSL activity with time postpartum.

In a second study by Hall and Muller (5), 45 samples of human milk were collected from 14 women from the fourth day to 71st week postpartum. One woman provided 25 of the samples, while most of the other women provided one sample each. The sampling times postpartum for different women were not very well distributed over the experimental period. While this study is of interest because of some of the other observations made in it, the data on BSSL activity with time postpartum cannot be analyzed statistically and would therefore be difficult to draw any conclusions from.

After completing the work for this manuscript, a third report on BSSL activity with time postpartum has been published. Mehta et al. (11) obtained repeated milk samples from mothers and analyzed for BSSL and bile salt-stimulated esterase (BSSE) activities. Their milk sampling and methods were very similar to ours. However, they observed no change in BSSL activity with time postpartum. They did observe a decrease in BSSE activity. They further characterized the two enzyme activities by gel filtration chromatography and gel electrophoresis and found that both activities had identical elution and migration patterns. This suggested that the lipase and esterase activities were associated with the same protein moiety. If the lipase and esterase activities are due to one enzyme, it is difficult to explain how the esterase activity is altered without a change in lipase activity. Further work is needed to explain the difference between our observations and those of Mehta et al. (11).

One suggested explanation for our observed decrease in BSSL activity with time postpartum was that the lipid from the milk, which increased with time postpartum, was significantly decreasing the specific activity of the triolein in our assay mixture. However, this was not the case. The milk used in our assay was diluted 150-fold. This meant that on the average 1.1% of the lipid in the assay was from milk lipid. The contribution of milk lipid ranged from 0.5 to 1.8% of the total lipid in the assay. The contribution of endogenous milk lipid was extremely small when compared to the 32% decrease in BSSL activity observed between 2 and 16 weeks postpartum.

In conclusion, our results would suggest that BSSL activity declines with time postpartum. From these results, it would appear that BSSL activity in milk is greatest when the infant's digestive system is least developed and that declining BSSL activity in breast milk would coincide with a maturing digestive capability in the infant. These results may be of clinical importance in view of the work by Alemi et al. (12). They improved fat digestion in very low-birth-weight infants by feeding a mixture of raw human milk and low-birth-weight formula. If this practice were to be expanded, based on our results, the best source of BSSL would be milk from mothers during the initial stages of lactation.

Finally, we did not observe a significant relationship between BSSL activity and lipid in the milk. We, therefore, could not extend to a broader spectrum of milk samples the observation by Hall and Muller (5) that BSSL activity and lipid concentration in milk were correlated.

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Fatty Acid Composition of Serum Lipids in Fasting Ponies

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ABSTRACT

Alterations in the fatty acid distribution of total lipid extracts and 4 of the major lipid subclasses of serum in ponies fasted overnight and for 4 and 7 days were determined. Although increases in 16:0, 16:1, and 18:3ω3 were observed, decreased amounts of 18:0 and 18:2ω6 combined to cause no significant change in the saturated to unsaturated fatty acid ratio in the total extracts. Phospholipid became somewhat preferentially enriched in saturated fatty acids due to a decrease in 18:1, although this response was variable. The free fatty acid and triglyceride fractions both showed increases in relative amounts of 18:3ω3 and a decrease in 18:0 and a concomitant change in the saturated to unsaturated fatty acid ratio. This endogenous alteration was most likely due to the mobilization of an increased proportion of polyunsaturated fatty acids from tissue sites with their subsequent incorporation into triglyceride by the liver. It probably reflects the type of forage diet on which the animals had been maintained prior to the study. The fatty acid composition of the cholesteryl ester fractions was unchanged during fasting but contained appreciable amounts of the 18:2ω6 fatty acid.

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Hyperlipidemia in equines presented for treatment of a variety of diseases associated with food deprivation has been described (1). Under these conditions, a greater than 50-fold increase in serum triglycerides and fatty livers were observed. Failure of these animals to eat, along with the subsequent mobilization of endogenous fat, was apparently responsible for the initiation of these changes. Bartley (2) reported other features of this hyperlipidemic response including increased serum total free fatty acid and cholesterol concentrations. He suggested that the lipoprotein elevated during fasting was the very low density fraction (pre-β). Morris et al. (3) found a tremendous increase in the pre-β lipoprotein fraction of fasted ponies, which appears unique for this species.

When clinically normal ponies are experimentally fasted for 3 or more days, VLDL concentrations appear to exceed by far the amount found in serum of other species, including man. Bauer (4) has found the increase in the pre-β band of plasma lipoproteins separated on agarose gel electrophoresis to be consistent with the very low density lipoprotein ($d < 1.006$ g/ml) isolated via ultracentrifugation. In addition, compositional analysis of all lipoprotein fractions separated ultracentrifugally indicated no appreciable differences among the LDL (1.006-1.063 g/ml) and HDL (1.063-1.21 g/ml) fractions in either fed or fasted animals. The serum VLDL concentrations of these fasted animals, however, increased 12-fold over fed VLDL levels and contained half as much protein and 50% more cholesterol than fed pony VLDL, whereas the relative triglyceride concentrations were nearly identical.

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Holman (5) in his studies of fatty acid profiles in human disorders has concluded that it is the relative proportion of the individual fatty acids within a lipid class which governs the availability of substrates at the surface of a membrane and not the concentration in the surrounding cytoplasm which determines the properties of the whole. Thus, in addition to the lipid and lipoprotein analysis of fasted ponies, the determination of the relative fatty acid distribution of the total lipids and 4 of the major subclasses of lipids in the serum of normally fed or fasted ponies is important in characterizing the hyperlipidemic response in fasting ponies. Also, since only limited information on the gas liquid chromatographic analysis of fatty acid methyl esters of serum from full-size horses exists (6,7) and since no systematic studies on the plasma fatty acid composition of either fed or fasted ponies have been previously reported, this study was undertaken.

METHODS

Experimental Animals

Six ponies, ranging in age from 1 to 6 years, were used in these studies. They were housed in individual stalls, indoors on concrete, and had free access to fresh water and trace mineralized salt at all times. On the day experiments were begun, 4 of the animals were selected for the fasted group while the 2 remaining ponies were used as additional control animals. Prior to beginning the experiments, the animals' diets consisted of locally available mixed grass pasture.

Fasting was begun at 5:00 p.m. and all blood

samples were obtained between 9:00 and 10:30 a.m. each subsequent day for 8 days for serum lipid analysis. Samples for lipid fractionation and fatty acid analysis were obtained on days 0 from all 6 animals and on days 4 and 7 from the 4 fasted animals. All samples were collected via jugular vena puncture, placed in tubes containing sodium citrate and kept at 4 C.

Sample Preparation and Analysis

Total lipid was extracted from plasma samples in 60 vol of $CHCl_3/CH_3OH$ (2:1, v/v). The lipid extracts were first partitioned with 4 vol of H_2O followed by 2 additional washes of the lower phase with $CHCl_3/CH_3OH/H_2O$ (3:48:47, v/v/v).

The major lipid classes were fractionated via thin layer chromatography using Silica Gel G coated glass plates (Brinkman Instruments, Inc., Westbury, NY) developed in hexane/diethyl ether/acetic acid (90:10:1, v/v/v). After development, the plates were sprayed with 0.2% 2,7-dichlorofluorescein in ethanol (w/v) and spots were outlined under longwave UV-light and collected. The lipid classes were identified with the aid of appropriate reference standards (NuChek Prep, Elysian, MN). Fatty acid methyl esters of total extracts or isolated lipid classes were prepared using 4% sulfuric acid in methanol (40 min, 90 C) and extracted with hexane.

Fatty acid methyl esters were analyzed on a 6 ft × 8 in. stainless steel column packed with 10% SP 2330 on 100/120 Chromosorb WAW (Supelco, Inc., Bellefonte, PA). Fatty acids were identified with appropriate methyl ester standards by comparing retention times. Operating conditions of the Hewlett-Packard 5840 gas liquid chromatograph equipped with a dual flame ionization detector were as follows: the injection port heater and flame ionization detector temperature were maintained at 250 C and 290 C, respectively. Nitrogen carrier-gas flow was adjusted to 20 ml/min with 15 ml/min hydrogen gas flow rate and 20 ml/min air flow rate. Column oven temperature was 190 C for first 20 min of analysis then temperature programmed at 7 C/min to 210 C. Identification of certain fatty acid isomers was additionally confirmed via combined GLC-mass spectrometry (data not shown).

RESULTS

The fatty acid composition of the total lipid extract from fasted ponies is presented in Table 1. The major fatty acid in overnight fasted animals (16 hr) was linoleic (36.9%) with approximately equal amounts (16.9-18.4%) distributed among palmitic, stearic and oleic acid. These 4 fatty acids comprised 90% of the total serum fatty acid distribution on day 0, while α-linolenic acid contributed ca. 3%. On day 4 of the experimental period, palmitic acid accounted for 31.6% of the total, whereas stearic and linoleic acids showed a relative decrease to 5.5% and 20.4%, respectively. Relative amounts of palmitoleic and α-linolenic acids were increased. On day 7 of the fasted period, this distribution was essentially unchanged from day 4. Total serum triglyceride in these fasted ponies increased by day 4 to an average of 733 mg/dl as compared with 28 mg/dl in the overnight fasted state, as reported previously (4). This change was accompanied by a mean 12-fold increase in serum VLDL in the fasted animals, but LDL and HDL concentrations remained unchanged. Although changes in the relative distribution of serum fatty acids occurred with fasting, absolute amounts of each fatty acid were increased in every case. The ratio of saturated to unsaturated fatty acids (s/u ratio) of the total lipid extract remained essentially constant during the experimental period as well.

TABLE 1

Effect of Fasting in Pony Serum Total Lipid Fatty Acid Composition[a]

Fatty acid	Days fasted 0 (n=6)	4 (n=4)	7 (n=4)
14:0	1.0 ±0.4	1.3 ±0.4	1.2 ±0.2
14:1	trace[b]	trace	trace
16:0	16.9 ±1.8	31.6 ±4.2[c]	29.6 ±1.7[c]
16:1	1.5 ±0.4	4.7 ±1.3[c]	5.7 ±1.0[c]
17:0	trace	trace	trace
18:0	17.5 ±2.4	5.5 ±0.6[c]	6.8 ±1.3[c]
18:1ω9	18.4 ±2.9	22.3 ±4.4	22.1 ±3.1
18:2ω6	36.9 ±2.9	20.4 ±6.1[c]	22.2 ±5.4[c]
18:3ω3	2.8 ±1.3	11.2 ± .64[c]	11.1 ±2.0[c]
20:0	trace	trace	trace
20:2	trace	trace	trace
20:4	trace	trace	trace
>22:4	2.6	2.2	1.7
s/u[d]	0.59± .05	0.63± .08	0.63± .07

[a]Values are weight percentages of fatty acid methyl esters (mean±SD).
[b]Trace refers to < 0.5%.
[c]$p < .001$ as compared to day 0 fatty acid.
[d]Ratio of saturated to unsaturated.

Changes in phospholipid fatty acid composition (Table 2) after fasting either 4 or 7 days included only slight changes in palmitic and linoleic acids which due to some variability in the response were not consistently significant. However, a 2-fold reduction in oleic acid was observed which caused a significant alteration of the s/u ratio at day 4 of the experiment. Variation in the response at day 7 caused this change to become less significant. One animal, which was largely responsible for this

TABLE 2

Effect of Fasting on Pony Serum Phospholipid Fatty Acid Composition[a]

Fatty acids	Days fasted 0 (n=6)	4 (n=4)	7 (n=4)
14:0	trace[b]	trace	trace
14:1	trace	0.7 ±0.6	trace
16:0	15.5 ±1.0	20.4 ±2.0[c]	19.3 ±3.4
16:1	1.1 ±0.2	1.1 ±0.2	1.7 ±1.1
17:0	1.0 ±0.5	0.9 ±0.2	0.6 ±0.1
18:0	26.6 ±1.7	27.5 ±2.8	26.1 ±2.5
18:1ω9	16.5 ±2.2	7.8 ±1.8[c]	8.6 ±1.5[c]
18:2ω6	31.1 ±1.0	34.4 ±1.2[d]	34.4 ±5.3
18:3ω3	trace	1.7 ±0.3	1.9 ±0.3
20:0	0.9 ±0.3	trace	trace
20:1	0.7 ±0.3	trace	trace
20:2	trace	trace	trace
20:4	trace	trace	trace
22:0	0.5 ±0.3	0.9 ±0.1	trace
20:3/22:1	trace	0.9 ±0.2	0.8 ±0.2
22:6	0.6 ±0.4	trace	0.5 ±0.4
24:0	1.0 ±0.5	0.7 ±0.4	1.0 ±0.3
24:1	1.7 ±0.9	1.4 ±0.5	1.4 ±0.6
s/u[e]	0.88±0.03	1.05±0.10[d]	0.98±0.12

[a]Values are weight percentages of fatty acid methyl esters (mean±SD).
[b]Trace refers to < 0.5%.
[c]$p < .001$ as compared to day 0 fatty acid.
[d]$p < .01$ as compared to day 0 fatty acid.
[e]Ratio of saturated to unsaturated.

TABLE 3

Effect of Fasting on Pony Serum Free Fatty Acid Composition[a]

Fatty acid	Days fasted 0 (n=6)	4 (n=4)	7 (n=4)
12:0	trace[d]	trace	trace
14:0	1.4 ±0.4	trace	0.7 ±0.2
14:1	trace[e]	trace	trace
16:0	19.9 ±2.2	20.0 ±1.6	17.7 ±3.1
16:1	3.1 ±0.5	3.7 ±1.3	3.5 ±0.7
17:0	trace	trace	trace
18:0	15.3 ±3.2	6.7 ±2.5[d]	10.7 ±1.8[d]
18:1ω9	26.6 ±4.8	32.5 ±6.8	27.3 ±2.5
18:2ω6	26.0 ±4.0	19.1 ±4.8[c]	19.8 ±3.9[c]
18:3ω3	5.4 ±4.6	14.7 ±2.0[d]	24.7 ±6.1[c]
s/u[f]	0.59± .08	0.40± .10[d]	0.38±0.05[c]

[a]Values are weight percentages of fatty acid methyl esters (mean±SD). Only trace amounts of individual fatty acids > 20:0 were found.
[b]Trace refers to < 0.5%.
[c]$p < .001$ as compared to day 0 fatty acid.
[d]$p < .01$ as compared to day 0 fatty acid.
[e]$p < .05$ as compared to day 0 fatty acid.
[f]Ratio of saturated to unsaturated.

variation, as previously reported (4), also had the smallest hyperlipemic response during the fasting period (only a 6-fold increase in serum triglyceride).

Alterations in the serum free fatty acid composition (Table 3) showed a marked increase in α-linolenic and a decrease in stearic acids. Linoleic acid was also decreased to a lesser extent. In addition, the s/u ratio decreased, indicating that fasted pony serum is relatively unsaturated fat enriched when compared to the control (overnight fasted) situation. It is interesting to note that the relative amount of α-linolenic acid was considerably greater at day 7 (25%) than at day 4 (15%) when compared to the day 0 (5%) situation. This is in contrast to the distribution of the other fatty acids which were essentially unchanged when days 4 and 7 were compared.

Tables 4 and 5 show the fatty acid distribution in the triglyceride and cholesteryl ester subclasses, respectively. The triglyceride fatty acid showed a decrease in stearic and an increase in α-linolenic acids on both days 4 and 7 of the fasting period. A preferential increase in α-linolenic acid was not seen on day 7 when compared to day 4 as in the case of the free fatty acids, however. No significant changes in the saturated to unsaturated fat ratios were observed in the cholesteryl ester fractions of fasted animals when compared with the fed state. The relative fatty acid distribution also remained unchanged. It should be noted, however, that most of the fatty acid contained in this lipid class was linoleate.

TABLE 4

Effect of Fasting on Pony Serum Triglyceride Fatty Acid Composition[a]

Fatty acid	Days fasted 0 (n=6)	4 (n=4)	7 (n=4)
14:0	2.0 ±0.9	1.3 ±0.7	1.6 ±0.4
14:1	0.8 ±0.5	trace[b]	0.5 ±0.1
16:0	32.4 ±3.6	37.8 ±3.8	37.0 ±3.7
16:1	4.7 ±0.5	5.5 ±1.8	5.6 ±1.7
17:0	trace	trace	trace
18:0	7.9 ±2.3	3.2 ±0.8[d]	3.4 ±0.9[d]
18:1ω9	30.6 ±5.9	25.4 ±5.2	25.5 ±3.5
18:2ω6	11.1 ±1.7	12.3 ±3.9	12.9 ±3.6
18:3ω3	5.0 ±1.5	12.3 ±2.1[c]	12.1 ±1.1[c]
s/u[c]	0.83±0.11	0.77±0.14	0.74±0.14

[a]Values are weight percentages of fatty acid methyl esters (mean±SD). Only trace amounts of individual fatty acids > 20:0 were found.
[b]Trace refers to < 0.5%.
[c]$p < .001$ as compared to day 0 fatty acid.
[d]$p < .01$ as compared to day 0 fatty acid.
[e]Ratio of saturated to unsaturated.

TABLE 5

Effect of Fasting on Pony Serum Cholesteryl Ester Fatty Acid Composition[a]

Fatty acid	Days fasted 0 (n=6)	4 (n=4)	7 (n=4)
14:0	0.7 ±0.5	trace	trace
14:1	trace[b]	trace	trace
16:0	9.2 ±3.0	12.1 ±1.5	11.0 ±0.9
16:1	2.6 ±0.6	2.8 ±0.7	2.3 ±0.5
17:0	-	-	-
18:0	2.1 ±1.2	1.4 ±0.2	0.8 ±0.1
18:1ω9	8.8 ±4.5	7.9 ±2.0	7.8 ±1.5
18:2ω6	74.1 ±6.2	72.5 ±4.1	73.7 ±4.2
18:3ω3	trace	0.7 ±0.3	1.0 ±0.6
s/u[c]	0.15±0.07	0.18±0.02	0.18±0.05

[a]Values are weight percentages of fatty acid methyl esters (mean±SD). Only trace amounts of individual fatty acids > 20:0 were found.
[b]Trace refers to < 0.5%.
[c]Ratio of saturated to unsaturated.

DISCUSSION

Earlier studies on equine lipids analyzed via gas chromatography were accomplished using serum (6,8) or milk (9) from the full-size horse. In addition, these animals were in the normally fed state. The present work concerns itself with the fatty acids of serum lipid subclasses from ponies both in the fed and fasted states and, as such, represents an initial characterization of pony serum lipids.

Generally, mammalian tissues contain lipids preferentially enriched in 16 and 18 carbon chain length fatty acids. This finding was also observed in the total lipid extracts of fed pony serum, and is consistent with a previous report in which serum lipid fatty acids were determined in the full-size horse (6). Although changes in the fatty acid composition occurred with fasting, a constant s/u ratio of the total extract was maintained. This ratio may be important for membrane integrity of various tissues. In the pony, fasting appears to cause a massive mobilization of tissue fatty acids with their subsequent presentation to the liver in large quantities. The pony appears unique among species in this response, since large amounts of serum VLDL triglyceride also appear in the circulation. Although it has not yet been determined whether increased production or decreased utilization of this VLDL triglyceride is responsible for the increase, it is reasonable to expect the fatty acid composition of that triglyceride to be similar to that of the fatty acid being mobilized from tissue sites.

Although a slight decrease in the relative amount of linoleic acid did occur in the free fatty acid fraction, this change may be due in part to utilization of this particular fatty acid via Δ6 desaturase activity and subsequent chain elongation. The s/u ratio of the serum free fatty acids in fasted animals indicates a shift from saturated to unsaturated fat being mobilized. This finding could be due to an increased utilization of saturated fatty acids in the presence of increased serum concentrations, a decreased utilization of unsaturated fatty acids under similar conditions, or may merely reflect the fatty acid distribution of the animals' triglyceride stores and/or previous diet. This latter explanation is most likely since leaf tissues (i.e., pastures, etc.) which contain 6-8% lipid on a dry weight basis are characterized by a high content of glycolipids and phospholipids with a high proportion of 18:3ω3 and 18:2ω6 fatty acids (10). In general, forages comprise a high proportion of 18:3ω3 fatty acids, whereas diets supplemented with cereal grains and oilseed concentrates contain a large proportion of 18:2ω6 fatty acids. It is expected then that ponies, maintained primarily on pasture, would have lipid stores which are relatively enriched in 18:3ω3 fatty acids.

An interesting finding in the phospholipid fractions is that they contain so little of the C20 and C22 carbon atom polyunsaturated acids. Low levels of these fatty acids have previously been reported in the full-size horse (6,8). In particular, only small amounts of arachidonic acid were present in the pony in spite of large amounts of the linoleic acid precursor in the serum lipids. Indeed, horse serum phospholipids contain much more linoleic acid than other herbivores studied (8), indicating the possibility of an inverse relationship in this species between these two acids in serum. Furthermore, linoleic acid enrichment of human endothelial cells in culture has been associated with a decrease in the arachidonic acid content of the cellular phospholipids and a reduction of prostacyclin (PGI_2) release (11). This finding is not peculiar to endothelial cells. It has also been reported in human skin fibroblasts (12) and mouse peritoneal macrophages (13). Also, in the human, a decrease in both percentage and total amounts of 20:4 in plasma free fatty acids has been observed when safflower oil (containing large amounts of linoleic acid) was fed (14). Investigations designed to explain further this relationship and its importance in prostaglandin synthesis would be most interesting.

Although the cholesteryl ester fatty acid distribution was not affected during fasting, it should be noted that linoleic acid made up most of the fatty acid contained in this fraction. With fasting, only the VLDL are appreciably increased and this lipoprotein has a low cholesterol content, 85% of

which is in the free form (4). In addition, after a 5-day fast, the cholesteryl esters increase no more than 20% in the pony (15). These small changes may help explain why no differences were seen between fed and fasted pony cholesteryl ester fatty acids. The large amount of linoleic acid seen in this fraction is consistent with previous findings in the full-size horse (7,8). Equine lecithin-cholesterol acyl transferase (LCAT) is very likely to be significantly involved in the production of these unsaturated cholesteryl esters. In the horse, this enzyme has been reported to show strong specificity for both lecithin linoleic acid as well as its β-position (7). The composition of serum cholesterol esterified by horse LCAT thus appears to be dependent upon the fatty acid specificity of the enzyme itself (7). Our observations in the pony support these results reported in the full-size horse.

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Enzymatic Hydrolysis of Fractionated Products from Oils Thermally Oxidized in the Laboratory

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ABSTRACT

Enzymatic hydrolysis of the acylglycerol products obtained from thermally oxidized vegetable oils was studied. Corn, sunflower and soybean oils were heated in the laboratory at 180 C for 50, 70 and 100 hr with aeration and directly fractionated by silicic acid column chromatography. By successive elution with 20%, then 60% isopropyl ether in *n*-hexane, and diethyl ether, the thermally oxidized oils were separated into three fractions: the nonpolar fraction (monomeric compounds), slightly polar fraction (dimeric compounds), and polar fraction comprising oligomeric compounds. Enzymatic hydrolysis with pancreatic lipase showed that the monomers were hydrolyzed as rapidly as the corresponding unheated oils, the dimers much more slowly, and the oligomeric compounds barely at all. Overall, the hydrolysis of the dimers was less than 23% of that for the monomers, with small differences among the oils. Longer heating periods resulted in greater reductions in hydrolysis of the dimeric compounds. These results suggest that the degree of enzymatic hydrolysis of the fractionated acylglycerol compounds is related to differences in the thermal oxidative deterioration, and amounts of polar compounds in the products.
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Heating of oils changes their composition and new compounds are formed. The conditions used for domestic frying leads to two typical groups of products (1-5); volatile breakdown derivatives, and nonvolatile oxidation products (NVOP). The latter portion includes monomeric, dimeric and polymeric compounds. The NVOP accumulate in the thermally oxidized oils and are subsequently ingested with the fried foods. Several workers have shown that thermally oxidized oils contain potentially harmful derivatives of fatty acids (6-9). In these instances, they were generally fed to animals in the form of methyl or ethyl esters, whereas in thermally oxidized oils they are essentially present as acylglycerols (10). In fact, the natural fatty acid methyl esters are not absorbed intact by the intestinal mucosa, even when they are fed with triacylglycerols to favor their micellar solubilization in the intestinal lumen (11).

The hydrolysis rate of normal fatty acid methyl esters by pancreatic lipase is considerably slower than that of the corresponding triacylglycerols (12,13), and thermally oxidized oil is not so rapidly hydrolyzed as the corresponding unheated oil (14). However, it was still not clear how well individual fractionated products would be hydrolyzed by pancreatic lipase. Acylglycerol products obtained from thermally oxidized vegetable oils heated continuously in the laboratory at a frying temperature of 180 C under aeration were compared with fractionated products from unheated oils.

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MATERIALS AND METHODS

Preparation of Heated Oils

Refined commercially available vegetable oils (corn, sunflower and soybean oils) were heated in open stainless steel beakers continuously at 180 ± 2 C for 50, 70 and 100 hr with aeration (400-600 ml/min).

Determination of Chemical Properties of Unheated and Thermally Oxidized Oils

For peroxide values, conventional iodometic titration with thiosulfate was used (15). Carbonyl values were determined by the method of Kumazawa and Oyama (16), and iodine values by the method of the Association of Official Analytical Chemists (17). Ester bonds were determined by the method of Snyder and Stephens (18) and methyl palmitate was used for the calibration curve.

Separation of Monomeric, Dimeric and Oligomeric Compounds

The unheated and thermally oxidized oils were fractionated on a silicic acid column by a modification of the method of Ota et al. (19). The silicic acid was Bio-Sil A (100/200 mesh) from BIO-RAD Laboratories, Mississauga, Ontario. It was activated at 120 C for 3 hr before the column was prepared. Silicic acid (20 g) was poured into the column (2 × 60 cm) with 5% isopropyl ether in *n*-hexane. After the column was washed with 200 ml

of 5% isopropyl ether in *n*-hexane, a measured quantity of ca. 1.0 g unheated or thermally oxidized oils was applied. Monomeric compounds were eluted with 125 ml of 20% isopropyl ether in *n*-hexane, dimeric compounds with 125 ml of 60% isopropyl ether in *n*-hexane, and oligomeric compounds with 200 ml of diethyl ether, successively. Each lipid class fractionated by silicic acid column chromatography was detected by thin layer chromatography (TLC), and good separation of monomeric, dimeric and oligomeric compounds was obtained. Glass plates were coated with Silica Gel G of 0.5 mm thickness, and the solvent systems were petroleum ether/diethyl ether/acetic acid (50:50:2 or 80:30:1, v/v/v) and *n*-hexane/benzene (40:60, v/v). The oligomeric fraction also contained thermal degradation products.

The chemical properties of the fractionated products were determined by the same methods as described earlier.

Hydrolysis of the Fractionated Products by Pancreatic Lipase

Enzymatic hydrolysis of the fractionated products from these oils, before and after heating, were determined with pancreatic lipase (20). A 100 mg sample of the product was suspended in 6.0 ml of 0.2 M Tris buffer (pH 7.6) containing 0.25 M $CaCl_2$. To this suspension, 100 mg of pancreatic lipase (Sigma Chemical Co., St. Louis, MO) refined with acetone and then diethyl ether was added. The reaction mixture was incubated at 37 C, and a time period of 13 min was selected based on results of preliminary experiments using triolein. Two ml of 2 N HCl was added to the reaction mixture, and it was extracted 3 times with 10 ml of diethyl ether. The combined extracts were washed with distilled water to remove the HCl. The hydrolysates were recovered from the upper layer, and then titrated with 0.02 N KOH in alcohol using 0.1% phenolphthalein in alcohol as an indicator. Control experiments without added enzyme were carried out under the same conditions, and the values obtained were deducted as blanks to estimate the acids released due to the hydrolysis procedure.

RESULTS

Analyses of Oils

Table 1 shows the chemical properties and the contents of acylglycerol compounds for the three unheated vegetable oils. There were no significant differences except for the iodine values, which were affected by the linolenic acid content of the soybean oil, and high linoleic acid level in the sunflower oil. The dimeric and oligomeric fractions also contained sterols and a very small amount of phospholipids, respectively, as seen by TLC.

Figure 1 shows the changes in total ester bonds of the thermally oxidized oils, and also changes in the amounts of triacylglycerol isolated by preparative TLC. Regarding total ester bonds in the thermally oxidized oils, no distinct differences were observed among the three oils, but small reductions were seen due to the length of heating, such as 2.0-2.5% for 70 hr, and 4.0-4.8% for 100 hr. In contrast, there were large reductions in the amount of triacylglycerol in the oils ranging from 21.7-27.4% for the 50-hr heating to 41.4-45.9% for the 100-hr heating.

After the oils were heated, the acylglycerol compounds were fractionated by silicic acid column chromatography (21,22). Thermal oxidation resulted in lower amounts of acylglycerol monomeric compounds (triacylglycerols, which are relatively nonpolar). For example, 70 hr of heating caused

TABLE 1

Analytical Data for Unheated Oils[a]

		Oils		
		Corn	Sunflower	Soybean
Peroxide value (meq/kg)		2.6 ±0.1	2.8 ±0.1	2.1 ±0.1
Acid value		0.20±0.03	0.23±0.03	0.24±0.03
Carbonyl value (meq/kg)		4.2 ±0.2	3.9 ±0.2	3.6 ±0.2
Iodine value		131.2 ±1.1	137.3 ±1.0	141.5 ±1.0
Acylglycerols	Monomeric (%)[b]	96.3 ±0.7	96.5 ±0.8	97.4 ±0.7
	Dimeric (%)[c]	2.3 ±0.5	2.2 ±0.5	1.2 ±0.6
	Oligomeric (%)[d]	1.4 ±0.3	1.3 ±0.3	1.4 ±0.3

[a]Mean ± SEM (n = 5).
[b]20% isopropyl ether in *n*-hexane.
[c]60% isopropyl ether in *n*-hexane.
[d]Diethyl ether.

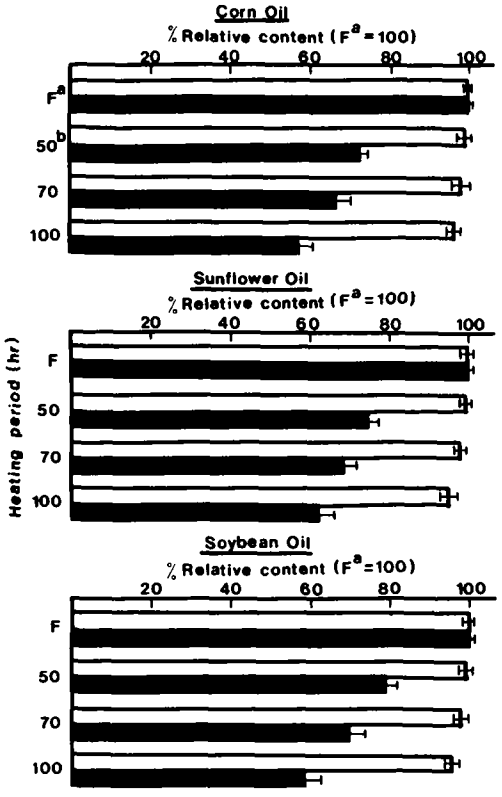

FIG. 1. Changes in ester-type lipid and triacylglycerol contents of thermally oxidized oil due to heating. □ ester-type lipid; ■ triacylglycerol. [a]F = unheated oils; [b]heating period (hr).

reductions of ca. 32%, and 100 hr caused ca. 38% reductions (Table 2). However, the acylglycerol dimeric and oligomeric compounds increased substantially during heating, dimers increased by 21.2% and oligomers by 13.6% at 70 hr. The composition of the oils changed gradually as oxidation reactions and polymerization took place to form dimeric and oligomeric compounds.

Analyses of Monomeric and Dimeric Compounds

Analytical data for the monomeric and dimeric compounds from the fractionation of the unheated and the thermally oxidized oils are shown in Tables 3 and 4, respectively. In general, the chemical values for monomeric compounds from the unheated oils were lower than those for the original oils (Table 1); iodine values decreased slightly after chromatography because polar sterols and phospholipids in the original oils were absorbed strongly on the silica gel columns. Heating decreased the iodine values significantly, whereas the acid and carbonyl values increased, the latter showing no further rise beyond 70 hr. Peroxide values increased in the early stages of heating, and then decreased as breakdown took place.

For the dimeric compounds obtained from unheated oils (Table 4), the carbonyl values were much higher, and the iodine values were much lower than for the original oils. As a result of heating, these effects became more pronounced with time. However, the carbonyl values at each stage of heating were not so high as those for the original thermally oxidized oils (103.8, 124.8 and 181.5 meq/kg for the 50-, 70- and 100-hr heating of corn oil is an example in Table 2). Therefore, as

TABLE 2

Analytical Data for Thermally Oxidized Oils[a]

		Oils[b]								
		Corn			Sunflower			Soybean		
		50	70	100	50	70	100	50	70	100
Peroxide value (meq/kg)		6.3	5.2	0.6	5.7	3.2	0.9	6.5	5.0	1.2
Acid value		0.34	0.75	1.00	0.25	0.46	0.64	0.24	0.52	0.58
Carbonyl value (meq/kg)		103.8	124.8	181.5	105.4	113.5	156.7	93.2	118.7	168.9
Iodine value		118.7	96.7	86.0	120.9	102.5	87.3	121.6	103.6	89.2
Acylglycerols	Monomeric (%)	72.9	65.2	58.6	73.8	64.2	56.7	76.2	66.2	59.6
	Dimeric (%)	16.7	20.3	23.3	18.2	22.7	25.3	15.3	20.6	22.8
	Oligomeric (%)	10.4	14.5	18.1	8.0	13.1	18.0	8.5	13.2	17.6

[a]Each value is an average of 5 determinations. The iodine values, and amounts of acylglycerol monomeric compounds were all significantly lower than for the unheated oils ($P < 0.05$). The carbonyl values, and the amounts of acylglycerol dimeric and oligomeric compounds all increased significantly ($P < 0.05$).
[b]Heating period (hr).

TABLE 3

Analytical Data for Monomeric Compounds from Unheated and Thermally Oxidized Oils[a]

	Oils[b,c,d]											
	Corn				Sunflower				Soybean			
	F	50	70	100	F	50	70	100	F	50	70	100
Peroxide value (meq/kg)	2.1	7.2	5.4	5.8	1.6	5.5	3.3	3.0	1.8	5.8	3.8	3.2
Acid value	-	0.08	0.12	0.36	-	0.02	0.08	0.21	-	0.02	0.06	0.16
Carbonyl value (meq/kg)	2.5	7.2	23.1[e]	22.2[e]	2.7	8.7	27.1[e]	30.9[e]	2.8	9.3	27.1[e]	26.5[e]
Iodine value	130.4	112.3[e]	102.6[e]	90.6[e]	132.3	116.1[e]	103.6[e]	91.3[e]	136.7	118.9[e]	105.3[e]	96.5[e]

[a]Each value is an average of 5 determinations.
[b]F = unheated oils.
[c]Heating period (hr).
[d]- = not detectable.
[e]Significantly different from the value for unheated oil ($P < 0.05$).

TABLE 4

Analytical Data for Dimeric Compounds from Unheated and Thermally Oxidized Oils[a]

	Oils[b,c]											
	Corn				Sunflower				Soybean			
	F	50	70	100	F	50	70	100	F	50	70	100
Acid value	0.03	0.62	1.03	1.12	0.02	0.25	0.49	0.72	0.02	0.65	0.75	0.81
Carbonyl value (meq/kg)	53.2	65.7	99.6[d]	132.3[d]	56.8	74.1	103.6[d]	136.8[d]	50.6	92.8[d]	126.3[d]	148.4[d]
Iodine value	112.2	93.7[d]	79.6[d]	68.7[d]	115.8	92.3[d]	77.4[d]	70.8[d]	118.0	97.8[d]	86.7[d]	75.7[d]

[a]Each value is an average of 5 determinations.
[b]F = unheated oils.
[c]Heating period (hr).
[d]Significantly different from the value for unheated oil ($P < 0.05$).

expected, the carbonyl values for the oligomeric compounds obtained from the heated oils were very high. For instance, even for the 50-hr heating, levels ranged from 183.7 meq/kg for thermally oxidized corn oil to 238.5 meq/kg for the heated soybean oil.

Enzymatic Hydrolysis of the Fractionated Products

Monomeric and dimeric compounds obtained from silicic acid column chromatography were hydrolyzed by pancreatic lipase after identification by TLC. The reaction mixture was incubated at 37 C and the amount of free fatty acid produced by hydrolysis at 13 min was always less than 60% (23) based on results of preliminary experiments using triolein. Shown in Table 5 are values for mg (as linoleic acid) of free fatty acid produced by hydrolysis of the monomers and dimers from each of the unheated and thermally oxidized oils. The dimeric compounds were hydrolyzed significantly less than the monomeric compounds from the same oil and conditions of heating. For the unheated oils, the hydrolysis of the dimers was ca. 27% that of the monomers. For the heated oils, there was a range from ca. 21% at 50-hr heating down to 15% at 100 hr of heating. The oligomeric compounds obtained from the thermally oxidized oils separated by column chromatography were barely hydrolyzed by the pancreatic lipase.

Figure 2 shows the hydrolysis of dimeric compounds obtained from unheated and thermally oxidized oils. In order to illustrate the relative hydrolysis as a percentage, the values for the unheated oils (mg free fatty acid produced) were normalized to 100 (F = 100). Hydrolysis of the heated dimeric compounds was more difficult than that of the dimers from the corresponding unheated oils; for example, 70 hr of the heating caused reductions of ca. 17-23%, and 100-hr heating caused reductions of ca. 35-38%. However, no significant differences were observed among the three thermally oxidized oils.

TABLE 5

Hydrolysis of Monomeric and Dimeric Compounds from Unheated and Thermally Oxidized Oils by Pancreatic Lipase[a]

Oil	Heating period (hr)	Free fatty acids produced (mg)[b]		Relative hydrolysis (%)
		Monomer[c]	Dimer[c]	
Corn	0	58.3 ± 0.8[d]	15.8 ± 0.8[d]	27.0
	50	61.6 ± 1.0[e]	13.2 ± 1.0[d,e]	21.0
	70	65.1 ± 1.2[f]	12.5 ± 1.2[e,f]	19.0
	100	68.0 ± 1.2[f,g]	10.1 ± 1.3[f]	15.0
Sunflower	0	59.7 ± 0.8[d]	16.1 ± 0.8[d]	27.0
	50	63.7 ± 1.0[e]	13.5 ± 1.0[d,e]	21.0
	70	67.6 ± 1.2[f]	12.3 ± 1.2[e,f]	18.0
	100	70.6 ± 1.2[g]	10.5 ± 1.3[f]	15.0
Soybean	0	58.9 ± 0.8[d]	16.4 ± 0.8[d]	28.0
	50	62.6 ± 1.0[e]	14.2 ± 1.0[d]	23.0
	70	66.3 ± 1.2[f]	13.5 ± 1.2[d,e]	20.0
	100	70.2 ± 1.2[g]	10.4 ± 1.3[f]	15.0

[a]Mean ± SEM (n = 5).
[b]Expressed as linoleic acid (mg).
[c]Values in each column with the same superscript are not significantly different from each other ($P < 0.05$). All corresponding values for the dimers were significantly lower than those for the monomers ($P < 0.01$).

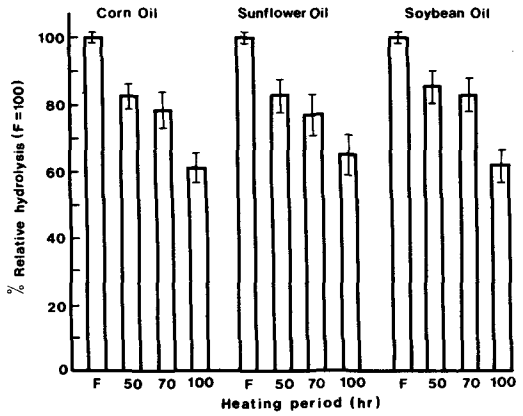

FIG. 2. Hydrolysis of dimeric compounds obtained from unheated and thermally oxidized oils by pancreatic lipase for 13 min (average of 5 determinations). F = unheated oils.

DISCUSSION

Used fats which have not been unreasonably abused contain less than 15% of dimers or oligomers. However, the presence of 1-3% of dimers also has been reported in freshly deodorized commercial vegetable oils (24-26). The current work was undertaken to determine the potential enzymatic hydrolysis of fractionated acylglycerol products obtained from thermally oxidized vegetable oils, heated with aeration in the laboratories at frying temperature. Results were compared with those for fractionated compounds from unheated oils.

The residual ratio of triacylglycerols in the heated oils decreased substantially (Fig. 1), and in proportion to the degree of heating, being lowest in corn oil, and in general followed by sunflower oil and soybean oil. These results indicate that thermal oxidative deterioration is related to the fatty acid composition. However, although there was an increase in dimeric and oligomeric compounds with the progress of heating (Table 2), the level of total ester bonds in the thermally oxidized oils did not change significantly. This would suggest that the ester linkage in the oils was not disturbed much under heating conditions without moisture. Cleavage of oxidation products with heating is competitive with both cyclization and polymerization, but recent work confirms that temperature is of primary importance. Cyclic monomer formation does not occur to any significant extent until the temperature approaches the thermal polymerization range of 200-300 C (27). Therefore, with the heating conditions used in this experiment, the fractionated products should be mostly composed of one or more acylglycerol molecules.

The monomeric compounds obtained from the thermally oxidized oils resulted in an increased pancreatic hydrolysis in comparison with that of the original unheated oils (Table 5). In other

biological systems such as SH-enzymes (28,29) and cytochromes (30), it has been generally accepted that inactivation damage can be caused by hydroperoxides, but no such effect should be expected here because the peroxide values were low, as shown in Tables 1 and 3. Another factor is that the polyunsaturated fatty acids, being more susceptible to oxygen attack during thermal oxidation, are converted to dimers and relatively inert oligomers via scission products. The fractionated monomers after heating contained considerably more saturated acids than the monomers from the unheated oils (31). This was confirmed also by the iodine values in Table 3. Pancreatic lipase activity on triacylglycerols is known to be influenced by the structure of the fatty acids and by their position on the triacylglycerol skeleton (32). Among the monomeric compounds esterified in the 1- or 3-position of the *sn*-glycerol produced during the heating of the oils, it seems likely that a certain amount could remain for preferential hydrolysis by pancreatic lipase.

Dimeric compounds of increased molecular size were hydrolyzed to a lesser degree (15-28%) than monomeric compounds (Table 5). In vivo studies by Ohfuji and coworkers (33) showed that rats fed thermally oxidized oils absorbed dimeric components. They suggested that the crosslinking in the dimers is via a C-C bond (8), which is hydrolyzed with more difficulty in rats. With the monomers, it is possible to hydrolyze readily 2 out of 3 of the ester bonds, whereas with the dimer which is larger, due to a number of different chemical entities, and C-C linkages, internal ester groups would not be available for hydrolysis, so still only a maximum of two exposed ester groups on the primary carbons can be hydrolyzed. Besides, much of the dimeric structure is more complex than indicated above (3), and intramolecular C-C linkages could form in addition to the intermolecular ones as a result of heating. This would inhibit hydrolysis even more, leading to the lower percentage values with longer heating periods in Figure 2. There was no appreciable enzymatic hydrolysis of oligomeric compounds obtained from the original oxidized oils. However, these compounds are more polar and complicated, and contain thermal degradation products capable of inactivating the enzyme. It would be of interest to pursue studies regarding the inactivating mechanisms of enzymes by thermally polymerized lipid components and their degradation products.

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Inhibition by Adenosine Triphosphate of Heart Microsomal Neutral Lipase Activity

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ABSTRACT

Triacylglycerol lipolysis was inhibited by palmitate in the isolated perfused normal rat heart. Acetate or acetylcarnitine could reproduce the inhibitory effects of palmitate. Since heart neutral lipase plays an important role in the lipolysis of heart triacylglycerols, the effects of acetylcarnitine, acetyl CoA and related metabolites on the microsomal neutral lipase activity were studied. ATP inhibited the enzyme activity in a concentration-dependent manner without a lag phase. AMP and adenylyl imidodiphosphate, two compounds structurally related to ATP but whose phosphate groups cannot be tranferred, did not inhibit the microsomal lipase activity. These results suggested that ATP inhibited the lipase activity through the transfer of its phosphate group. It is proposed that cellular ATP concentration is a determinant of tricylglycerol lipolysis in the heart.
Lipids 18:408-411, 1983.

INTRODUCTION

Free fatty acids (FFA) are a major source of energy for the heart muscle, over 60% of the oxygen consumption of the heart being accounted for by fatty acid oxidation (1,2). The important sources of FFA for the heart are perfusate (or blood) FFA, circulating lipoproteins and heart endogenous triacylglycerols (TG)(1,2). When the work done by the heart is increased (e.g., epinephrine treatment, an increase in perfusion pressure or filling pressure), heart endogenous TG lipolysis is increased as indicated by increased glycerol release (3) or TG fatty acid oxidation (4). Previous work demonstrated inhibition of TG lipolysis in the isolated heart when perfused with palmitate (4,5). The present study was undertaken to explore the biochemical mechanisms responsible for the inhibition by palmitate of TG lipolysis in the heart muscle.

METHODS AND MATERIALS

Normal male Sprague-Dawley rats (290 ± 20 g) maintained on Wayne chow and tap water were used. The isolated heart was washed free of blood by perfusing through the aortic canula for 3 min with Krebs-Henseleit buffer (KHB), pH 7.4, containing 9 mM glucose. The heart was then perfused for 30 min by the Langendorff method (6) in a recirculation perfusion with 50 ml of Krebs-Henseleit buffer, pH 7.4, containing 3% fatty acid-free bovine serum albumin (KHBA) and appropriate substrates. Heart TG determination was done as described (7).

Microsomal neutral TG lipase activity was determined as follows. Rats were injected ip with nembutol (60 mg/kg). The isolated heart was perfused for 15 min at 37 C (single pass perfusion) with Krebs-Henseleit bicarbonate buffer containing 1% fatty acid-free bovine serum albumin and 10 units/ml heparin. The heparin perfusion removes the endothelial lipase activity (8,9), without affecting the heart neutral lipase activity (9). The heart was washed free of buffer by flushing with ca. 30 ml of ice-cold saline. The ventricle was homogenized in a buffer containing 0.25 M sucrose and 10 mM phosphate buffer, pH 7.0 (sucrose-phosphate buffer). The homogenate was centrifuged at 4 C at $5,000 \times g$ for 20 min. The supernate was centrifuged at $17,000 \times g$ for 30 min and the resulting supernate was centrifuged at $100,000 \times g$ for 60 min. The sedimented microsomes were washed once and suspended in the sucrose-phosphate buffer.

The incubation mixture contained 50 mM tris. HCl buffer, pH 7.4, 5 mM Mg^{2+} (as chloride) and 0.75 μmol of trioleylglycerol [$9{:}10^3$] (1.5 μC_i) sonicate. After preincubation at 37 C for 5 min, the reaction was started by adding microsomes (ca. 2 mg protein) in a total volume of 0.7 ml. After 30 min of incubation, FFA were extracted (10) and radioactivity was counted and corrected for efficiency. Triolein sonicates were prepared by drying the radioactive triolein in a conical centrifuge tube, adding nonradioactive triolein in 0.5% triton-X-100 containing 5 mg/ml purified bovine serum albumin (11) and sonicating the mixture at 2 C for 30 sec (Biosonik IV, Bronwill Instruments, Rochester, NY).

Trioleylglycerol, palmitic acid (Applied Science, State College, PA), triolein [$9{:}10^3$H, 111 C_i/mmole] (New England Nuclear Corp., Boston), ATP, AMP acetyl DL-carnitine (Sigma), adenylyl imidodiphosphate [AMP-PNP](Boehringer Mannheim, Indianapolis), and fatty acid-free bovine serum albumin (Miles Laboratories, Elkhart, IN) were obtained from the designated sources.

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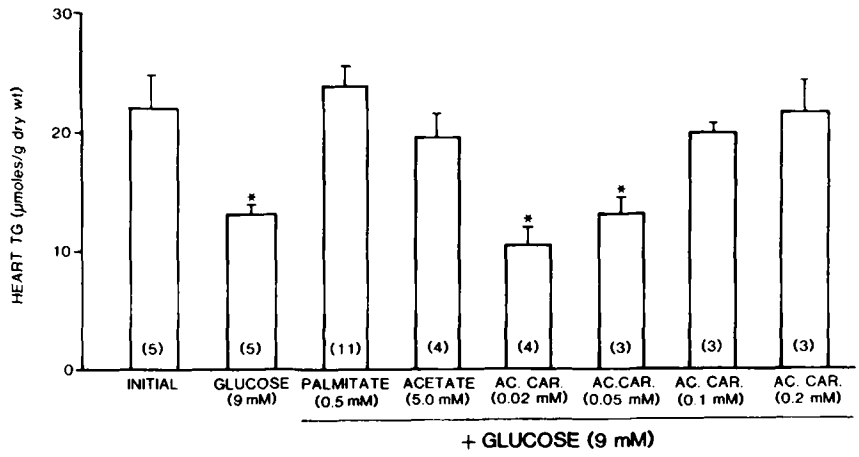

FIG. 1. Inhibition of endogenous triacylglycerol lipolysis by palmitate, acetate and acetyl carnitine (Ac. Car.) in the isolated perfused heart.
*$p < 0.05$ compared to Initial.

RESULTS

Inhibition of TG Lipolysis by Palmitate, Acetate and Acetylcarnitine (Fig. 1).

Perfusion of the isolated heart with KHBA containing 9 mM glucose resulted in rapid TG lipolysis. Inclusion of 0.5 mM palmitate in the perfusate blocked TG lipolysis. To determine the chain length requirement of fatty acid, hearts were perfused with 5 mM acetate instead of palmitate. Acetate blocked TG lipolysis. Since acetyl CoA is a common intermediate in acetate and palmitate metabolism and since acetyl CoA is transported across the mitochondrial membrane as acetylcarnitine, the experiments on the inhibition of TG lipolysis were carried out with acetylcarnitine. The inhibition of TG lipolysis by acetylcarnitine was related to the concentration of the inhibitor.

Effect of Various Intermediates on Heart Neutral Lipase Activity

The results suggested that acetylcarnitine or metabolite(s) was an effective inhibitor of TG lipolysis through the inhibition of TG lipase activity. Heart muscle contains several lipase activities (3,12). Among these, the neutral lipase activity appears to play an important role (13-15). The effects of acetylcarnitine and some major metabolites on the heart microsomal neutral lipase activity were studied. Acetylcarnitine (4 mM), acetyl CoA (250 μM), citrate (5 mM), succinate (5 mM), malate (5 mM), aspartate (5 mM), NADH (1 mM) and NADPH (1 mM) were not inhibitory up to the concentrations indicated. Severson and Hurley (16) observed that acetylcarnitine and acetyl CoA had no inhibitory activity on the rat heart cytosolic neutral lipase activity.

Inhibition of the Neutral Lipase Activity by ATP (Fig. 2)

Since oxidation of palmitate, acetate and acetylcarnitine by the perfused heart results in ATP synthesis, the effect of ATP on microsomal lipase activity was studied. ATP produced a concentration-dependent inhibition of the microsomal neutral lipase activity (Fig. 2B). The inhibition was immediate and continued throughout the incubation period of 45 min (Fig. 2A). The relatively high concentrations (ca. 10 mm) required to produce 50% inhibition could be due to the fact that crude microsomes, which exhibit high ATPase activity, were used in the present experiments. The inhibition by ATP was the same in presence of 5 mM Mg^{2+} or 20 mM Mg^{2+}, indicating that the inhibition was not due to the chelation of Mg^{2+} by ATP.

To test the specificity of the inhibitor, the experiments were repeated with AMP, a metabolite with close structural similarity to ATP but without transferable phosphate groups. AMP had no inhibitory activity (Fig. 2B). The experiments were repeated with AMP-PNP, a structural analog of ATP, but in which an NH grouping replaces the terminal bridge oxygen of the triphosphate chain (17). This alteration in structure renders the phosphate groups unavailable for enzymic transfer (17). AMP-PNP did not inhibit lipase activity (Fig. 2B).

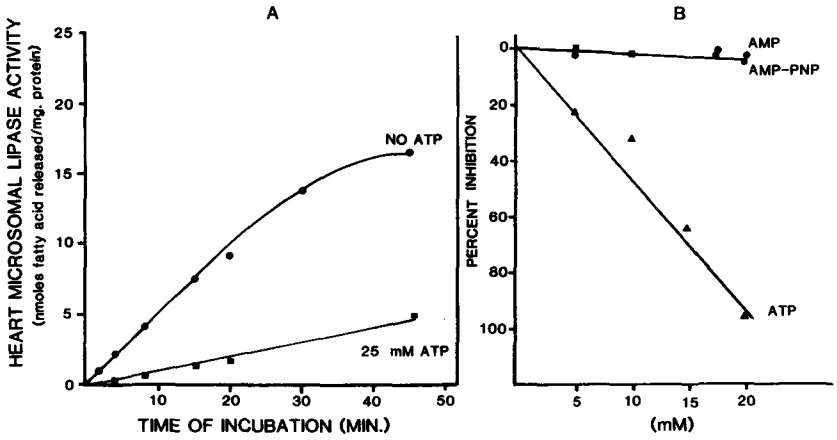

FIG. 2. ATP inhibition of heart microsomal neutral lipase activity as a function of time (A) and concentration (B).

These results suggested that ATP inhibited the neutral lipase activity through the transfer of its phosphate group.

DISCUSSION

The results of this study demonstrated inhibition of TG lipolysis in the isolated perfused rat heart by palmitate, acetate and acetylcarnitine. These observations suggested that acetyl CoA or a metabolite played a role in the inhibition. The biochemical mechanism responsible for the inhibition of TG lipolysis was studied using heart subcellular fractions.

Heart muscle contains several lipolytic activities (3,12). The major ones are the endothelial lipoprotein lipase (18), neutral lipase found in the microsomal and soluble fractions and an acidic lipase found in the lysosomes (3,12). Although the relative physiological importance of these lipases in the lipolysis of intracellular TG is not established, the neutral lipase appears to play an important role (13-15). Stam and Hulsmann (15) observed that an increase in heart neutral lipase activity was associated with an increase in heart TG content following trierucate feeding in rats or in vitro perfusion of the normal rat heart with Intralipid®. Their work also suggested that this increase in neutral lipase activity was due to the induction of new enzyme protein.

In our experiments, acetylcarnitine, acetyl CoA and several key tricarboxylic acid cycle intermediates did not inhibit the heart neutral lipase activity.

The activity of numerous enzymes (19,20) including that of the rat adipose tissue TG lipase (21) is known to be modulated through the phosphorylation and subsequent dephosphorylation of the enzyme, ATP acting as a phosphate donor. In our studies, the inhibitory effect of ATP could not be reproduced by two structurally related compounds, namely AMP and AMP-PNP, whose phosphate groups could not be transferred. These results suggested that ATP inhibited the heart neutral TG lipase activity through the transfer of its phosphate group, possibly resulting in the phosphorylation of the enzyme. This suggestion is supported by the demonstrated presence of a protein kinase activity in heart microsomes (22).

Results of earlier studies suggest that a reduction in intracellular ATP concentration may stimulate endogenous TG lipolysis in the isolated perfused rat heart. For example, the increased rate of TG lipolysis in the perfused heart in response to increased work (2,4) is associated with decreased cellular ATP concentration (23). Hron et al. (24) observed inhibition of endogenous TG lipolysis when the heart was perfused with ketone bodies. Since ketone bodies are readily oxidized by the myocardium to produce energy (2), the observations of Hron et al. (24) could be explained by the inhibition of TG lipolysis by ATP generated as a result of ketone body metabolism (23). The increased rate of lipolysis in the isolated perfused heart of diabetic rats (25) is associated with decreased ATP concentration in the diabetic heart (26).

We propose that cellular ATP concentration is a determinant of heart TG lipolysis. Such a proposal would predict that any substrate capable of maintaining stable ATP concentration in the perfused heart would block heart endogenous TG lipolysis. Regulation of TG lipase by ATP concentration in the heart cell would result in the coordination of the mobilization of stored energy (TG) with the availability of a readily utilizable form of energy, namely ATP.

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Fatty Acid Composition of Tissue Phospholipids and Prostaglandin Excretion in Hyperlipidemia Induced in Rats by Implantation of the Mammotropic Pituitary Tumor MtT-F_4

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ABSTRACT

A mammotropic pituitary tumor, MtT-F_4, was implanted into male Fisher 344 rats for a period of 4 weeks. This tumor induced growth retardation, hyperlipidemia, hepatic hypertrophy and adrenal hyperplasia. Lipids were extracted from various tissues. In tumor-bearing rats, phospholipid concentration was found to be increased in plasma, spleen and testis. Distribution among the various phospholipid classes was similar to that of controls except in liver and heart, where phosphatidylcholine was increased at the expense of phosphatidylinositol and phosphatidylserine. The main difference was in the fatty acid composition of major phospholipids. The proportion of $\omega 6$ fatty acids was lower and that of docosahexaenoic acid of the $\omega 3$ series (22:6ω3) was higher in most tissues, especially in plasma, liver, heart and kidney. Concurrently, the urinary excretion of two endogenous metabolites of PGI_2 (2,3-dinor-6-keto-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-2,3-dinor-$PGF_{1\alpha}$) was found to be increased significantly in tumor-bearing rats. The results raise the hypothesis that hormonal changes induced by the MtT-F_4 tumor accelerate the conversion of arachidonic acid (20:4ω6) to prostaglandins. This effect, perhaps coupled with a diversion of linoleic acid (18:2ω6) towards other metabolic processes, would account for a partial depletion of membrane phospholipids in 18:2ω6 and for the reduced production of longer chain $\omega 6$ unsaturated acids from 20:4ω6, creating a state of "relative essential fatty acid deficiency." As a result, the metabolism of $\omega 3$ fatty acids is altered towards an enhanced production of 22:6ω3 which accumulates in the lipids of cell membranes to compensate for the depletion of unsaturated acids of the $\omega 6$ series.
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INTRODUCTION

The mamotropic pituitary tumor, MtT-F_4, implanted in Fisher 344 rats, secretes large amounts of growth hormone, prolactin and ACTH (1). The secretion of ACTH induces marked adrenal hyperplasia and the release of corticosteroids with secondary hypertension (2). Rats bearing this tumor also develop hyperlipidemia secondary to enhanced hepatic lipid synthesis and adipose tissue lipolysis (3-5). Exposure of cells to this endogenous hyperlipidemia is likely to affect the lipid composition of their membranes which in turn could alter their permeability as well as the activities of membrane-bound enzymes. Hence, it is possible that some of the biological effects induced by tumor implantation could be the consequence of a modification in the fatty acid composition of membrane structural phospholipid (PL) component. Furthermore, since membrane PL are the source of the unsaturated fatty acids which serve as precursors in the biosynthesis of prostaglandins (6), any alteration in the fatty acid composition could also influence prostaglandin metabolism. With this in mind, we undertook the present study to examine the effect of MtT-F_4 tumor implantation on membrane PL composition of various tissues and on prostaglandin metabolism. Because the tumor-bearing rats develop hypertension (2) and since PGI_2 may play a role in the modulation of blood pressure (7), we chose to monitor the urinary excretion of two endogenous metabolites of PGI_2 (2,3-dinor-6-keto-$PGF_{1\alpha}$ and 6,15-diketo-13,14-dihydro-2,3-dinor-$PGF_{1\alpha}$).

Since it is generally accepted that the urinary excretion of the major metabolite(s) of a particular prostaglandin in a reliable index of the overall synthesis of a prostaglandin in vivo (8,9), we felt that these measurements would provide general information on the biosynthetic capacity of these animals with regard to this aspect of the prostaglandin system.

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MATERIALS AND METHODS

Animals

Six male Fisher 344 rats weighing 100 g were implanted with MtT-F_4 tumor and 6 control rats were subjected to a sham operation (3-5). All animals were maintained on Purina rat chow and given water ad libitum. They were sacrificed 4

weeks after the operation. Overnight fasted animals were killed by exsanguination under ether anesthesia. Blood was withdrawn from the heart into a test tube containing EDTA (1 mg/ml blood). Plasma and red blood cells (RBC) were separated by centrifugation at 1,000 × g for 10 min. RBC were washed twice with 0.9% NaCl. Liver, heart, kidney, testis, spleen, epididymal fat pad, adrenals, tumor cortex and muscle from hind leg (mainly gastrocnemius) were removed, washed with ice-cold saline, blotted dry, weighed and stored at −20 C until analysis.

Extraction and Fractionation of Lipids

Plasma cholesterol (CH) (10) and triglycerides (TG) (11) were measured enzymatically. Plasma PL were determined by the method of Bartlett (12). The homogenized tissues and RBC were extracted with chloroform/methanol (2:1, v/v) according to the method of Folch et al. (13). Aliquots of extracts were assayed for total phosphorus (12). The lipids extracted from adipose tissue and tumor cortex were assayed directly for total fatty acid composition after saponification. Total PL of plasma and adrenals were first separated by thin layer chromatography (TLC) by means of a solvent system of hexane/diethyl ether/glacial acetic acid (80:20:1, v/v) and the phospholipids recovered from the plate were kept for analysis of their fatty acid composition. Aliquots of other tissue lipid extracts were separated by TLC into 5 major PL fractions: phosphatidylcholine (PC), phosphatidylserine plus phosphatidylinositol (PS + PI), phosphatidylethanolamine (PE), sphingomyelin (Sph) and lysophosphatidylcholine (LPC) using a solvent system (14) of chloroform/methanol/glacial acetic acid/water (50:25:8:4, v/v). Our preliminary results had shown that Sph and LPC contained little polyunsaturated fatty acid (PUFA) and their fatty acid composition was not significantly affected by the tumor. Hence, in this study, these fractions were not analyzed. Only the fatty acids of 3 major glycerophospholipid fractions (PE, PS + PI, and PC) were converted to methyl esters (FAME) (15) and analyzed by gas liquid chromatography (GLC).

Analyses of Fatty Acid Composition

FAME were analyzed on a Hewlett-Packard gas chromatograph (Model 5840) equipped with flame ionization detector (FID) and automatic integrator. A fused silica capillary column (20 m × 0.2 mm id) of Carbowax 20M (Hewlett-Packard) was used for the identification of individual FAME. The GLC conditions were as follows: injection port temperature, 260 C; FID temperature, 300 C; nitrogen flow, 2 ml/min. The column temperature was programmed from 110 to 210 C at 3 C/min. For the quantitation of FAME, a glass column (180 cm × 4 mm id) packed with 10% silar-10C on 100-120 mesh Gas-Chrom Q (Applied Science) was used. The column was flushed with nitrogen at a flow rate of 30 ml/min, and the analyses were performed isothermally (180 C). Individual FAME were identified by comparing their retention times to those of standard mixtures (Applied Science). Fatty acid compositions were presented as weight percentages of total FAME.

Measurement of Urinary Prostaglandin Metabolites

Prior to the sacrifice, 3 rats each from the control and from the tumor-bearing rats were placed in individual Econo polycarbonate metabolic cages. Three consecutive 24-hr urinary collections were made. Two urinary metabolites of prostaglandin I_2 (prostacyclin), i.e., 2,3-dinor-6-keto-$PGF_{1\alpha}$ and 6,15-diketo-2,3-dinor-13,14-dihydro $PGF_{1\alpha}$, were measured by gas chromatography-mass spectrometry (GC-MS). The analytical procedure was based on a stable isotope dilution method (16). To an aliquot of the total 24-hr urine were added 100 ng each of the 2 deuterated metabolites as internal standards. The urine was acidified with conc HCl to pH 3 and eluted through a "Clin Elut" column (Analytichem) with dichloromethane/ethyl acetate (8:2, v/v). The eluate was washed with 0.05 M Tris buffer (pH 8.0). The aqueous phase was discarded and the organic solvent was evaporated to dryness under a stream of nitrogen. The residue was then dissolved in 2-3 drops of pyridine and 1 ml of 0.05 M sodium borate buffer (pH 8.0) was added to the sample. After standing at room temperature for 15 min, the mixture was extracted twice with ethyl acetate and the organic phase was discarded. The aqueous phase was acidified to pH 3 and the metabolites were extracted into dichloromethane. After evaporation of the organic solvent, the residue was converted into O-methyloxime-trimethylsilyl ether/methyl ester derivatives and analyzed on a Hewlett-Packard 5985B GC-MS by selected ion monitoring (SIM) of the M-31 fragments. A column (180 cm × 2 mm id) packed with 3% SP-2250 on Supelcoport (100-120 mesh) was used and flushed with helium at a flow rate of 30 ml/min. The oven temperature was maintained at 235 C. The ion source temperature was 200 C and the electron energy 70 eV.

RESULTS

General

Four weeks after tumor implantation, animals developed hyperlipidemia (Table 1); plasma CH and PL concentrations were increased 7-fold (232 vs 31 and 464 vs 67 mg/dl, respectively) and plasma

TABLE 1

Effect of MtT-F_4 Tumor Implantation on Body and Tissue Weights and on Plasma Lipids

	Control	MtT-F_4	p
Total body weight (BW) (g)	204	154	<0.001
Liver (% BW)	2.63	5.97	<0.001
Heart (% BW)	0.29	0.40	<0.001
Kidney (% BW)	0.29	0.53	<0.001
Testis (% BW)	0.50	0.47	NS
Spleen (% BW)	0.12	0.08	<0.001
Adrenal (% BW)	0.02	0.17	<0.001
Plasma cholesterol (mg/dl)	31	232	<0.001
Plasma triglycerides (mg/dl)	34	120	<0.001
Plasma phospholipid (mg/dl)	67	464	<0.001

TG 3.5-fold (120 vs 34 mg/dl). The growth of tumor-bearing rats was significantly stunted. Their liver, kidney and adrenal weights were increased. The heart weight was normal (0.62 vs 0.59 g) but its relative weight (% BW) was increased significantly. The weight of testes was decreased in proportion to the body mass; both absolute and relative weights of spleen were subnormal in tumor-bearing rats.

Phospholipid Distribution

In tumor-bearing rats, the concentration of total phospholipids in most of the tissues examined did not differ from that in controls (Table 2), except for spleen and testis where it was increased. In PL composition, there was an increase in PC at the expense of PS + PI in liver and heart. In the spleen, Sph and LPC were increased, and PE and PI + PS were reduced. In muscle, both PE and PC were increased and Sph decreased.

Fatty Acid Patterns of Tissue Phospholipids

Liver. Fatty acids of liver PL are shown in Table 3. In PE, the presence of the tumor significantly increased the proportions of palmitate (16:0) and docosahexaenoate (22:6ω3), whereas it reduced those of oleate (18:1), linoleate (18:2ω6) and arachidonate (20:4ω6). In PC, the proportions of stearate (18:0) and 22:6ω3 were raised and those of 18:1, 18:2ω6 and 20:4ω6 were reduced.

Heart. In rat heart (Table 4), the tumor significantly increased the proportion of 22:6ω3 in all PL classes. The proportion of 18:2ω6 was reduced in both PE and PC, whereas that of 20:4ω6 was reduced in PE and PS + PI.

TABLE 2

Concentration and Distribution of Phospholipids in Tissues from MtT-F_4 Tumor-Bearing (n = 6) and Control (n = 6) Rats

Tissue	Total phospholipids (mg/g tissue)	% of lipid phosphorus				
		PE	PI + PS	PC	Sph	LPC
Liver						
Control	25.6	24.8	8.3	58.5	8.0	0.4
MtT-F_4	23.8	20.6[a]	7.1[d]	63.8[b]	8.1	0.4
Heart						
Control	24.4	29.1	15.8	35.9	12.4	6.9
MtT-F_4	25.3	29.8	14.2[d]	39.6[a]	12.3	4.2
Kidney						
Control	42.1	29.3	12.1	31.9	18.0	8.9
MtT-F_4	35.1	29.5	11.2	31.8	18.1	9.4
Testis						
Control	21.6	43.7	7.6	41.6	5.3	1.8
MtT-F_4	26.6[a]	41.2	7.1	43.2	6.7	1.9
Spleen						
Control	21.3	24.8	13.8	40.4	11.5	10.1
MtT-F_4	30.5[c]	19.4[b]	11.4[d]	40.5	15.1[b]	13.5[d]
Muscle						
Control	16.7	26.4	3.0	39.2	25.3	-
MtT-F_4	20.4	31.4[d]	3.3	50.4[a]	14.9[a]	-

[a,b,c,d]Underlined values significantly different from control values at $p<0.001$, $p<0.01$, $p<0.02$ and $p<0.05$, respectively.

TABLE 3

Percentage Composition of Major Fatty Acids in Liver Glycerophospholipids of MtT-F$_4$ Tumor-Bearing (n=6) and Control (n=6) Rats

	PE		PS+PI		PC	
Methylester	Control	MtT-F$_4$	Control	MtT-F$_4$	Control	MtT-F$_4$
16:0	18.2	21.8[d]	10.9	10.6	22.7	23.5
18:0	29.4	30.7	41.4	41.4	22.7	27.0[b]
18:1	3.6	2.1[a]	0.9	0.5	4.9	2.3[a]
18:2ω6	6.5	1.9[a]	2.2	1.5	11.3	4.3[a]
20:4ω6	24.4	17.0[a]	33.5	32.3	25.8	21.8[b]
22:5ω3	2.0	0.7[a]	0.9	0.5[d]	0.8	0.4[a]
22:6ω3	13.6	24.1[a]	2.3	4.9[d]	6.3	16.9[a]

[a,b,d]Underlined values significantly different from control values at $p<0.001$, $p<0.01$ and $p<0.05$, respectively.

TABLE 4

Percentage Composition of Major Fatty Acids in Heart Glycerophospholipids of MtT-F$_4$ Tumor-Bearing (n=6) and Control (n=6) Rats

	PE		PS+PI		PC	
Methylester	Control	MtT-F$_4$	Control	MtT-F$_4$	Control	MtT-F$_4$
16:0	12.8	12.2	8.1	10.3	19.1	17.1
18:0	24.6	26.2	40.5	37.4	24.2	24.2
18:1	5.1	6.0	4.9	5.0	8.1	6.0[b]
18:2ω6	7.7	3.0[c]	6.7	6.2	12.9	4.6[a]
20:4ω6	15.2	11.8[a]	28.4	22.3[d]	24.1	27.6
22:4ω6	0.5	1.1[c]	-	-	0.4	0.5
22:5ω3	3.0	2.4	1.9	2.5	2.7	3.2
22:6ω3	23.8	32.4[a]	6.0	7.2[d]	7.2	14.1[b]

[a,b,c,d]Underlined values significantly different from control values at $p<0.001$, $p<0.01$, $p<0.02$ and $p<0.05$, respectively.

Kidney. In kidney PE (Table 5), the proportion of palmitoleate (16:1) and 18:2ω6 was reduced, while that of 22:6ω3 was increased. In PS+PI, only 22:6ω3 was increased. In PC 18:2ω6 and 20:4ω6 were reduced, while 18:1 and 22:6ω3 were increased.

Testis. Fatty acids of testis PL are shown in Table 6. In this tissue, tumor implantation increased the proportion of 22:6ω3 in all PL classes but had little effect on other fatty acids.

Spleen. In spleen PL (Table 7), the tumor reduced the proportion of 20:4ω6 in PE, 18:1 and 18:2ω6 in PS+PI, but increased 22:6ω3 in both fractions. The tumor had little effect on the fatty acid pattern of spleen PC.

Muscle. Fatty acids of muscle PL are shown in Table 8. In tumor-bearing rats, the proportion of 18:1 was increased in all classes and that of 18:2ω6 in PC, and of 20:4ω6 and 22:5ω3 in PE and PS+PI was reduced.

Red blood cells. Fatty acids of PL in RBC are shown in Table 9. The tumor significantly reduced the proportion of 18:2ω6 in PE and PC and that of 20:4ω6 in PS+PI and PC. On the other hand, the proportion of 22:6ω3 in all PL classes was increased.

Other tissues. Fatty acids of total PL from plasma and adrenal are shown in Table 10. In plasma PL, the tumor implantation significantly increased the proportions of 18:0 and 22:6ω3 and reduced those of 18:1 and 18:2ω6. In adrenal PL, the tumor increased the percentage of 22:6ω3 but reduced the proportion of 20:4ω6. The fatty acid pattern of a total lipid extract from the tumor cortex is also shown in Table 10. There was no significant accumulation of 22:6ω3.

TABLE 5

Percentage Composition of Major Fatty Acids in Kidney Glycerophospholipids of MtT-F_4 Tumor-Bearing (n=6) and Control (n=6) Rats

	PE		PS+PI		PC	
Methyl ester	Control	MtT-F_4	Control	MtT-F_4	Control	MtT-F_4
16:0	15.5	15.9	10.5	12.0	38.8	39.8
18:0	26.6	26.1	44.4	41.5	16.4	16.2
18:1	4.6	6.3	6.2	6.4	12.0	13.3[a]
18:2ω6	7.5	5.3[d]	4.8	4.4	10.0	6.3[a]
20:4ω6	35.0	33.9	25.8	27.9	18.1	15.3[c]
22:6ω3	0.8	6.3[a]	2.0	3.8[d]	1.9	6.8[a]

[a,c,d]Underlined values significantly different from control values at $p<0.001$, $p<0.02$ and $p<0.05$, respectively.

TABLE 6

Percentage Composition of Major Fatty Acids in Testis Glycerophospholipids of MtT-F_4 Tumor-Bearing (n=6) and Control (n=6) Rats

	PE		PS+PI		PC	
Methyl ester	Control	MtT-F_4	Control	MtT-F_4	Control	MtT-F_4
16:0	31.7	30.0	26.3	27.3	41.3	41.0
18:0	7.8	8.1	24.3	24.9	5.3	5.9
18:1	6.6	6.7	7.4	8.4	17.3	19.1
18:2ω6	3.6	3.1	2.0	2.5	3.3	3.1
20:4ω6	18.2	18.6	22.7	18.6[d]	10.6	9.8
22:5ω6	23.5	22.3	14.1	12.2	17.6	15.6
22:6ω3	2.0	4.4[a]	0.6	2.0[b]	1.0	2.6[a]

[a,b,d]Underlined values significantly different from control values at $p<0.001$, $p<0.01$ and $p<0.05$, respectively.

TABLE 7

Percentage Composition of Major Fatty Acids in Spleen Glycerophospholipids of MtT-F_4 Tumor-Bearing (n=6) and Control (n=6) Rats

	PE		PS+PI		PC	
Methyl ester	Control	MtT-F_4	Control	MtT-F_4	Control	MtT-F_4
16:0	14.4	15.9	7.9	6.2	63.7	63.4
18:0	24.2	24.6	49.1	51.8	22.1	21.5
18:1	10.9	9.5	14.2	11.5[d]	7.4	7.0
18:2ω6	5.7	3.5	5.8	3.4[b]	2.6	2.4
20:4ω6	34.8	29.9[d]	15.7	17.3	3.5	3.6
22:4ω6	2.4	3.6	1.6	1.5	-	-
22:5ω3	2.5	2.1	1.2	1.5	-	-
22:6ω3	2.4	7.9[d]	1.5	3.3[c]	-	-

[b,c,d]Underlined values significantly different from control values at $p<0.01$, $p<0.02$ and $p<0.05$, respectively.

TABLE 8

Percentage Composition of Major Fatty Acids in Muscle Glycerophospholipids of MtT-F$_4$ Tumor-Bearing (n=6) and Control (n=6) Rats

	PE		PS+PI		PC	
Methyl ester	Control	MtT-F$_4$	Control	MtT-F$_4$	Control	MtT-F$_4$
16:0	9.1	9.9	5.2	7.3	35.8	36.1
18:0	28.2	27.5	40.1	40.5	9.9	9.9
18:1	10.3	13.3[a]	12.7	15.6	11.3	13.9[b]
18:2ω6	5.8	5.3	3.9	3.9	18.1	12.6[a]
20:4ω6	10.1	9.2[d]	19.3	14.8[c]	12.6	12.0
22:4ω6	-	-	1.1	0.8	-	-
22:5ω3	5.6	3.8[b]	3.4	2.1[b]	2.3	2.1
22:6ω3	28.2	29.0	9.9	9.8	8.2	9.9

[a,b,c,d]Underlined values significantly different from control values at $p<0.001$, $p<0.01$, $p<0.02$ and $p<0.05$, respectively.

TABLE 9

Percentage Composition of Major Fatty Acids in Red Blood Cell Glycerophospholipids of MtT-F$_4$ Tumor-Bearing (n=6) and Control (n=6) Rats

	PE		PS+PI		PC	
Methyl ester	Control	MtT-F$_4$	Control	MtT-F$_4$	Control	MtT-F$_4$
16:0	12.5	15.3	3.5	5.6	39.8	38.4
18:0	12.0	10.9	24.4	24.4	19.1	18.8
18:1	17.1	17.7	5.5	6.9[a]	10.1	10.5
18:2ω6	3.8	3.2[c]	4.7	4.3	15.2	9.2[a]
20:4ω6	39.0	36.6	55.3	49.1[b]	11.6	14.3[a]
22:4ω6	5.5	5.1	-	-	-	-
22:5ω3	4.5	5.2	0.9	0.5	0.3	0.1
22:6ω3	4.0	5.3	3.9	5.4[c]	1.2	5.0[a]

[a,b,c,d]Underlined values significantly different from control values at $p<0.001$, $p<0.01$, $p<0.02$ and $p<0.05$, respectively.

Urinary Excretion of Prostaglandin Metabolites

The differences between the urinary excretion of PGI_2-metabolites in the control and in the tumor-bearing rats are shown in Table 11. The urinary metabolites of PGI_2 were significantly increased in tumor-bearing rats. The daily excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ was increased by 52% (151.6 vs 99.8 ng/24-hr urine) and that of 6,15-diketo-2,3-dinor-13,14-dihydro-$PGF_{1\alpha}$ by 68% (91 vs 54.3 ng/24-hr urine).

DISCUSSION

In mammals, there are 3 common families of PUFA, characterized by the position of the double bond closest to the terminal methyl group as ω3, ω6 and ω9 series. The fatty acids of the ω9 series are endogenously synthesized from oleic acid (18:1ω9), the ω6 fatty acids are derived from linoleic acid (18:2ω6), and those of ω3 series from linolenic acid (18:3ω3). Linoleic and linolenic acids cannot be synthesized by the animal body and are provided by the diet. They are not interconvertible but both acids are elongated and desaturated by the same enzymes and give rise to separate fatty acid families (ω3 and ω6).

The fatty acid analyses of tissue phospholipids (Tables 3-10) in tumor-bearing rats revealed a remarkable decrease in the proportions of ω6 fatty acids (18:2ω6 and 20:4ω6) and a significant in-

TABLE 10

Major Fatty Acids in Total Lipids (TL) of Epididymal Fat and Tumor Cortex and in Total Phospholipids (PL) of Plamsa and Adrenal of MtT-F_4 Tumor-Bearing Rats (n=6) and Control Rats (n=6)

	% Fatty acid methyl esters						
	16:0	18:0	18:1	18:2ω6	20:4ω6	22:4ω6	22:6ω3
Plasma-PL							
Control	23.6	25.2	11.0	12.0	19.9	-	2.5
MtT-F_4	20.9	28.5[c]	5.7[b]	6.1[a]	22.6	-	12.7[a]
Adrenal-PL							
Control	8.1	37.8	6.6	3.0	40.8	1.7	0.4
MtT-F_4	7.4	40.6	6.0	2.9	34.8[b]	3.2	3.1[a]
Epididymal fat-TL							
Control	27.2	2.8	25.2	29.5	0.6	-	-
MtT-F_4	30.2[c]	4.1[a]	28.4[a]	25.5[a]	0.4	-	-
Tumor cortex-TL	27.2	17.9	18.8	7.4	14.4	1.4	5.2

[a,b,c]Underlined values significantly different from control values at $p<0.001$, $p<0.01$ and $p<0.02$, respectively.

TABLE 11

Urinary Excretion of Two Metabolites of PGI_2 Expressed as ng per 24-hr Urine Collection

	Assays[c]			
Compound	1	2	3	Mean
2,3-dinor-6-keto $PGF_{1\alpha}$				
Control	83.6 ± 5	136.1 ± 0	79.6 ± 9	99.8 ±27
MtT-F_4	114.4[d] ±27	186.8[a] ± 177	153.6[d] ± 33	151.6 ±39
6,15-diketo-2,3-dinor-13,14-dihydro $PGF_{1\alpha}$				
Control	47.4 ± 4	77.0 ± 9	38.4 ± 4	54.3 ±19
MtT-F_4	75.4[d] ±24	104.6[b] ± 12	93.0[d] ± 36	91.0[d] ±26

[a,b,d]Underlined values significantly different from control values at $p<0.001$, $p<0.01$ and $p<0.05$, respectively.

[c]Each value represents mean ± SD of 3 individual rats.

crease in the proportion of the ω3 fatty acid 22:6ω3. The reduction of ω6 fatty acids was not the result of overt EFA deficiency, since there is no change in the triene:tetraene ratio (17) of tissue lipids in tumor-bearing rats. Docosahexaenoic acid (22:6ω3) is known to be important for the growth of rainbow trout (18) and constitutes an important component of phospholipids in animal tissues such as retina (19), brain (20), heart muscle (21) and liver (22). But the exact biological function of this fatty acid is not well established. The mechanism which elevates the proportion of 22:6ω3 in the tumor-bearing rats is also not clear.

However, the pronounced increase of 22:6ω3 in this animal was not unique. Wood (23) has demonstrated a remarkable increase of 22:6ω3 in the liver phospholipids of hepatoma host rats. Similar increase in liver PE has also been reported in choline-deficient rats (24).

There was no trace of 22:6ω3 in the diet. Thus, the increased proportion of 22:6ω3 cannot be ascribed to the diet. Direct output of 22:6ω3 from the tumor cell and its deposition in tissue PL is unlikely, since the proportion of 22:6ω3 in tumor cortex was low. A rapid turnover of the ω3 fatty acids in tissues would provide the most likely explanation. Normally, when diets rich in 18:3ω3 are given to rats, the amount of this fatty acid in tissue PE and PC remains negligible, whereas 20:5ω3, an intermediate preceding a rate-regulating step in the formation of 22:6ω3 (25), rises sharply (26). In this study, 18:3ω3 in Purina chow given to

the animals represented only 4.7% of the fatty acid intake. Furthermore, the proportion of 20:5ω3 in tissue PL of tumor-bearing rats was not increased. If indeed the turnover of ω3 fatty acids in tumor-bearing rats is accelerated, it is conceivably through the lifting of the regulation imposed on the conversion of 20:5ω3 to 22:5ω3 at the rate-limiting step, and this would result in high levels of 22:6ω3.

To obtain an idea of the overall amounts of unsaturated acids in the membranes, we calculated the concentration (μmol/g tissue weight) as well as the absolute amounts (μmol) of 18:2ω6, 20:4ω6 and 22:6ω3 in the PL fraction of three major organs of control as well as of implanted rats (Table 12). In spite of an average 60% increase in absolute weight for liver, kidney and heart, the combined phospholipid pool size is reduced by 37.7% for 18:2ω6 and is increased only by 25% for 20:4ω6 in implanted vs control animals. This further supports the notion of "relative EFA deficiency" in the tumor rats which have a 24.5% lower body weight at the end of the experiment. The only unsaturated fatty acid that follows the large increase in weight of these 3 organs is 22:6ω3, the pool size of which is raised 234% in the PL fraction.

The reduced proportion of PUFA of the ω6 series in the tissue phospholipids of the tumor-bearing rats may be related in part to an increased in vivo production of prostaglandins, as suggested by the observed increase in the urinary excretion of two endogenous metabolites of PGI_2 (Table 11). The cause of this increased PGI_2 biosynthesis is not known. Gudbjarnason et al. (21) have studied the membrane PL in rat myocardium. After either nicotine treatment to induce the secretion of norepinephrine or repeated administration of norepinephrine to rats, they have demonstrated effects similar to those we have observed here: an increase in the proportion of 22:6ω3 and a corresponding decrease in those of 20:4ω6 and 18:2ω6. Their results implied that the modification of fatty acid composition in tissue PL is under the influence of hormones. Since the tumor-bearing rats exhibit hypertension and high plasma catecholamine concentrations (Buu and Küchel, personal communication), the increased excretion of prostaglandin metabolites could be due here also to a hormonal effect. Moreover, the PGI_2 increase could represent a defence mechanism against the rise in blood pressure. Indeed, PGI_2 is the principal prostaglandin synthesized by the blood vessels and is thought to play a significant role in the modulation of vascular reactivity and blood pressure (9). On the other hand, the observed increase in PGI_2 formation may be one of the various metabolic anomalies induced by the tumor. Recently, an increased production of PGE_2 has been docu-

TABLE 12

Concentrations (μmol/g Tissue Weight) and Total Amounts (μmol) of Linoleic, Arachidonic and Docosahexaenoic Acids in the Major Phospholipid Fractions of Liver, Heart and Kidney of Control and MtT-F$_4$ Tumor-Bearing Rats

		18:2ω6		20:4ω6		22:6ω3	
		Control	MtT-F$_4$	Control	MtT-F$_4$	Control	MtT-F$_4$
Liver	PE	1.10	0.25	4.13	2.22	2.30	3.15
	PS+PI	0.12	0.07	1.90	1.46	0.13	0.22
	PC	4.51	1.74	10.30	8.83	2.52	6.84
Total μmol/g		5.73	2.06	16.33	12.51	4.95	10.21
Total per organ (μmol)		30.74	18.94	87.61	115.01	26.56	93.87
Heart	PE	1.46	0.60	2.88	1.38	4.50	6.52
	PS+PI	0.68	0.60	2.92	2.14	0.62	0.68
	PC	3.02	1.22	5.62	7.38	1.68	3.76
Total μmol/g		5.16	2.42	11.42	10.90	6.80	10.96
Total per organ (μmol)		4.05	1.49	6.71	6.67	3.98	6.76
Kidney	PE	2.47	1.46	11.51	9.36	0.26	1.74
	PS+PI	0.65	0.46	3.50	2.92	0.27	0.40
	PC	3.58	1.88	6.48	4.55	0.68	2.02
Total μmol/g		6.70	3.80	21.49	16.83	1.21	4.16
Total per organ (μmol)		7.93	6.20	25.43	27.48	1.58	6.79
Total all 3 tissues (μmol)		42.72	26.63	119.75	149.19	32.12	107.42
Percent changes (C vs T)		−37.7%		+24.6%		+234.4%	

mented in rabbits bearing a VX2 carcinoma (27,28).

We are faced with the need to explain a relative decrease in 18:2ω6 and 20:4ω6 in membrane phospholipids with a very low proportion of 18:2ω6 in plasma PL. This tissue depletion is most obvious in those organs of the tumor rats that increased in weight (liver, heart, kidney) and where the ω6 acids did not rise in proportion to the increase in size and weight but even decreased (18:2ω6). This deficiency may be due to a decreased availability or to an increased utilization of EFA. The control rats and the tumor rats were fed the same diet and there was no evidence of gross malabsorption in either group, so there is very little argument to support the view that an absorption defect was responsible. Hence, we postulate that an increased utilization is the most likely explanation. This could be due to an enhanced catabolism through β-oxidation, and/or to an increased utilization of arachidonic acid for its diverse metabolic functions including formation of prostaglandins through the cyclooxygenase pathway, formation of leucotrienes and other more abundant 12-hydroxy-derivatives (12-HETE) through the lipoxygenase pathway, formation of lipoic acid and perhaps of some other unknown coenzymes. We believe the kinetics of the various enzymes responsible for desaturation, elongation or β-oxidation could be altered in the presence of the large amounts of hormones secreted by the tumor or by endocrine glands stimulated by the tumoral hormones. Part of the EFA pool may be immobilized in adipose tissue triglycerides (Table 10) and perhaps in plasma triglycerides and cholesteryl esters as well (not measured). In this context, the proportion of EFA derived into the production of PG and other 20:4ω6 metabolites could become a critical factor contributing to the direct depletion of membrane phospholipids EFA, especially if more of the unsaturated acids are also pushed towards oxidation at a higher rate (which is not unlikely in hypertensive, tachycardiac rats with high catecholamine levels and failure to thrive). Furthermore, an increased membrane formation in muscle and other tissues (cardiac, renal and liver hypertrophy) could also contribute to the sequence of events leading to the depletion. We would like to put the emphasis here on the role of an accrued synthesis of PG and other 20:4ω6 metabolites because their major donors are constituted by membrane PL (especially PI and PC) and because their total body production has been grossly underestimated in the past, as pointed out by Nugteren (29). This author showed that, in man, the total urinary excretion of prostanoic and prostadienoic acid homologues was of the order of 300 μg per 24 hr. He pointed out that only a relatively small part of the prostaglandins synthesized in vivo appears ultimately as tetranor-prostadienoic acids in the urine. Indeed, the yield from orally given uniformly labeled ^{14}C-prostaglandin E_2 is 14-20% (29), whereas for the intravenous administration of PGE_2 and $F_{2\alpha}$ the yields were 15-30% (30,31). Nugteren estimated that the total prostaglandin production in the adult human was of the order of 1-2 mg per 24 hr. This figure is far from being negligible and does not even take into account the other metabolites of arachidonic acid through the lipoxygenase

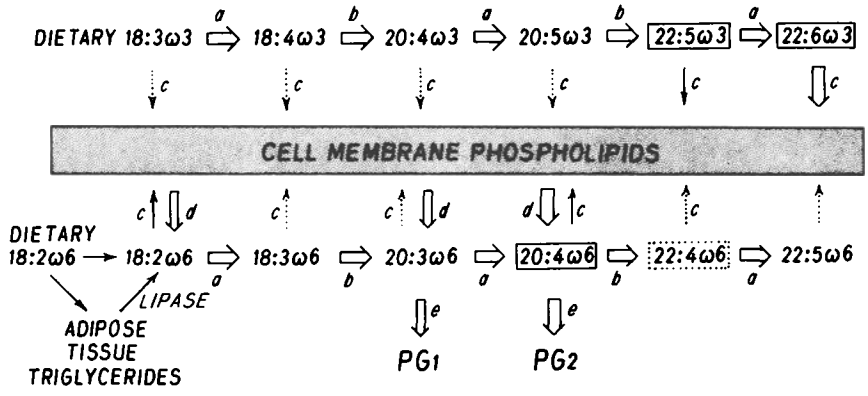

FIG. 1. Proposed effects of MtT-F_4 tumor on the metabolic pathways of ω3 and ω6 fatty acid families. Fatty acids enclosed in solid boxes represent the major products of each pathway in most tissues, and that in the dotted box represents the fatty acid found mainly in tissues such as adrenal, lung, brain, spleen and testis. Large arrows (*a* desaturation and *b* elongation) indicate the steps activated by the secretion of the tumor; *c* indicates the deposition of fatty acids into the cell membrane phospholipids, *d* represents the activity of phospholipase, and *e* represents the activity of cyclooxygenase.

pathway such as 12-hydroxyeicosatetraenoic acid which is a major metabolite in platelets.

On the basis of these observations, we propose that in the tumor-bearing rats there is a general increase in the cyclooxygenase activity which promotes the conversion of C_{20} ω6 fatty acids into prostaglandins (Fig. 1). This, perhaps coupled with an enhanced oxidation of essential fatty acids and further diversion of 20:4ω6 into the lipoxygenase pathway and the production of other metabolites, could explain the partial depletion in linoleic acid. Secondarily, this would lead to a reduction in the elongation and further desaturation of 20:4ω6. In turn, the relative deficiency in 20:4ω6 in cell membranes would stimulate the metabolism of PUFA of the ω3 series by reducing the competition for the common enzyme systems. The fact that 20:5ω3 is not as good a substrate for the cyclooxygenase as is 20:4ω6 (32-34) would explain its preferential conversion into 22:6ω3 that accumulates in the tissue phospholipids of tumor-bearing rats. This attractive hypothesis links for the first time the excessive accumulation of 22:6ω3 in membranes to a relative deficit in 20:4ω6 caused by enhanced prostaglandin production and warrants further studies to put it to the test. Since the tumor produces hormones which may be elevated in several pituitary disorders in man, it is important to study further the exact role of hormones in bringing about these anomalies of membrane composition in the MtT-F$_4$ rat model.

A partial depletion in membrane 18:2ω6 attributable to diversion of the latter towards other metabolic processes would constitute another example of a "relative and selective EFA deficiency state," a concept we have advanced from our studies of fatty acid profiles in Friedreich's Ataxia (35) and in zinc-deficient rats (36). This notion implies that low level of EFA occurring in the absence of an increase in the triene:tetraene ratio (which is the current criterion for defining EFA deficiency) may be responsible for important biological effects, some of which could be deleterious. The situation here is quite distinct from that of the fatty acid deficient model where no EFA is fed to the animal. In the latter, the substrate, i.e., 20:4ω6, is not available so that a depressed prostaglandin synthesis is not only expected but even needed to spare EFA for incorporation into membrane PL and preservation of membrane stability. In our model, the substrate is available, 18:2ω6 is fed in the diet, but the low levels of 18:2ω6 and 20:4ω6 in membrane PL might reflect overutilization of EFA. The enhanced prostaglandin synthesis, according to our hypothesis, is one of the causes of, and not the consequence of, this "relative EFA deficiency." The secondary partial destabilization of membranes is compensated by incorporation of 22:6ω3 into membrane PL. This cannot occur in the true EFA deficiency model because the precursor, 18:3ω3, is not provided.

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Modification of Microsomal Lipid Composition and Electron Transport Enzyme Activities in Isovalerate-Supplemented cells of Novel *Tetrahymena* ISO

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ABSTRACT

Tetrahymena ISO cells, which have an unusually high level of *iso* odd-numbered fatty acids, were grown medium supplemented with various concentrations of isovalerate. There was a marked increase in the total proportion of *iso* odd-numbered fatty acids in supplemented whole cells (28.9 → 70.3%) and microsomes (37.7 → 84%), with a corresponding decrease in normal fatty acids, although no significant alteration of phospholipid composition was observed during 11 hr isovalerate-supplementation. Microsomal palmitoyl-CoA and stearoyl-CoA desaturase activities in isovalerate-supplemented cells decreased by 45.7% and 30.6% during 11 hr, respectively. NADH-cytochrome c reductase and NADH-ferricyanide reductase activities as well as the content of cytochrome b_{560ms}, which is similar to mammalian microsomal cytochrome b_5, were reduced in microsomes from 11 hr-supplemented cells, whereas NADPH-cytochrome c reductase activity was constant. It is suggested that the alteration of the cross-sectional area of lipid molecules in the bilayer, which results from the replacement of normal fatty acids with *iso*-15:0 and *iso*-17:1, would result in the decline of palmitoyl- and stearoyl-CoA desaturation in the isovalerate-supplemented cells, in order to maintain membrane fluidity at a functional level.
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INTRODUCTION

Modification of membrane lipids has been produced in bacterial system and animal cells by a number of procedures (1,2).

The development of these techniques for membrane lipid modification now makes it possible to carry out enzymatic and physical studies in membrane biochemistry (3). All these modification techniques have been found to be effective in *Tetrahymena* cells, which have a proven ability to alter their membrane lipid composition in response to growth condition (4). As previously reported (5), a novel *Tetrahymena* ISO has a much higher content of *iso*-15:0(10%) and *iso*-17:1 (18.1%), which are ususally minor components in the classical strain of *Tetrahymena pyriformis*. This unusual strain prompted us to investigate the biological significance of *iso* odd-numbered fatty acids in the membrane. Although Conner and Reilly (6) have reported that adding isovalerate to *T. pyriformis* W, which has a low concentration of branched chain fatty acids, caused an increase of *iso* odd-numbered fatty acids in phospholipids, we have extended the *iso* fatty acid manipulation experiment to study the influence of *iso* odd-numbered fatty acids on the physical properties and membrane functions in the *Tetrahymena* ISO cell, which has a high level of *iso* odd-numbered fatty acids (*iso*-15:0, *iso*-17:1) in membrane phospholipids.

In this communication, we examine the effects on lipid composition and the activities of microsomal desaturase and electron transport enzymes of feeding to *Tetrahymena* ISO cells isovalerate, which is a precursor for *iso* odd-numbered fatty acids.

MATERIALS AND METHODS

The novel cells which we designate as *Tetrahymena* ISO were provided by Dr. S. Hutner, Haskins Laboratories at Pace University, New York. This strain is similar in gross morphology to *T. pyriformis*, but the exact species identity has not yet been determined. The cells of *Tetrahymena* ISO were grown in proteose-peptone basal medium as previously (7). When the cell density reached 5×10^4 cells/ml, isovalerate solution (pH 7.2) was added to the growth medium. The concentrations of isovalerate in the culture medium were 2 mM, 4 mM and 10 mM. Cell fractionation was performed using the procedure of Nozawa and Thompson (7). Lipids were extracted by the method of Bligh and Dyer (8), and the resultant lipid solutions were stored in chloroform/methanol (6:1,v/v) at −20 C. Lipid phosphorus was determined by the procedure of Bartlett (9) as modified by digestion with perchloric acid according to Marinetti (10). Phospholipids were detected with a molybdenum blue reagent as described by Dittmer and Lester (11). Further identification of the spot was performed by spraying the developed chromatograms first with Dragendorff reagent, then with ninhydrin, and by cochromatography with reference substances pur-

chased from Sigma Chemical Co. (St. Louis, MO) and P.L. Biochemicals (Milwaukee, WI). Estimation of phospholipid distribution was done according to the method of Rouser et al. (12), using the solvent system: chloroform/acetic acid/methanol/water (75:25:5:2.2, v/v) Fatty acid methyl esters prepared by Morrison's method (13) were chromatographed on a 2 m glass column packed with 10% diethylene glycol succinate (DEGS) on 80/100 mesh Chromosorb W at 185 C, and 2% OV-1 on 80/100 mesh Chromosorb W at 140 C, respectively, using a JEOL Model JGC-1100 gas chromatograph. For *iso* and *anteiso* fatty acid determination, fatty acid methyl esters were analyzed on a 3 m column of 25% ethylene glycol succinate (EGS) at 190 C after hydrogenation with platinum black under an H_2 stream, and branched fatty acids were identified by authentic standards. The gas chromatograph-mass spectrometer (Model RMU-6MG, Hitachi Ltd., Tokyo) and 002 type Detalizer (Model HITAC 1011) were employed for unknown fatty acid identification. Fatty acid methyl esters were chromatographed on a 30 m glass capillary column of FFAP at 150-220 C or 1 m column of 5% DEGS at 165 C, respectively. Electron energy was 20 eV, ion current, 100 μA and accelerator voltage, 3.2 kV. The temperature of the ion source was 160 C (5). The numbers of double bonds of unsaturated fatty acids were determined by the method as previously described (14). Fatty acid methyl esters were separated by thin layer chromatography on Silica Gel G plates containing 10% $AgNO_3$ by developing in ether/hexane (1:9, v/v). Each spot obtained was scraped from the chromatoplate and immediately extracted three times with a mixture of petroleum ether/diethyl ether (1:1, v/v) and analyzed by gas liquid chromatography. Authentic standards such as 14:0, 16:0, 18:0, 16:1Δ^9, 18:1Δ^9 and fatty acid methyl esters from *T. pyriformis* W (15) were used for comparison of retention times and for cochromatography. Microsomal palmitoyl-CoA and stearoyl-CoA desaturase activites were determined as previously described (16).

The incubation mixture contained, in a final volume of 0.5 ml, a suitable amount of microsomes (0.35 mg of protein for assay of palmitoyl-CoA desaturase, 0.1 mg for assay of stearoyl-CoA desaturase), 50 nmol of NADH, 0.1 M potassium phosphate buffer (pH 7.2) and 20 nmol of either [1-^{14}C] palmitoyl-CoA (1 Ci/mol) or [1-^{14}C] stearoyl-CoA (1 Ci/mol). The samples were preincubated for 1 min at 28 C prior to the addition of microsomes to initiate the reduction. The procedure for analysis of reaction products was essentially as previously described (17). NAD(P)H-cytochrome c and NADH-ferricyanide reductase activities were measured at 25 C by the changes in absorbance at 550 nm and 420 nm, respectively. Either NADH or NADPH (100 nmol), 20 nmol of cytochrome c, (or 500 nmol of potassium ferricyanide) in 0.1 M potassium phosphate buffer (pH7.4) were incubated in a final volume of 1.0 ml. Reduction of cytochrome c and ferricyanide was recorded in a Hitachi 356 two-wavelength double beam spectrophotometer, and the activities were calculated using respective extinction coefficients of 19.6 $mM^{-1}cm^{-1}$ (18) and 1.02 $mM^{-1}cm^{-1}$(19). *Tetrahymena* microsomal cytochrome b_{560ms} content was determined by measuring the reduced minus oxidized difference spectrum taking the extinction difference of the cytochrome between 425 and 410 nm as 216 $mM^{-1}cm^{-1}$ as previously described (20). Protein was determined by the method of Lowry et al. (21) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Figure 1 illustrates the effects of supplementation with various concentrations of isovalerate on *Tetrahymena* ISO cell growth. The cell population decreased with increasing concentration of isovalerate. However, the ciliates were morphologically normal, although reduced motility was observed with the 10 mM concentration of isovalerate. Isovalerate supplementation at any level did not appear to influence the amount of lipids per cell.

The phospholipid composition of *Tetrahymena* ISO grown in the presence of 4 mM isovalerate is shown in Table 1. *Tetrahymena* cell membranes

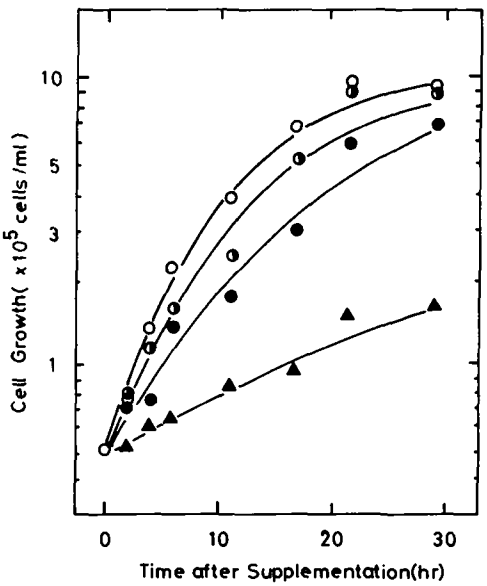

FIG. 1. Effect of isovalerate supplement on cell growth. Control (unsupplemented, ○), 2 mM (◑), 4 mM (●) and 10 mM (▲) of isovalerate were supplemented.

TABLE 1

Phospholipid Composition of Control and Isovalerate-supplemented *Tetrahymena* Cells

Phospholipids	Control	Isovalerate-supplemented 4 hr	Isovalerate-supplemented 11 hr
Lysolecithin	4.3 ± 0.4[b]	2.0 ± 1.2	1.2 ± 0.6
Lecithin	25.3 ± 1.6	28.1 ± 1.9	29.1 ± 0.7
Lysophosphatidylethanolamine plus lysoglyceroaminoethyl phosphonate[a]	3.3 ± 0.4	2.4 ± 1.1	3.5 ± 2.7
Phosphatidylethanolamine	39.7 ± 1.4	36.4 ± 1.0	40.0 ± 0.5
Glyceroaminoethylphosphonate	21.9 ± 1.5	20.4 ± 2.3	17.4 ± 3.0
Cardiolipin	4.9 ± 0.4	5.7 ± 1.6	6.2 ± 1.0

[a]Includes also ceramide-aminoethylphosphonate.
[b]Values represent mean ± SD (%) obtained from 3 different experiments.

TABLE 2

Fatty Acid Composition of Control and Isovalerate-Supplemented *Tetrahymena* ISO Cells and Microsomes[a]

Fatty acid	Whole cells		Microsomes	
	Control	Isovalerate (11 hr)	Control	Isovalerate (11 hr)
iso-13:0	0.8 ± 0.0[d]	5.6 ± 1.2	1.7 ± 0.1	10.7 ± 0.8
n-14:0	8.2 ± 1.0	2.2 ± 0.2	7.6 ± 1.3	1.5 ± 0.1
iso-15:0	10.0 ± 1.0	24.8 ± 0.4	10.7 ± 0.5	34.4 ± 1.4
n-16:0	5.3 ± 1.0	2.1 ± 0.9	4.5 ± 0.5	0.3 ± 0.1
n-16:1$^{\Delta 9}$	11.6 ± 0.6	1.2 ± 0.2	11.1 ± 0.8	6.0 ± 0.3
iso-17:1$^{\Delta ?}$	18.1 ± 1.8	39.9 ± 1.5	25.3 ± 0.6	38.9 ± 1.7
n-18:0	1.7 ± 0.8	1.5 ± 0.5	3.7 ± 0.1	2.4 ± 0.2
n-18:1$^{\Delta 9}$	3.6 ± 0.8	trace	3.7 ± 0.2	0.8 ± 0.0
n-18:2$^{\Delta 6,11}$	2.2 ± 0.3	1.4 ± 0.4	3.7 ± 0.3	1.5 ± 1.0
n-18:2$^{\Delta 9,12}$	6.9 ± 1.3	2.0 ± 0.2	5.0 ± 1.0	trace
n-18:3$^{\Delta 6,9,12}$	22.2 ± 0.4	7.8 ± 0.8	17.2 ± 1.3	2.8 ± 0.9
Others[b]	9.4 ± 0.3	11.5 ± 0.2	5.8 ± 0.5	0.7 ± 0.1
Total *iso* fatty acid	28.9	70.3	37.7	84.0
Unsaturation index[c]	118	71	109	57

[a]The cells were grown in the presence of 4 mM isovalerate and were harvested at 11 hr-supplementation.
[b]Includes *n*-12:0, *n*-15:0, *iso*-16:2, *iso*-17:0 as minor components.
[c]The unsaturation index is defined as Σ((number of double bonds of each fatty acid) × (mol% of each fatty acid)).
[d]Values represent mean ± SD (%) obtained from 3 different experiments.

contain 3 major phospholipids, lecithin, phosphatidylethanolamine and glyceroaminoethylphosphonate which is characteristic of this cell. After 11 hr, the relative proportion of phospholipids in whole cells and microsomes (data not shown) are not affected significantly by adding isovalerate. In contrast, Table 2 shows major changes in the fatty acid composition of supplemented cells. There was quite a similarity in fatty acid composition between whole cells and microsomes. Major fatty acids of the control cells are 16:0, 18:3, *iso*-15:0 and *iso*-17:1. The latter 2 *iso* fatty acids are, if present, usually minor components in the classical strains of *Tetrahymena pyriformis*. Within 11 hr after 4 mM isovalerate supplementation, there were marked increases in the total percentage of *iso* odd-numbered fatty acids of whole cells (28.9 → 70.3%) and microsomes (37.7 → 84.0%) with compensatory decreases of normal fatty acids. Especially, a drastic increase of *iso*-13:0, *iso*-15:0 and *iso*-17:1

was observed in supplemented whole cells and microsomes, corresponding to a decrease of *n*-16:1 and *n*-18:3 after 11 hr. The unsaturated index of microsomes from 11 hr-supplemented cells is almost one-half that of control microsomes.

As we have reported (22), lowering of the environmental temperature consistently induced the activity of microsomal desaturase enzyme and followed to produce a higher ratio of unsaturated to saturated fatty acids in cell membranes. Moreover, the increase of microsomal desaturase activities was primarily associated with the alteration of microsomal electron transport enzymes such as, NAD(P)H-cytochrome c reductase, NADH-ferricyanide reductase and cytochrome b_{560ms}, which is similar to but not identical with mammalian cytochrome b_5(20). Therefore, in order to investigate the drastic decrease of unsaturation index in isovalerate-supplemented cells, we focused on microsomal desaturase activites and electron tranport enzymes. Table 3 shows the microsomal desaturase activities and electron transport enzyme activities such as NAD(P)H-cytochrome c reductase, NADH-ferricyanide reductase and cytochrome b_{560ms}, which may be involved in the desaturase systems. As previously reported (17), palmitoyl-CoA and stearoyl-CoA desaturase systems in *Tetrahymena* microsomes requires 0_2 and NAD(P)H and also are inhibited by KCN but not by CO. The desaturase activities (stearoyl-CoA and palmitoyl-CoA) in isovalerate-supplemented cells are lower than those of control cells. In addition, decreases of NADH-cytochrome c (19.8% decrease), NADH-ferricyanide (15.7% decrease) reductase and the content of cytochrome b_{560ms} (22.2% decrease) were observed in microsomes from isovalerate-supplemented cells, although there was no alteration of NADPH-cytochrome c reductase. Since NADH was more effective for desaturase activity than NADPH as cofactor (23), NADPH-cytochrome c reductase which would link to cytochrome b_{560ms}, might play a minor role in desaturase activity. In consequence, the large decrease of unsaturated fatty acids in supplemented cells might be ascribed to the decrease of the microsomal electron transport enzymes which would participate in microsomal fatty acyl-CoA desaturation.

Since cultures of *Tetrahymena* respond to supplementation with several short-chain fatty acid precursors, sodium propionate, isobutyrate, α-methyl-*n*-butyrate and tris-isovalerate (24,25), it is speculated that this *Tetrahymena* ISO cell can utilize isovalerate as a precursor for *iso* odd-numbered fatty acids more efficiently than other *Tetrahymena* cells. The branched structures of *iso*-fatty acids, like the double bonds of unsaturated fatty

TABLE 3

Desaturase and Reductase Activities in Microsomes from Control and Isovalerate-Supplemented *Tetrahymena* Cells[a]

Enzymes		Control (100%)	Isovalerate (%)
Palmitoyl-CoA desaturase	(nmol/min/mg)	2.30±0.10[b]	1.25±0.20 (54.3)
Stearoyl-CoA desaturase	(nmol/min/mg)	6.11±0.13	4.24±0.10 (69.4)
NADH-cyt c reductase	(μmol/min/mg)	0.106±0.020	0.085±0.010(80.2)
NADPH-cyt c reductase	(μmol/min/mg)	0.029±0.000	0.028±0.000(96.6)
NADH-ferricyanide reductase	(μmol/min/mg)	1.72±0.27	1.45±0.12 (84.3)
Cytochrome b_{560ms}	(nmol/mg)	0.095±0.010	0.074±0.018(77.8)

[a]The cells were grown in the presence of 4 mM isovalerate and were harvested at 11 hr-supplementation.
[b]Values represent mean±SD obtained from 3 different experiments.

The microsomal fractions were prepared from control and 11 hr-isovalerate (4 mM)-supplemented cells. Desaturase activities of palmitoyl-CoA and stearoyl-CoA were determined as described in Materials and Methods. The reaction mixture (0.5 ml) contained 0.1 mM NADH, 0.1 M potassium phosphate buffer (pH 7.2), 20 μM palmitoyl-CoA (or stearoyl-CoA) and 0.35 mg (0.1 mg for stearoyl-CoA) of microsomal protein. After the mixture was preincubated for 1 min at 28 C, the assay tubes were incubated at 28 C for 3 min. The activities of NADH-cytochrome c, NADPH-cytochrome c and NADH-ferricyanide reductase were measured spectrophotometrically at 28 C. Details for each enzyme assay were described in Materials and Methods. Values represent the mean obtained from 3 different experiments.

acids, increase the average cross-sectional area of lipid molecules in the bilayer and tend to make the membrane fluid (26). To maintain membrane fluidity at a functional level, the content of unsaturated fatty acids should be decreased, and this is accomplished in part by a decrease in activity of microsomal electron transport proteins.

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Effects of Lysophosphatidylcholine on Jejunal Water and Solute Transport in the Rat in vivo[1]

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ABSTRACT

The effects of lysophosphatidylcholine on jejunal water and solute transport were studied in vivo in the rat. Five mM lysophosphatidylcholine significantly reduced absorption of water, electrolytes and glucose ($P<0.05$) and 10 mM lysophosphatidylcholine induced net fluid secretion. The effects of 10 mM lysophosphatidylcholine were significantly reduced in the presence of 5 mM phosphatidylcholine ($P<0.05$) and 2 mM cholesterol ($P<0.05$). The fractional absorption of lysophosphatidylcholine decreased with increasing concentration of the detergent in the perfusion solution. Increasing concentrations of taurocholate in the perfusion solutions potentiated the effects of lysophosphatidylcholine ($P<0.01$), although 10 mM taurocholate by itself had no significant effect on intestinal water and electrolyte transport. The data establish that lysophosphatidylcholine, a zwitterionic detergent, affects intestinal transport in the same way as bile acids, fatty acids and synthetic cationic or nonionic detergents. By comparison with the response of the human jejunum to taurodeoxycholate, it is likely that lysophosphatidylcholine generated during the normal process of digestion has an effect on intestinal water and solute transport in man. *Lipids* 18:428-433, 1983.

INTRODUCTION

Dihydroxy bile acids and long-chain fatty acids inhibit intestinal transport of water and electrolytes and induce intestinal fluid secretion in man and experimental animals (1-6). In addition, they reduce absorption of organic solutes in the small intestine (2,3,5). These effects have been attributed to the detergent properties of these compounds (4,6). Lysophosphatidylcholine (1-acyl-*sn*-glycero-3-phospho-choline) is a zwitterionic detergent normally present in postprandial small intestinal contents (7,8). It shares with dihydroxy bile acids and fatty acids the ability to interfere with the integrity of biological membranes, such as red blood cells (9,10) and the gastric mucosa (11-14). The molecular basis for the action of lysophosphatidylcholine on biological and artificial membranes is the subject of very active investigation (15). If one could therefore establish that this compound affects intestinal transport processes the same way as dihydroxy bile acids and fatty acids, the results and conclusions from investigations of the effects of lysophosphatidylcholine in other systems would also be applicable to the effects of detergent compounds on the intestinal mucosa. Moreover, since previous studies have demonstrated that anionic (1-6,16), cationic (16) and nonionic (16) detergents affect intestinal water and solute transport, it was reasonable to expect that a zwitterionic detergent might have similar effects. We, therefore, tested the effects of lysophosphatidylcholine on water and solute transport in the rat small intestine.

[1]The work has been published in abstract form: Gastroenterology 79: 1091 (1979).

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METHODS

Materials

Lysophosphatidylcholine from egg yolk was purchased from Sigma Chemical Company, St. Louis, MO. It was 98% pure by thin layer chromatography (TLC) and was used as supplied. Its fatty acid composition was 29.7% stearic acid and 70.3% palmitic acid by gas liquid chromatography. Lyso-1-[palmitoyl-1-^{14}C]-phosphatidylcholine, [1,2-^{3}H] polyethylene glycol-4000 and [1,2-^{14}C]-polyethylene glycol-4000 were purchased from New England Nuclear, Boston, MA. Egg phosphatidylcholine (Sigma Chemical Company, St. Louis, MO) was further purified by column chromatography (17). The final product was greater than 98% pure by TLC (17). Taurocholate (>97% pure) was purchased from Calbiochem-Behring, San Diego, CA, and cholesterol (>99% pure) from NuChek Prep, Elysian, MN.

Experimental Model

Male Sprague-Dawley rats (Holtzman Co., Madison, WI) weighing 300-350 g were used after an overnight fast. Under general anesthesia with pentobarbital and ether, 40 cm of jejunum, starting at the ligament of Treitz, were isolated and cannulated proximally and distally and returned into the abdominal cavity with the blood supply intact. Control and test solutions were kept at 37 C and perfused at 42 ml/hr. The temperature of the animals was kept at 37 C by heat lamps controlled by a rectal thermometer. Each solution was perfused for 2 hr. The first hour was used for equilibration, followed by six 10-min sampling

periods. The mean values of the six sampling periods constituted one data point.

Composition of Perfusion Solutions

Perfused were isotonic electrolyte solutions of the following basic compositions (in mM): Na 134, K 5, Cl 139, glucose 11.2, polyethylene glycol-4000 (PEG) 5 g/l with ^{3}H-PEG 25 μCi/L or ^{14}C-PEG 5 μCi/L. After addition of the test compounds, osmolality was adjusted to 280 mosmol/L with mannitol and pH to 7.5 with 0.1 N NaOH. Three groups of experiments were performed. Various test compounds were added depending on the experimental design (see below).

Analytical Procedures

Lysophosphatidylcholine and PEG concentrations (18) were determined by measuring the respective isotopes in the perfusion solutions and collected samples. For isotope determinations, 1 ml of sample was mixed with 10 ml of a scintillation cocktail composed of toluene and emulsifier (Ready Solv-HP, Beckman Instruments, Inc., Fullerton, CA) and counted in a liquid scintillation counter (Beckman LS-255) with two windows. Quench correction was made by external standardization. Counts per minute were converted into disintegrations per minute with a computer program which corrected for quenching and spillover of ^{14}C into the tritium channel (19). Spillover of tritium into the ^{14}C channel was less than 1%. Total phospholipid recovery was determined as total phosphate recovery (20). The difference between the phospholipid recovery and the recovery of lysophosphatidylcholine by the isotope technique was attributed to release of phospholipids from the mucosa. Glucose was determined by the glucose oxidase method (Boehringer-Mannheim Corp., NY), Na and K by flame photometry and chloride by electrometric titration with a silver nitrate solution. The integrity of lysophosphatidylcholine after perfusion was checked by TLC (17).

Calculations and Statistical Analysis

Net water and solute movements were calculated using standard formulas from the change in PEG and solute concentrations (21). Absorption rates were expressed as ml/hr per 40 cm, or as μmol/hr per 40 cm of jejunum. The differences in net movement of water and solutes were statistically evaluated by paired or unpaired t-tests. Linear regressions were calculated by the method of least squares (22).

RESULTS

Effects of 5 and 10 mM Lysophosphatidylcholine

In 8 rats, perfusion of a control solution was followed by 5 mM and 10 mM lysophosphatidylcholine. Five mM lysophosphatidylcholine reduced water absorption significantly ($P<0.05$) (Table 1). In the presence of 10 mM lysophosphatidylcholine, net water movement was reduced to zero. Concomitantly, glucose absorption was reduced in a dose-dependent manner ($P<0.01$). A linear relationship existed between net movement of water and glucose absorption ($r=0.83$; $P<0.01$) (Fig. 1). In this figure, we included data on glucose absorption obtained during control periods and during perfusion of 10 mM lysophosphatidylcholine in experiment 3. Data on the absorption of lysophosphatidylcholine are only available in 4 animals. Absorption rates of lysophosphatidylcholine increased only slightly with the increase of the phospholipid concentration in the perfusion solution from 5 mM to 10 mM. When solute absorption rates were expressed as fractional absorption (%), a linear relationship existed between glucose absorption and absorption of lysophosphatidylcholine ($r=0.76$; $P<0.02$) (Fig. 2). Throughout the experiment, a net gain of phospholipid was observed. This was 3.8 ± 1.9 μmol/hr per 40 cm during the control period and increased to 48.7 ± 5.2 μmol/hr/40 cm ($P<0.001$ vs control) during the perfusion with 5 mM lysophosphatidylcholine and 37.6 ± 10.5 μmol/hr per 40 cm ($P<0.001$ vs control) during perfusion with 10 mM lysophosphatidylcholine. No breakdown of lysophosphatidylcholine was observed when the collected samples were checked by TLC.

Net changes in electrolyte movement were similar to the changes in water movement (Table 1). Absorption of sodium was significantly reduced ($P<0.05$). Perfusion with detergent resulted in net

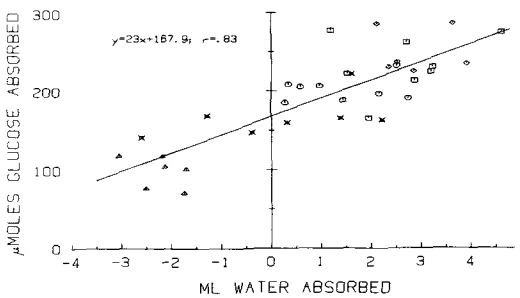

FIG. 1. Relationship between net water movement and glucose absorption under the influence of lysophosphatidylcholine. Perfused were isotonic electrolyte solutions containing 11.2 mM glucose (⊡), or in addition 5 mM (o) or 10 mM lysophosphatidylcholine (⊡⊡), 10 mM taurocholate (◈) or 10 mM taurocholate + 10 mM lysophosphatidylcholine (Δ). The test segment was 40 cm of jejunum, perfusion rate 42 ml/hr. Data are from experiments 1 and 3 (Tables 1 and 3).

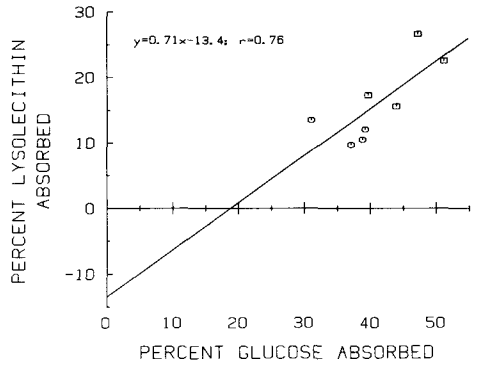

FIG. 2. Relationships between fractional absorption of glucose and lysophosphatidylcholine. Isotonic electrolyte solutions containing 11.2 mM glucose and 5 mM (□) or 10 mM (o) lysophosphatidylcholine were perfused through 40 cm of rat jejunum at 42 ml/hr.

secretion of potassium ($P<0.05$). The standard errors in the movement of chloride were rather large and the only significant difference observed was between perfusion of 5 and 10 mM lysophosphatidylcholine.

Effect of Phosphatidylcholine on Fluid Secretion Induced by Lysophosphatidylcholine

Because lysophosphatidylcholine in the postprandial contents is generated from phosphatidylcholine and because phosphatidylcholine blocks the effects of dihydroxy bile acids on water transport in the jejunum (2) and the gallbladder (23), we tested the combined effects of 10 mM lysophosphatidylcholine and 5 mM phosphatidylcholine in 4 rats. To solubilize phosphatidylcholine, control and test solutions contained in addition of 5 mM taurocholate. Taurocholate at this concentration has no effect on water transport (4,24). Four solutions were perfused: (1) a control solution, (2) 10 mM lysophosphatidylcholine, (3) 10 mM lysophosphatidylcholine plus 5 mM phosphatidylcholine, (4) 5 mM phosphatidylcholine (Table 2). Phosphatidylcholine reduced the effects of lysophosphatidylcholine on water and glucose absorption ($P<0.05$). Five mM phosphatidylcholine by itself had no significant effect on water transport.

Effects of Cholesterol on Fluid Secretion Induced by Lysophosphatidylcholine

Because cholesterol reduces the secretory effects of dihydroxy bile acids and fatty acids (25), and because it protects against the damaging effects of lysophosphatidylcholine in red cells and liposomes (26), we tested its effects on fluid secretion induced by lysophosphatidylcholine. Preliminary studies had indicated that the effects of lysophosphatidylcholine were not completely reversible. We therefore had to resort to unpaired experiments. Group A tested the effects of 10 mM lysophosphatidylcholine (n=6), group B the effects of 10 mM lysophosphatidylcholine plus 2 mM cholesterol (n=5), in the sequence Control I—Test—Control II (Table 3). All solutions contained 10 mM taurocholate to facilitate solubilization of the cholesterol. Ten mM lysophosphatidylcholine induced fluid secretion ($P<0.01$). The effects were only partially reversible, since fluid absorption in Control II was less than in Control I ($P<0.05$). Associated with these effects were induction of secretion of sodium, chloride and potassium ($P<0.01$) and a reduction in glucose absorption ($P<0.01$). When cholesterol

TABLE 1

Effect of Lysophosphatidylcholine on Water and Solute Transport in the Rat Jejunum

Net movement per 40 cm jejunum[a]		Control	Lysophosphatidylcholine 5 mM	Lysophosphatidylcholine 10 mM
H_2O	(ml/hr)	2.7± 0.4	1.4± 0.4[c]	0.2± 0.7[d]
Glucose	(μmol/hr)	233.7± 13.2	201.7± 5.7[c]	166.8± 9.9[d,e]
Na^+	(μmol/hr)	180.7± 73.9	13.7± 55.0[c]	−97.9± 102.2[c]
K^+	(μmol/hr)	−3.8± 5.3	−12.6± 4.0[c]	−13.5± 4.2[c]
Cl^-	(μmol/hr)	253.3± 87.0	315.9± 36.5	143.5± 60.6[c]
Lysophosphatidylcholine	(μmol/hr)[b]	-	44.9± 66.3	47.5± 3.9
Phospholipid	(μmol/hr)[b]	−3.8± 1.9	−48.7± 5.2[f]	−37.6± 10.5[f]

Results are mean (±SE) from studies in 8 rats. Each solution was perfused at 42 ml/hr through 40 cm of intestine.
[a]Minus sign indicates net secretion.
[b]Data from studies in 4 rats.
[c]$P<0.05$ vs control.
[d]$P<0.02$ vs control.
[e]$P<0.05$ vs lysophosphatidylcholine 5 mM.
[f]$P<0.001$ vs control.

TABLE 2

Influence of Phosphatidylcholine on the Effects of Lysophosphatidylcholine in the Jejunum

Test conditions	Net movement per 40 cm jejunum	
	Water (ml/hr)[a]	Glucose (μmol/hr)
Control	3.5 ± 1.1	207 ± 27
Lysophosphatidylcholine 10 mM	−1.1 ± 0.7[b]	102 ± 17[d]
Lysophosphatidylcholine 10 mM + phosphatidylcholine 5 mM	0.9 ± 0.1[c]	132 ± 21[c,d]
Phosphatidylcholine 5 mM	1.9 ± 1.0[c]	172 ± 34

Results are mean ± SE from studies in 5 rats. Each solution was perfused at 42 ml/hr through 40 cm of intestine. All solutions contained 5 mM taurocholate.

[a]Minus sign indicates net secretion.

[b]$P<0.05$ vs control.

[c]$P<0.05$ vs lysophosphatidylcholine.

[d]$P<0.005$ vs control.

TABLE 3

Effect of Lysophosphatidylcholine (LYS) in the Absence and Presence of Cholesterol (CHOL) on Water and Solute Transport in the Rat Jejunum

Test conditions	Net movement per 40 cm jejunum[a]				
	H_2O (ml/hr)	Glucose (μmol/hr)	Na (μmol/hr)	Cl (μmol/hr)	K (μmol/hr)
Control I (n = 6)	2.9 ± 0.3	250.1 ± 11.5	229.6 ± 52.5	444.4 ± 72.4	−1.9 ± 1.7
LYS 10 mM	−2.2 ± 0.2[b]	98.3 ± 8.2[b]	−415.0 ± 31.1[b]	−91.0 ± 33.7[b]	−25.7 ± 2.1[b]
Control (II)	0.9 ± 0.3[c,e]	165.2 ± 11.6[c,d]	18.3 ± 41.0[c,d]	267.7 ± 37.6[d]	−20.5 ± 2.9[c,d]
Control I (n = 5)	2.8 ± 0.4	212.7 ± 18.0	225.9 ± 48.8	326.4 ± 30.6	−1.3 ± 1.0
LYS 10 mM + CHOL 2 mM	−1.5 ± 0.3[b,e]	91.8 ± 14.0[b]	−295.0 ± 41.3[b,e]	3.1 ± 32.5[b,e]	−18.2 ± 2.7[b,e]
Control (II)	0.7 ± 0.2[b,d]	136.9 ± 8.3[c,d]	7.7 ± 32.3[b,d]	223.1 ± 37.1[c,d]	−14.8 ± 1.9[b]

Results are mean ± SE. Each solution was perfused at 42 ml/hr through 40 cm of jejunum. All solutions contained 10 mM taurocholate.

[a]Minus sign indicates net secretion.

[b]$P<0.01$ vs Control I.

[c]$P<0.05$ vs Control I.

[d]$P<0.01$ vs LYS 10.

[e]$P<0.05$ vs LYS 10 (unpaired t-test).

was added to the perfusion solutions, similar results were obtained; the magnitude of the fluid and electrolyte secretion, however, was significantly less ($P<0.05$) than during perfusion with 10 mM lysophosphatidylcholine alone.

DISCUSSION

The studies indicate that the actions of lysophosphatidylcholine on intestinal transport resemble those of bile acids and fatty acids. It had an adverse effect on absorption of electrolytes, water and glucose. Linear relationships existed between the changes of water and solute movement; this is similar to what has been observed in the presence of deoxycholate and fatty acids (27). As in the case of bile acids (2,23), the effects of lysophosphatidylcholine are diminished in the presence of phosphatidylcholine. The inhibitory effects of cholesterol are also qualitatively similar to those observed in the presence of bile acids and fatty acids (25).

During perfusion with lysophosphatidylcholine, phospholipids were released into the lumen. This has also been observed during jejunal perfusion with ricinoleic acid in the hamster (6) and in man (28). It has been interpreted as evidence for membrane damage (6). If membrane damage were directly responsible for the change in water and solute transport, one would expect an increase in phospholipid release during perfusion of the higher

concentrations of lysophosphatidylcholine parallel with the increased effect on water and solute transport. Phospholipid secretion, however, did not appreciably change or actually decreased with an increase in the detergent concentration. A possible explanation would be that "cell damage" and effect on water and solute transport are not directly related. This explanation is supported by the observation that mucosal damage induced by chenodeoxycholate occurs independently from fluid secretion induced by this bile acid (29).

To measure absorption of lysophosphatidylcholine, we used ^{14}C-labeled palmitoyllysophosphatidylcholine, while the bulk consisted of a mixture of 70.3% palmitoyl- and 29.7% stearoyl-lysophosphatidylcholine. In view of other studies on the partitioning and uptake of lipids by the intestinal mucosa (30), we believe that the difference in absorption rates between the C-16 and C-18 compound is relatively small and that the isotope measurements represent an accurate assessment of lysophosphatidylcholine absorption. The absorption rate of lysophosphatidylcholine did not significantly increase with its concentration in the perfusion solution. This observation is comparable to our observations for the fractional absorption of fatty acids, which also decreased with increasing fatty acid concentration (27). Possible explanations for this phenomenon are the increase in micellar size resulting in a decrease in the rate of diffusion across the unstirred water layer (31) or saturation of the intestinal metabolic pathways (32) for lysophosphatidylcholine. Because of the close relationship between the change in the fractional absorption of glucose and phosphatidylcholine, however, we believe that the "apparent saturation" of lipid absorption is an expression of a general reduction in solute absorption induced by the detergent (27).

Phosphatidylcholine reduced the effects of lysophosphatidylcholine; this is similar to its effect on bile acid induced fluid secretion (2,23) and is in keeping with in vitro observations, that it inhibits red cell lysis by lysophosphatidylcholine (26). The mechanism is thought to be due to expansion of micellar size and concomitant reduction of the monomer concentration of the detergents (2). At a similar molar ratio, phosphatidylcholine blocked the effects of TDC completely in the human jejunum. The incomplete protection against lysophosphatidylcholine is probably due to the result of a carry-over effect (see Table 3) because water absorption during the subsequent perfusion with phosphatidylcholine did not reach the level of the initial control period either.

Cholesterol reduced the effects of lysophosphatidylcholine on water and electrolyte transport. This corresponds to the observation that the effects of lysophosphatidylcholine on the permeability of red blood cells and liposomes are mitigated by cholesterol (26), and to the observation that cholesterol inhibits the secretory effect of taurodeoxycholate and oleic acid in the human jejunum (25). The effect was not as dramatic as in the human intestine. This quantitative difference is best explained by the observation that the protective effect of cholesterol varies with the detergent used; cholesterol, for instance, abolished the effect of oleic acid completely, inhibited the effects of taurodeoxycholate to a significant degree and had no effect on fluid secretion induced by linolenic acid (26). It is thought that the protective effect of cholesterol is related to its ability to stabilize lipid bilayer membranes (26).

The magnitude of the changes in water movement induced by 10 mM lysophosphatidylcholine varied between the 3 experimental groups from 0.2 ± 0.7 ml/hr in group 1 to -2.2 ± 0.2 ml/hr in group 3 ($P<0.01$ vs group 1) with the fluid secretion observed in group 2 in between. Since the solutions differed only by the content of taurocholate (none in group 1, 5 mM in group 2 and 10 mM in group 3) the data suggest that taurocholate might have potentiated the effects of lysophosphatidylcholine. Taurocholate by itself has no effect on water transport if one compares control absorption in experiments 1, 2 and 3.

The relative potency of lysophosphatidylcholine in its effect on intestinal function and, therefore, the relevance of these studies for events in the human intestine can only be appreciated by comparing the effects of lysophosphatidylcholine with those of taurodeoxycholate. In the rat jejunum, 5 mM taurodeoxycholate has no effect on water and solute transport (33) while 5 mM lyso significantly reduced water and electrolyte absorption. In contrast to its ineffectiveness in the rat intestine, 5 mM taurodeoxycholate produces significant fluid secretion in the human jejunum (25). The postprandial concentrations of lysophosphatidylcholine in the human jejunum range from 2 mM to 4 mM (7,8). In view of the greater susceptibility of the human jejunum to taurodeoxycholate, it should also be more susceptible to the effects of lysophosphatidylcholine. Lysophosphatidylcholine, therefore, could have a significant effect on intestinal transport in the postprandial phase under physiological conditions. Its effects on the other hand, are mitigated by phosphatidylcholine and cholesterol.

ACKNOWLEDGMENTS

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Dietary treatment	Δ^9-Desaturase activity (nmol mg prot^{-1} 20 min^{-1})	Δ^6-Desaturase activity (nmol mg prot^{-1} 20 min^{-1})	Δ^5-Desaturase activity (nmol mg prot^{-1} 20 min^{-1})
PO	0.5 ± 0.4[b]	2.5 ± 0.7	4.6 ± 1.4
HPO + 4.6 cal % 18:2	2.2 ± 1.0[d]	2.0 ± 0.4	5.4 ± 2.4
HCO + 4.6 cal % 18:2	1.0 ± 0.3[c]	1.9 ± 0.4	3.4 ± 0.8
HHO + 4.6 cal % 18:2	0.8 ± 0.5	1.7 ± 0.5[c]	3.0 ± 0.9[c]
RSO	1.2 ± 0.5[c]	3.4 ± 0.8	6.0 ± 2.6

[a]The partially hydrogenated oils were supplemented with safflower oil.
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METHODS

Synthesis of Mixed-Acid Phosphatidylcholines and High Pressure Liquid Chromatographic Analysis of Isomeric Lysophosphatidylcholines

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ABSTRACT

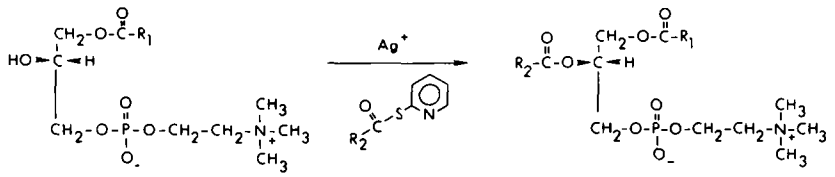
A new method for the synthesis of mixed-chain phosphatidylcholines is reported. Silver ion catalyzed acylation of lysophosphatidylcholines by 2-thiopyridyl esters occurs rapidly (10 min) at room temperature in organic solvents. Yields of isomerically pure mixed-chain phosphatidylcholines (> 98% isomeric purity) are generally greater than 80%. The reaction proceeds with only 1.5- to 2-fold excess of thiopyridyl ester, thus offering some advantages over existing procedures when precious acylating agents are used. The major disadvantage of the procedure is its sensitivity to water. Phosphatidylcholines having hydroxy fatty acyl groups are prepared by protection of the hydroxyl as the levulinate ester, conversion to the 2-thiopyridyl ester, acylation, and removal of the levulinate with hydrazine. For purification of lysophosphatidylcholines, a reverse-phase high pressure liquid chromatographic method for separation of 1-acylglycerophosphocholines from 2-acylglycerophosphocholines was developed.
Lipids 18:434-438, 1983.

Interest in membrane structure and function has stimulated an extensive search for synthetic methods leading to specific phospholipids. The synthesis of mixed-acyl phosphatidylcholines (PC) **2**, in particular, has been the subject of several recent publications and partial syntheses utilizing 1-acyl-*sn*-glycero-3-phosphocholine (lysoPC), **1**, prepared by phospholipase A_2 catalyzed hydrolysis of phosphatidylcholine have been reported (1-5). Our interest in phospholipid oxidation has led us to seek alternate methods of phospholipid synthesis. We report here a method that allows the efficient synthesis of mixed-acyl PC with high purity. Our method may be particularly useful when a lysoPC must be acylated with a precious fatty acid.

Existing methods for mixed-acyl PC synthesis rely primarily on acylation of lysoPC with acid anhydrides. Either *p*-dimethylamino pyridine (2) or 4-pyrrolidinopyridine (1) may be used as catalyst. Yields are excellent and the reaction is convenient to carry out. The pyrrolidine catalyst is preferred, since less migration of acyl substituents has been reported with this catalyst (1). On the other hand, the rate of acylation is relatively slow unless a large excess of anhydride is used and a 5-fold excess of this reagent is typically utilized. Furthermore, one equivalent of the acyl substituent in the anhydride is wasted since fatty acyl carboxylate is the leaving group of the acylating agent. For this reason, and also because we have found the acid anhydride method less than ideal for the synthesis of phospholipids having hydroxy fatty acyl substituents, we searched for alternate synthetic approaches to mixed-acyl PC.

The method reported here relies on a silver-ion catalyzed acylation of lysoPC with 2-pyridinethiol fatty acid esters (Scheme 1) (6-9).

A variety of diacyl PC have been prepared by this approach and the method would appear to be one of general utility. For example, phospholipids (2) containing the following substituents have been prepared: **3**, $R_1 = R_2 = 16{:}0$; **4**, $R_1 = 18{:}0$, $R_2 = 16{:}0$; **5**, $R_1 = 16{:}0$, $R_2 = 18{:}2$; **6**, $R_1 = 16{:}0$, $R_2 = 18{:}1$; **7**, $R_1 = 16{:}0$, $R_2 =$ 12-OH stearate; **8**, $R_1 = 16{:}0$, $R_2 =$



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ricinoleate.

During the course of these synthetic studies, we also developed reverse-phase high pressure liquid chromatographic (HPLC) methods for the analysis and isolation of 1-acyl lysoPC (1) and 2-acyl lysoPC. This HPLC analysis is convenient and may be generally useful in the analysis and preparation of pure lysoPC isomers.

EXPERIMENTAL

Materials

Phosphatidylcholines were obtained from Avanti Biochemicals (Birmingham, AL) as were lysophosphatidylcholines. Palmitic acid (NuChek Prep, Elysian, MN), linoleic acid (Sigma Chemical Co., St. Louis, MO), 12-hydroxystearic acid (Pfaltz and Bauer, Inc., Stamford, CT; Supelco Inc., Bellefonte, PA), and ricinoleic acid (Sigma) were used without further purification.

Phosgene in benzene came from MC&B and 2-pyridine thiol was obtained from Aldrich (Milwaukee, WI).

HPLC

Simple diacyl phosphatidylcholines were purified by reverse-phase HPLC on a Waters μ-Bondapak C-18 column with methanol/water (97:3, v/v). PC having acyl groups with hydroxy substituents (7 and 8) were purified on the same column using methanol/water (95:5) as solvent.

LysoPC were analyzed by reverse-phase HPLC on a Waters μ-Bondapak C-18 column with methanol/water (85:15). Detection for PC and lysoPC was by ultraviolet (UV) at 214 nm.

Synthesis of 2-Pyridinethiol Esters

The method of Corey and Clark (10) was used. Thus, 2-thiopyridyl chloroformate was prepared from 2-pyridinethiol and phosgene. Commercial phosgene in benzene was found suitable and the reaction was carried out as described (10), with the exception that toluene solvent was replaced by benzene. The 2-thiopyridyl chloroformate was converted to the thiopyridyl ester by reaction with free fatty acid in the presence of triethylamine to scavenge HCl. Fatty acyl pyridinethioesters were purified by flash chromatography on silica (Waters Prep 500, 1 in. od column with refractive index detection). Nuclear magnetic resonance spectra were consistent with assigned structures of all 2-pyridinethiol esters prepared (10). A typical procedure for conversion of 2-thiopyridyl chloroformate to the 2-pyridinethiol palmitic acid ester is described below.

Palmitic acid (512 mg, 2.0 mmol) and triethylamine (0.35 ml, 2.2 mmol) were taken up in dry diethylether at 0 C under argon. A solution (CH_2Cl_2) of 2-thiopyridyl chloroformate (0.49 g, 2.5 mmol) was added, and the milky mixture was stirred for 30 min at 0 C, diluted with 50 ml ether, and 0.2 g anhydrous $MgSO_4$ was then added. The solution was filtered, and the pale yellow filtrate was evaporated. The yellow solid that resulted was flash chromatographed on silica (Prep 500, 10% ethyl acetate/hexane on 200-400 mesh silica). Typical yields were 55-80%.

LysoPC Preparation

The procedure of Mason et al. was used with minor modifications (1). The lysoPC was dried exhaustively by pumping at high vacuum.

Phosphatidylcholine Syntheses

The synthesis of 1-stearoyl-2-palmitoyl-*sn*-glycerophosphocholine is typical and is presented below.

Silver perchlorate (5-9 equiv) was dried for 20 hr (dark, P_2O_5, high vacuum) at room temperature and an additional 2 hr at refluxing ethanol temperature. The 2-thiopyridyl palmitic acid ester (1.5-5 equiv) was dried overnight at room temperature under high vacuum. Freshly prepared lysoPC (one equivalent) was dried for 4 hr at room temperature under high vacuum. Benzene (2.5 ml/mmol lysoPC) was distilled into an oven-dried (argon) flask. The lysoPC was quickly added followed by solid thioester and $AgClO_4$. All additions were made in less than one min. After 10 min stirring under argon, thin layer chromatography showed complete reaction. Solvent was removed under vacuum and the crude white solid was suspended in 2.5 ml $CHCl_3$ and chromatographed on silica (Prep 500, $CHCl_3$/MeOH/H_2O, 6:2.5:.3). Yield for 1-stearoyl-2-palmitoyl-*sn*-glycerophosphocholine was greater than 95% if appropriate precautions to exclude water were taken. PC of highest purity may be obtained by reverse-phase HPLC (vide infra) of material obtained from silica chromatography. If the procedures outlined above were followed without drying the 2-thiopyridyl ester or the lysoPC, yields of PC product were low (20%).

Synthesis of 1-Palmitoyl-2-(12-hydroxystearoyl)-*sn*-glycerophosphocholine

Protection of hydroxy as levulinate ester. Levulinic anhydride (1.5 g, 7 mmol) was added to 0.9 g (3 mmol) 12-hydroxystearic acid in 6 ml pyridine under argon and the mixture stirred 16-20 hr. Pyridine was removed in vacuo, the residue was taken up in chloroform/water and acidified with 1 N HCl to pH 4. The aqueous phase was extracted 3 times with chloroform, the chloroform extracts

were combined, dried, and the solvent removed. Chromatography on silica gel (Prep 500, 20% ethyl acetate in hexane, 0.7% acetic acid) gave the pure 12-levulinate, **9**.

Synthesis of phospholipid and removal of protective groups. The levulinate ester of 12-hydroxystearic acid, **9**, was converted to the 2-thiopyridyl ester and 1-palmitoyl lysoPC was acylated by this reagent as described above. Removal of levulinate from the protected PC to give 1-palmitoyl-2-(12-hydroxystearoyl)-*sn*-glycerophosphocholine was carried out as described by vanBoom and Burgers (11) and Hassner et al. (12). The reaction was worked up by dilution of the pyridine/acetic acid solvent mixture with cold 1 N aq HCl and extraction with $CHCl_3/CH_3OH$ (2:1). Removal of the $CHCl_3$ followed by chromatography gave the pure phospholipid.

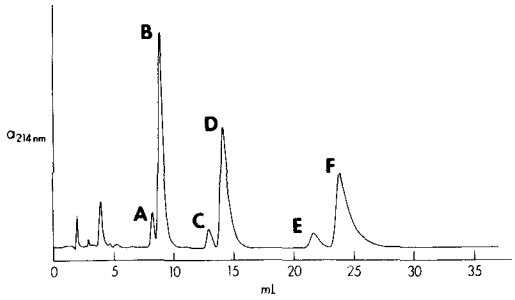


FIG. 1. HPLC trace for chromatography of lysoPC. Solvent was methanol/water (85:15), flow rate 1 ml/min, column Waters μ-Bondapak C-18. Fractions **A-F** as identified in text. Separation was achieved only with this column. Other highly coated columns proved ineffective in the separation. Compounds separated were as follows: **A**, 8.3 ml, 2-myristoyl-*sn*-glycero-3-phosphocholine; **B**, 8.9 ml, 1-myristoyl-*sn*-glycero-3-phosphocholine; **C**, 12.9 ml, 2-palmitoyl-*sn*-glycero-3-phosphocholine; **D**, 14.1 ml, 1-palmitoyl-*sn*-glycero-3-phosphocholine; **E**, 21.6 ml, 2-stearoyl-*sn*-glycero-3-phosphocholine; and, **F**, 23.8 ml, 1-stearoyl-*sn*-glycero-3-phosphocholine.

RESULTS AND DISCUSSION

HPLC of Lysophosphatidylcholines

The most efficient synthesis of mixed-acyl phosphatidylcholines available before this work was initiated, involved acylation of lysoPC by acid anhydrides. One of the problems associated with this synthetic approach is the rearrangement of 1-acyl lysoPC to the 2-acyl lysoPC isomer during the course of acylation. Nitrogen bases used as catalysts for the acylation also assist in acyl migration. A recent publication reports on the use of ^{31}P NMR to monitor this rearrangement and the details of migration catalysis have been worked out in this elegant study (13). During the course of our studies, we sought to analyze the isomeric purity of lysoPC and we have developed HPLC methods for their analysis.

A reverse-phase liquid chromatogram of a mixture of lysoPC is presented in Figure 1. This lysoPC mixture contains myristoyl, palmitoyl, and stearoyl lysoPC, and the fractions **A-F** have been assigned as follows: **A**, 8.3 ml, 2-myristoyl-*sn*-glycero-3-phosphocholine; **B**, 8.9 ml, 1-myristoyl-*sn*-glycero-3-phosphocholine; **C**, 12.9 ml, 2-palmitoyl-*sn*-glycero-3-phosphocholine; **D**, 14.1 ml, 1-palmitoyl-*sn*-glycero-3-phosphocholine; **E**, 21.6 ml, 2-stearoyl-*sn*-glycero-3-phosphocholine; and, **F**, 23.8 ml, 1-stearoyl-*sn*-glycero-3-phosphocholine. Freshly prepared lysoPC (snake venom) contains only the 1-acyl lyso compounds (**B, D,** and **F**) and upon standing, the 2-acyl substituted isomers are formed (**A, C,** and **E**).

Commercial samples of lysoPC are quite variable in purity. Some samples contained no detectable 2-acyl lysoPC, while others showed as much as 8% of this isomer. We find, in fact, a direct correlation of lysoPC purity and isomeric PC purity of products of the acylation procedures we have developed. The quality of commercial samples depends dramatically on history in transit and storage. The HPLC analysis reported here should allow for easy and direct analysis of commercial or laboratory samples.

The 2-acyl lysoPC isomers isolated by HPLC have ^1H NMR spectra consistent with their proposed structures. For example, in $CDCl_3$ for 2-palmitoyl-*sn*-glycero-3-phosphocholine (Fraction **C**), the *sn*-2-methine proton appears at 4.95 δ, characteristic of an acyl substituted methine, and inconsistent with an acyl substituted methylene (14). In DMSO-d_6, the *sn*-2-methine proton appears at 4.75 δ as a quintet and the primary -OH is observed at 5.7 δ as the expected triplet.

We should note that other reverse-phase HPLC columns were not found suitable for lysoPC separations. The utility of the column appears to relate directly to the amount of C-18 bonded phase present. The 10 μ column successfully used in these studies does not contain a highly coated silica and it was only with this column material that a successful separation was achieved. Even 5 μ or 3 μ spherical bead columns that are highly coated are not suitable for the separation of lysoPC isomers. The differences in solubility of the isomeric lysoPC is also noteworthy. Whereas the 2-acyl lysoPC were soluble in benzene, DMSO, chloroform, and methanol, the 1-acyl lysoPC were readily dissolved only in methanol.

TABLE 1

Synthetic Phosphatidylcholines from Acylation of Lysophosphatidylcholine with 2-Thiopyridyl Esters

Phosphatidylcholines	Isomeric impurity[a] (%)	F_1[b] (%)	F_2 (%)	Yield[c] (%)
1,2-Palmitoyl-*sn*-glycerophosphocholine	-	-	-	98
1-Stearoyl-2-palmitoyl-*sn*-glycerophosphocholine	1.5	49.3	50.7	95
1-Palmitoyl-2-linoleoyl-*sn*-glycerophosphocholine	1.8	49.0	51.0	93

[a]Amount of mixed-chain impurity, analysis as described in ref. 1.
[b]Fraction of fatty acids at the 1 and 2 positions; see ref. 1.
[c]Yield based on lysoPC.

Phosphatidylcholine Synthesis

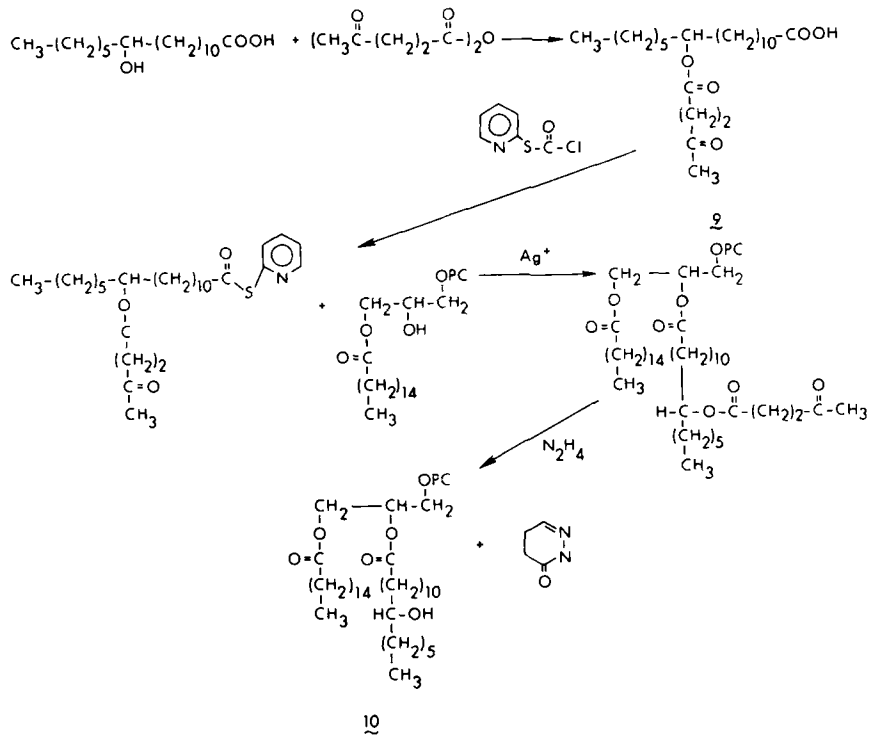
The yield and isomeric purity of 3 PC prepared by 2-thiopyridyl ester acylation of lysophosphatidylcholines are presented in Table 1. Yields are 90% or greater and the isomeric impurity present was comparable to that obtained by the method of Mason et al. (1). In fact, we have prepared both of the mixed-chain PC reported in Table 1 by acylation of lysoPC with acid anhydride in the presence of 4-pyrrolidine-pyridine catalyst, and the isomeric purity obtained by the 2-thiopyridyl ester method was slightly better in each case than that obtained by the acid anhydride method. Yields reported here are for material purified by normal-phase HPLC. If PC of highest purity are required, we recommend additional reverse-phase HPLC chromatography as described in the Experimental section.

1-Palmitoyl-2-(12-hydroxystearoyl)-glycerophosphocholine, 10

Several approaches to the synthesis of PC having acyl groups containing hydroxyl functions were attempted. Silyl protecting groups for the hydroxyl (*t*-butyldimethylsilyl) were utilized, but the best protecting group found for the free hydroxyl was the levulinate ester (11,12). While silyl protected hydroxy fatty acid derivatives could be used to acylate lysoPC, we found removal of the *t*-butyldimethyl silyl group to lead to impurities that could not be removed even by reverse-phase HPLC. The preferred synthetic approach to hydroxy fatty acyl phosphatidylcholines is illustrated in Scheme 2. Protection and removal of the protecting group are easy and convenient, and yields for the overall sequence of acylation with protected hydroxy fatty acid and deprotection with hydrazine were on the order of 50%. Isomeric impurity in lecithin synthesized by the route in Scheme 2 was 1.3%. The levulinate protecting group could also be used with the acid anhydride method of PC synthesis. That is, the levulinate ester of 12-hydroxystearic acid could be converted to its anhydride and used to acylate lysoPC. This acylation appeared to be unusually slow, however, and overall yields for the acylation-deprotection sequence were on the order of 30%.

Hydroxy fatty acid phosphatidylcholines have been isolated from natural sources (15,16), but the chemistry and biochemistry of these novel species have not been extensively explored. One might anticipate that these compounds would have unusual effects related to membrane structure and ion transport. The methods reported here for synthesis of PC and hydroxy-PC thus provide compounds of unique and novel structure for further study.

A comparison of the acyl anhydride and 2-thiopyridyl ester methods of PC synthesis should be made at this point. The 2-thiopyridyl ester method is fast and efficient with product isomeric purity equivalent to the acid anhydride method. In the case of precious or slow reacting acid anhydride acylating groups, the 2-thiopyridyl method appears to have some advantages. A great excess of thiopyridyl ester is not required and valuable fatty acid is not used as the leaving group. Yields of the levulinate derivatives described here were better than the anhydride method. The one major disadvantage noted thus far for the 2-thiopyridyl ester method is the great sensitivity to moisture. This detracts from the convenience of the method. Nevertheless, this new method is significantly



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different from current methodology and it thus provides a potentially useful alternative to existing procedures.

ACKNOWLEDGMENTS

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COMMUNICATIONS

Waxes and Volatile Oils in *Hypericum ericoides* (Guttiferae)

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ABSTRACT

A wax isolated from an hexane extract of *Hypericum ericoides* L. was shown to contain only an aldehydic and an alcoholic fraction. The aldehydic fraction was found to be largely *n*-octacosanal (~ 77%), with minor amounts of other linear homologous aldehydes, whereas the alcoholic fraction was almost pure *n*-octacosanol. From the same hexane extract, a volatile oil was also isolated and studied by combined gas chromatography-mass spectrometry (GC-MS). Monoterpene, sesquiterpene and aliphatic straight chain (C_8-C_{11}) hydrocarbons, sesquiterpene alcohols and aliphatic long-chain acids (C_8, C_9, C_{10}, C_{11}, C_{12}, C_{14}, C_{16} and C_{18}) were found. Biogenetic relationships are discussed.
Lipids 18:439-442, 1983.

INTRODUCTION

Specimens of the genus *Hypericum* have been used for a long time in folk medicine (1,2). Modern studies have been focused on the activity of extracts of these plants against certain viruses and bacteria and on their possible applications as medicines for various diseases (1,2). Mathis and Ourisson have published a detailed investigation of the distribution of essential oils (3-5) and hypericin (6) in *Hypericum* species. Hypericin has been found responsible for the phototoxicity experimented by cattle which have ingested aerial parts of these plants (7). Phenolic compounds are also amply represented in the genus. We have recently isolated several xanthonoids and flavonoids from *Hypericum ericoides* (1,2,8,9) and *H. balearicum* (10). We wish now to report the results of investigations on the hexane extract of *H. ericoides*, which contained several aliphatic straight chain hydrocarbons, alcohols, aldehydes and carboxylic acids, as well as some monoterpene and sesquiterpene compounds.

MATERIALS AND METHODS

Aerial parts (stems, leaves and flowers) of *H. ericoides* L. (ca. 8 kg) were air-dried and extracted with hexane. By steam-distillation of the extract, essential oils (ca. 65 g, ~12% of the whole extract) were separated. The residue was dissolved in hot hexane (2 l) and cooled to room temperature. A waxy solid (ca. 420 g, ~80% of the whole extract) precipitated out.

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An aliquot of the wax was chromatographed on silica gel (Merck G, Type 60) and eluted with hexane/benzene mixtures. Hexane/benzene (4:1, v/v), eluted an aldehydic fraction A (ca. 55%) and hexane/benzene (1:4, v/v) eluted an alcoholic fraction B (ca. 35%). Other more polar minor components (ca. 10%) were not investigated. The wax did not contain any carboxylic acids in detectable proportion, as inferred from an infrared (IR) spectrum.

The essential oil was subdivided into a neutral fraction C (ca. 96%) and an acid fraction D (ca. 4%) by repeated extraction of an ethereal solution with 5% aqueous NaOH. The neutral fraction C was also subdivided into a hydrocarbon fraction C_1 (ca. 60%) and an oxygenated fraction C_2 (ca. 40%) by column chromatography on silica gel and elution with hexane and ether, respectively. The hydrocarbon fraction was separated into mono- and sesquiterpenes by fractional distillation in vacuo. The oxygenated fraction contained only sesquiterpenes.

Gas chromatographic (GC) analyses were performed on a gas chromatograph with flame ionization detector, using a 2 m × 3 mm column, packed with 2% SE-30 on Chromosorb W/AW-DMCS. For the GC-MS analyses, a 100 m × 0.02 in. id capillary column, packed with SF-96, was used. For the hydrocarbon fraction of the volatile oil, an initial oven temperature of 80 C was held for 12 min and then a heating rate of 1 C/min was employed (final temperature 200 C).

The mass spectrometer was a Hitachi-Perkin Elmer RMU-6L apparatus, operating at 70 eV. ^{1}H NMR spectra were recorded at 90 MHz in a R-32

Perkin Elmer apparatus.

RESULTS

Fraction A, isolated from the wax by column chromatography, was a white solid melting in the interval 65-67 C. It formed a crystalline derivative with 2,4-dinitrophenylhydrazine and showed characteristic IR bands for an aliphatic aldehyde: 2720(w) and 1720(ms) cm^{-1}. A weak unresolved signal at δ 9.3 was also visible in the ^1H NMR spectrum ($CDCl_3$), which had typical features for a fully saturated, straight chain aldehyde. The mass spectrum gave the highest mass peak at m/e 408, consistent with a molecular formula $C_{28}H_{56}O$, as required by *n*-octacosanal, but the somewhat low melting point led us to suspect the presence of a mixture of homologous compounds. To clarify this point, we oxidized the product with Jones reagent (CrO_3) and methylated the oxidation product with diazomethane. The resulting methyl esters were analyzed by GC and the retention times compared with those of authentic samples. It can be seen (Table 1) that methyl *n*-octacosanoate is clearly the most abundant component and, hence, *n*-octacosanal is the major component in the original aldehyde mixture. No olefinic components were found.

Fraction B was a white crystalline solid, mp 80-81 C, with characteristic IR bands for a saturated long-chain alcohol: 3340 (broad) 2920, 2840, 1460, 1060, 730 cm^{-1}. The mass spectrum was consistent with that of *n*-octacosanol, the highest mass peak being at m/e 392 ($M^+ - H_2O$). This was confirmed by CrO_3 oxidation and methylation as above, giving practically pure methyl *n*-octacosanoate as evidenced by GC. Literature mp for *n*-octacosanol: 83-84 C (11), 80-81 C (12). By the usual methods, an acetate, mp 66-67 C, lit. 65-66 C (11), 65-68 C (12); a benzoate, mp 61-62 C, lit. 61-64 C (12) and a phenylurethane, mp 91-92 C, were also prepared.

TABLE 1

Fatty Aldehydes[a] of the Wax

Component[b]	%	Component[b]	%
C_{15}	1.0	C_{21}	0.3
C_{16}	3.6	C_{22}	7.4
C_{17}	3.2	C_{23}	0.2
C_{18}	3.8	C_{24}	0.3
C_{19}	2.0	C_{28}	77.0
C_{20}	1.2		

[a]Analyzed as the methyl esters of the corresponding carboxylic acids.

[b]Carbon number of the chain: only saturated straight chain isomers were found.

All derivatives gave the expected combustion analyses (13). For the not previously reported phenylurethane, the following values were found: C, 79.77; H, 12.00; N, 2.58. Calc. for $C_{35}H_{63}NO_2$: C, 79.40; H, 11.91; N, 2.65. Typical IR bands (KBr pellet) were found at 3300, 1700, 1590, 1525, 1225, 745, 720 and 690 cm^{-1}.

The components of the monoterpene hydrocarbon fraction were analyzed by GC-MS as described in the Materials and Methods section. Reference spectra (14-19) and, in most cases, comparisons with authentic samples were utilized for structure attributions (Table 2). As can be seen, some aliphatic nonterpenic hydrocarbons were also found in this fraction. The sesquiterpene hydrocarbon fraction was analyzed by an identical procedure, the results being also presented in Table 2.

The composition of the oxygenated fraction C_2 was rather simple: only two major and two minor peaks were detected by GC. Only one of the major components could be identified as β-eudesmol by GC and MS comparison with an authentic sample.

The acid fraction D was analyzed by methylation and GC-MS analysis of the methyl esters: benzoic acid and the saturated *n*-alkanoic C_8, C_9, C_{10}, C_{11}, C_{12}, C_{14}, C_{16} and C_{18} acids were unequivocally determined by comparison with authentic samples. C_{12} and C_{16} were the predominant components in the mixture.

DISCUSSION

Our results might be helpful for providing chemotaxonomic relationships between species in a yet not completely known genus *Hypericum*. A wide spectrum of natural products is known to be biosynthesized from carboxylic acids (20,21); linear hydrocarbons originate usually by enzymatic decarboxylation of carboxylic acids, whereas aldehydes and alcohols are formed from carboxylic acids by reduction. Other secondary transformations include dehydrogenations, hydroxylations, epoxydations, etc., of the aliphatic chain. All these metabolites are involved in the biogenesis of cutin and suberin, two noncarbohydrate polymers associated with wood and plant cuticles (22). We found only small amounts of low molecular weight (C_8-C_{11}) hydrocarbons and low to medium molecular weight (C_8-C_{18}) carboxylic acids in the steam volatile fraction, but great amounts of predominantly high molecular weight, nonvolatile aldehydes and alcohols. It would appear that the enzymatic system which reduces carboxylic acids to aldehydes and alcohols works efficiently only with high molecular weight components. No low molecular weight aldehydes were found in our studies in contrast to other *Hypericum* species (3-5). Moreover, the enzymatic decarboxylation

TABLE 2

Fatty and Terpene Hydrocarbons in the Essential Oil

Component	Relative retention times (to *n*-octane)	Abundance[a]	Reference spectra	Comparison[b]
n-Octane[c]	1	T	16	+
n-Nonane[c]	1.44	L	16	+
n-Decane[c]	2.26	L	16	+
n-Undecane[c]	3.62	L	16	+
	Monoterpenes			
α-Pinene	1.67	M	16, 17	+
β-Pinene	2.01	L	16, 17	+
Limonene	2.57	L	16, 17	+
	Sesquiterpenes			
α-Copaene	6.73	L	14	+
Caryophyllene	7.05	L	14, 15	+
γ-Muurolene	7.48	M	14	+
α-Curcumene	7.75	H	14	−
Calamenene	7.85	L	18, 19	+
δ-Cadinene	7.89	M	14, 15 18, 19	+
α-Muurolene	8.03	L	14, 15	+
Calacorene	8.03	L	18	−

[a]Letters indicate approximate relative abundances: H,high (10-20%); M,medium (5-10%); L,low (1-5%); T,trace (<1%).

[b]The sign (+) indicates direct comparison (GC and MS) with an authentic sample. The sign (−) indicates comparison only with literature mass spectra.

[c]Trace amounts of 5 other branched C_9-C_{10} saturated hydrocarbons were also found but they could not be unequivocally identified.

system seems not to be very efficient, working only to a small extent on low molecular weight acids (C_9-C_{12}). The reduction to alcohols works apparently only with the C_{28} component, a most interesting finding. Mathis and Ourisson (23) had already shown the presence of mixtures of the C_{24}, C_{26} and C_{28} alcohols in the wax of *H. perforatum*. Also of interest are the predominance of pinenes in the monoterpene hydrocarbon fraction, the practical absence of monoterpene alcohols and the low concentration (trace amounts) of branched (unidentified) hydrocarbons. Mathis and Ourisson (3-5) pointed out that specimens of the Euhypericum section (to which *H. ericoides* belongs) growing in dry climatic conditions afford essential oils with clear predominance of pinenes in the monoterpene hydrocarbon fraction. The oil from species of that section is usually rich in sesquiterpenes but poor in monoterpene alcohols. This is also the case here, the sesquiterpene fraction being of specially varied composition with the most represented carbon frameworks of the bisabolene and cadinene types. Caryophyllene and α-copaene are also present but not humulene (3-5). All these components, with the exception of caryophyllene and β-eudesmol, have been said to be biogenetically derived (24) from *cis,trans*-farnesyl pyrophosphate by appropriate cyclization.

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Stimulation of Bile Acid Synthesis by Dibutyryl Cyclic AMP in Isolated Rat Hepatocytes

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ABSTRACT

Freshly isolated rat hepatocytes were used to examine the effects of dibutyryl cyclic AMP on the incorporation of ^{14}C-acetate and ^{14}C-cholesterol into bile acids. After an initial lag period, both precursors were incorporated into cholic and chenodeoxycholic acids at a linear rate for the subsequent 60 min. An apparent stimulation of bile acid formation from ^{14}C-acetate by dibutyryl cyclic AMP was complicated by the concomitant inhibition of cholesterol synthesis. In experiments with ^{14}C-cholesterol, dibutyryl cyclic AMP (1 mM) increased the labeled cholic and chenodeoxycholic acids in the medium by 83 and 224%, respectively, but cellular levels of labeled bile acids were unchanged. As a result, the nucleotide stimulated the overall incorporation of ^{14}C-cholesterol into cholic acid by 39% and into chenodeoxycholic acid by 123%. The mean ratio of labeled cholic to chenodeoxycholic acid declined from 55:45 in control cells to 41:59 in cells incubated with dibutyryl cyclic AMP. The results demonstrate that label incorporation can be used to study the regulation of bile acid synthesis in isolated hepatocytes. We propose that dibutyryl cyclic AMP enhances bile acid production by phosphorylating, and thus stimulating the activity of, cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis.
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INTRODUCTION

Extensive studies have characterized the pathways for bile acid synthesis (1), and several factors are known to exert long-term effects on the formation of bile acid. For example, biliary diversion and cholestyramine feeding enhance the production of bile acids, probably because the reduced pool of circulating bile acids increases the synthesis of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis (2). Relatively little is known about the short-term regulation of bile acid synthesis, although Mitropoulos et al. showed that the administration of cholesterol or mevalonate to rats rapidly increased bile acid synthesis (3).

Most studies on the control of bile acid synthesis have utilized intact animals, perfused liver or liver slices. Isolated hepatocytes, which have provided considerable information on the regulation of other metabolic pathways in the liver (4-6), have been used by a few workers to investigate bile acid synthesis (7-11). However, these prior experiments with hepatocytes have not examined the incorporation of labeled precursors into bile acids and have provided little insight into factors that exert rapid effects on bile acid synthesis. The present study demonstrates that dibutyryl cyclic AMP (Bt_2cAMP) stimulates the incorporation of ^{14}C-acetate or ^{14}C-cholesterol into bile acids by isolated rat hepatocytes. A brief account of this work has been presented (12).

MATERIALS AND METHODS

^{14}C-Acetate (45-60 mCi/mmol) and ^{14}C-cholesterol (59.4 mCi/mmol) were obtained from New England Nuclear, Inc. Purified bile acids were purchased from Applied Science Laboratories, Inc. Dibutyryl 3',5'-cyclic AMP, monosodium salt, was bought from Sigma Chemical Co. Cholylglycine hydrolase (4000-5000 units/mg protein) was supplied by Dr. P.P. Nair, Sinai Hospital, Baltimore, MD.

Isolation of Hepatocytes

Fed, male, Sprague-Dawley rats, weighing about 125 g when purchased, were used for the isolation of hepatocytes after 1 wk of adaptation to altered lighting conditions (dark cycle from 4 AM to 4 PM). Rats had access to Purina rat chow and water, ad libitum. Hepatocytes were prepared between 10 and 11:30 AM by our published method (13) with the exception that the perfusate, a modified Krebs-Ringer bicarbonate buffer, was Ca^{2+}-free and contained 20% (v/v) steer red blood cells which were washed 5 times with 0.9% NaCl solution. The isolated hepatocytes were washed 3 times with modified Krebs-Ringer bicarbonate buffer containing Ca^{2+} (2.4 mM) and 1% bovine serum albumin (incubation buffer). Cell viability was assessed by dye exclusion as previously described (13). Hepatocytes with damaged cell membranes were counted as nonviable even if they excluded the dye. Cell viability, initially 90 to 95%, fell to 85 to 90% after 3 hr of incubation.

Incubation Conditions

Freshly isolated hepatocytes (3 to 6 million) were suspended in 3 ml of incubation buffer containing 1

μCi of ^{14}C-acetate or ^{14}C-cholesterol (added in 10 μl of ethanol) that was freshly purified by thin layer chromatography (TLC) using benzene/acetone (1:1). Incubations were carried out in 25 ml siliconized plastic flasks which were shaken at 120 oscillations per min in a water bath shaker at 37 C under an atmosphere of 95% O_2:5% CO_2. Cells were separated from the incubation medium by centrifugation for 5 min at 920 g at room temperature in an International table top centrifuge. Each experiment, done in triplicate, included a zero time incubation, where cells and medium were separated at 0 C immediately following the addition of label. Incorporation into bile acids was calculated by subtracting zero time control values from those obtained after a period of incubation.

Isolation of Bile Acids

Bile acids were isolated separately from the cells and incubation medium. Cell pellets were sonicated after suspension in 0.5 ml water at pH 11. The cell sonicate or medium (at pH 11.0) was heated for 15 min in a boiling water bath, cooled, and extracted 3 times with redistilled diethyl ether (5 ml) to remove neutral lipids and most of the ^{14}C-cholesterol. With ^{14}C-acetate as substrate, the heating step was omitted. The pooled ether extracts were washed once with 1 ml water at pH 11.0, and the water wash was added to the aqueous solution. The volume and pH of the medium or cell sonicate were adjusted to 3 ml and to pH 7 to 7.4, respectively. Three ml of acetate buffer (0.1 M, pH 5.6) was added, and the pH of the 6 ml mixture was checked to be between 5.6 and 5.8. The addition of 1.5 ml of 100 mM β-mercaptoethanol and 1.5 ml of 60 mM disodium EDTA (both in 0.1 M acetate buffer, pH 5.6) was followed by adding 0.1 ml cholylglycine hydrolase. After the mixture was incubated at 37 C for 2 hr with gentle shaking, the pH was adjusted to 1, cholic and chenodeoxycholic acids (20 μg of each) were added as carriers, and the mixture was extracted 3 times with 5 ml of ethyl acetate. The ethyl acetate extracts were dried with anhydrous sodium sulfate and evaporated to dryness under nitrogen. 0.4 ml methanol and 0.2 ml saturated methanolic HCl were added to the residue, and the mixture was incubated overnight at room temperature. The solvents were evaporated under nitrogen. The bile acid methyl esters were applied to a prewashed Silica Gel G plate and separated by successive development in the solvent systems benzene/acetone (95:5) and benzene/acetone (1:1) to separate the methylated bile acids from each other and from any radioactive cholesterol or oxidation products of cholesterol formed during the incubation or extraction steps. Cholesterol moved to the solvent front and the R_f's for cholic and chenodeoxycholic acids were 0.19 and 0.50, respectively. Bile acid spots were identified under UV light after spraying with 8-hydroxy-1,3,6-pyrenetrisulfonic acid. Each individual bile acid zone was scraped from the plate into a scintillation vial and counted for radioactivity in 10 ml of Betaflour in a liquid scintillation counter with a counting efficiency of 67% for ^{14}C.

Validation of Measurement of Labeled Bile Acids

The conditions utilized to isolate the primary bile acids are similar to those reported by several investigators for the isolation of bile acids from biological tissues and fluids (14). The efficiency of bile acid recovery by our method was determined by adding ^{14}C-taurochenodeoxycholic acid or ^{14}C-taurocholic acid to an incubation flask with suspended hepatocytes, immediately separating cells from incubation medium and carrying out our standard procedure for the quantitation of label in bile acids from the medium. We recovered 76% and 70% of the label in the chenodeoxycholic and cholic acid spots, respectively.

We also carried out experiments to determine the amount of label contaminating the spots identified as cholic and chenodeoxycholic acids on TLC plates. Hepatocytes were incubated with labeled cholesterol; bile acids extracted from the medium and pure radioactive bile acid standards were methylated and isolated from the TLC plates after development in our system. After the radioactivity was counted in aliquots, the remainder was reapplied on another TLC plate and developed in ethyl acetate/acetone (70:30). Bile acid spots were isolated and counted. Between 80 and 95% of the label applied to the second TLC plate was recovered in spots with R_f values identical to that of the labeled bile acid standards.

RESULTS

After an initial lag period of 30 min, the rate of incorporation of ^{14}C-cholesterol into medium bile acids (Fig. 1) and into total (cells plus medium) bile acids (data not shown) was linear for the next 60 min. A similar time course was observed for the incorporation of ^{14}C-acetate into medium bile acids. In experiments with ^{14}C-cholesterol, the cell sonicate contained about 20% of the radioactivity recovered in bile acids from medium and cells. When the amount of free bile acids was estimated by omitting the usual treatment with cholylglycine hydrolase, conjugated bile acids accounted for about 90% of the labeled bile acids in the incubation medium. These results suggest that both labeled acetate and cholesterol are suitable precursors for studies of the synthesis of bile acids in isolated rat hepatocytes.

Studies on the regulation of bile acid synthesis by Bt_2cAMP were initially carried out with ^{14}C-

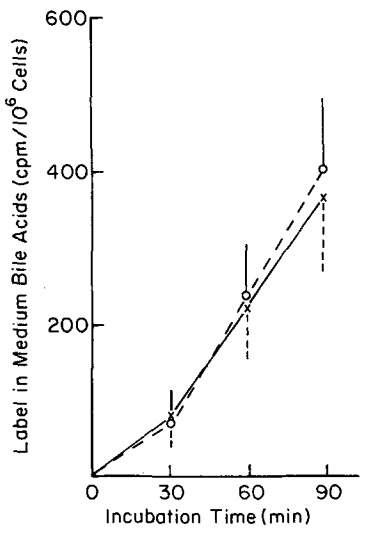

FIG. 1. Representative time course for ^{14}C-cholesterol incorporation into medium bile acids. Hepatocytes (6 million) were incubated in 3 ml of buffer with 1 μCi of label for various periods. X——X, chenodeoxycholic acid; O----O, cholic acid. The points in the figure represent mean ± S.E.M. from 3 experiments.

acetate. Such experiments are complicated by the fact that Bt$_2$cAMP inhibited cholesterol synthesis by about 25% (6). To correct for this problem, the effects of Bt$_2$cAMP were determined by comparing the radioactivity in secreted bile acids as a percent of total radioactivity in cholesterol plus bile acids in the presence and absence of the cyclic nucleotide. Bt$_2$cAMP more than doubled the percentage of label incorporated into the bile acids recovered in the incubation medium. In 3 experiments, the percentage label (mean ± standard error of mean or S.E.M.) in cholic acid was 2.2 ± 0.7 in control cells and 5.4 ± 0.9 in cells incubated with Bt$_2$cAMP. Incorporation into chenodeoxycholic acid was 8.3 ± 1.3 with Bt$_2$cAMP compared with 3.2 ± 1.0 in control cells. Since incorporation into cellular bile acids was not measured in these experiments, the results do not distinguish whether Bt$_2$cAMP enhanced the overall synthesis or only the secretion of bile acids. It is noteworthy that bile acids were a quantitatively significant product from ^{14}C-acetate, particularly in the presence of Bt$_2$cAMP where the secreted bile acids accounted for almost 15% of the radioactivity incorporated into cholesterol.

Because of the difficulties in interpreting the effects of Bt$_2$cAMP in experiments with ^{14}C-acetate, subsequent studies were carried out with labeled cholesterol. As shown in Table 1, 1 mM Bt$_2$cAMP stimulated the incorporation of ^{14}C-cholesterol into cholic and chenodeoxycholic acids in the incubation medium by 83 and 224%, respectively. Bt$_2$cAMP had no effect on the amount of labeled bile acid recovered from the cells. As a result, Bt$_2$cAMP produced a statistically significant increase ($p < 0.01$) in the incorporation of ^{14}C-cholesterol into cholic and chenodeoxycholic acids by 39 and 123%, respectively. Sodium butyrate (1 mM) had no effect on bile acid synthesis from either precursor. Bt$_2$cAMP had a relatively greater stimulatory effect on the formation of chenodeoxycholic acid compared with cholic acid. Thus, in 12 experiments, the ratio of labeled cholate: labeled chenodeoxycholate was 55:45 in control cells but 41:59 in cells incubated with Bt$_2$cAMP. This difference is significant with $p < 0.05$ by Student's two-tailed "t" test.

TABLE 1

Effect of Bt$_2$ cAMP on the Incorporation of ^{14}C-Cholesterol into Cholic and Chenodeoxycholic Acids

	Label Incorporation, as percent of Control	
	Cholic acid	Chenodeoxycholic acid
Incubation medium	183 ± 29*	324 ± 34*
Cell sonicate	88 ± 20	96 ± 11
Cells plus medium	139 ± 19*	223 ± 31*

Hepatocytes (3 to 6 million) were incubated for 1 hr with 1 μCi of ^{14}C-cholesterol in the presence and absence of 1 mM Bt$_2$cAMP. Counts recovered in each bile acid are expressed as the percent of the corresponding controls (incubated in the absence of Bt$_2$cAMP), taken as 100%. In the control incubations, radioactivity was 288 ± 88 cpm/10^6 cells in cholic acid and 251 ± 69 cpm/10^6 cells in chenodeoxycholic acid in the incubation medium; label was 74 ± 21 cpm/10^6 cells in cholic acid and 65 ± 15 cpm/10^6 cells in chenodeoxycholic acid in the cell sonicates. The results are the mean ± S.E.M. from 12 different experiments.

*Value statistically different from control at $p < 0.01$ by student's t test.

DISCUSSION

Two studies (7,8) that reported the synthesis of bile acids in rat hepatocytes utilized an enzymatic assay with hydroxysteroid dehydrogenase to quantitate the amount of bile acids secreted into the medium. Other workers (9,15) have questioned the validity of results obtained with this assay procedure. Furthermore, the fact that bile acid synthesis required supplementation of the incubation medium with Krebs cycle intermediates, such as succinate, suggest that damaged cells were used in one (8) of these studies, since hepatocytes with intact plasma membranes are considered impermeable to succinate (16). Additionally, Whiting and Edwards (9) suggested that the reported (8) stimulation of secretion by succinate could be from dehydrogenase activity unrelated to bile acid levels. Two studies (9,10) utilized gas liquid chromatographic techniques to measure bile acid synthesis in combined extracts from cells and medium. Both showed

that cholic acid was the major bile acid produced. One of these studies (10) used cultured hepatocytes and heavily fortified incubation medium. Recently, Botham et al. (11) reported the use of radioimmunoassay techniques to measure the amount of conjugated bile acids secreted into the incubation medium. Our findings demonstrate the incorporation of labeled precursors into bile acids from a Krebs-Ringer buffer containing no special additions.

Initial experiments with ^{14}C-acetate as precursor showed that Bt_2cAMP increased the relative incorporation of label into medium bile acids. Since simultaneous inhibition of cholesterol synthesis by Bt_2cAMP complicated the interpretation of these experiments, subsequent studies were carried out with ^{14}C-cholesterol. As shown in Table I, Bt_2cAMP also produced a significant stimulation of the incorporation of ^{14}C-cholesterol into both cholic and chenodeoxycholic acids.

Mere demonstration of increased incorporation of labeled precursors into bile acids by the addition of Bt_2cAMP can not be construed as proof that this agent stimulates the synthesis of bile acids. Thus, the cyclic nucleotide may enhance the cellular uptake of labeled precursors, increase their specific activity in a critical intracellular pool, or facilitate the transfer of cholesterol to cholesterol 7α-hydroxylase. We have found, however, that Bt_2cAMP does not alter the uptake of labeled acetate or cholesterol from the incubation medium (data not shown). Since Bt_2cAMP inhibited acetate incorporation into cholesterol in the present and previous studies (6), while stimulating the incorporation of ^{14}C-acetate into bile acids, it is highly unlikely that Bt_2cAMP exerts both actions by altering the specific activity of the labeled acetate available to the microsomal enzymes which are rate-limiting for both biosynthetic pathways. The possibility that Bt_2cAMP facilitates the transfer of cholesterol to 7α-hydroxylase can not be dismissed. On the other hand, it should be pointed out that Bt_2cAMP enhanced the formation of bile acids both from cholesterol newly synthesized from labeled acetate and from endogenous ^{14}C-cholesterol taken up by the cell. Taken together, these considerations strongly suggest that the observed effects of Bt_2cAMP on the incorporation of labeled precursors does result from a stimulation of bile acid synthesis.

Earlier experiments, which showed that Bt_2cAMP inhibited ^{14}C-acetate incorporation into cholesterol by isolated rat hepatocytes (6), stimulated studies on the regulation of HMG CoA reductase. Elegant work has now shown that the activity of HMG CoA reductase is inhibited by covalent phosphorylation of the enzyme and stimulated by removal of phosphate groups (17). This was the first example of control of the activity of a microsomal enzyme by altering its phosphorylation state. Recent reports have provided evidence for activation of two other microsomal enzymes, glucose-6-phosphate, and acyl CoA: cholesterol acyl transferase, by covalent phosphorylation (18,19). Studies by Sanghvi et al. (20), and in our laboratory (21), have shown that treatment of rat liver microsomes with alkaline phosphatase inhibits the activity of cholesterol 7α-hydroxylase; enzyme activity can be partially or completely restored by subsequent incubation of the microsomes with MgATP, cAMP, and protein kinase. Based on these observations, we propose that Bt_2cAMP enhances overall bile acid synthesis in isolated rat hepatocytes by stimulating a cAMP-dependent protein kinase to phosphorylate and activate cholesterol 7α-hydroxylase. It is, of course, possible that other mechanisms may also play a role in the effects of Bt_2cAMP since the activities of both HMG CoA reductase (22,23) and cholesterol 7α-hydroxylase (24,25) are modulated by cytosolic proteins in the absence of added ATP. The fact that Bt_2cAMP stimulated label incorporation into bile acids in the incubation medium, but not within cells, suggests that Bt_2cAMP may also increase the conjugation or secretion of bile acids, or both, but no studies have been carried out to test this possibility.

Little is known concerning the factors that determine the relative rates of synthesis of cholic and chenodeoxycholic acids. We have no explanation for the relatively greater synthesis of chenodeoxycholate from labeled cholesterol, and particularly from ^{14}C-acetate, when the results of the present study are compared with those of two workers (10,11). Amuro et al. (26) have recently reported that human hepatoblastoma cells in culture predominantly synthesized chenodeoxycholic acid. They did not explain the relatively greater synthesis of chenodeoxycholic acid. It is possible that we are measuring another co-migrating dihydroxy bile acid such as muricholic acid along with chenodeoxycholic acid. Treatment of rats with thyroid hormone, which stimulates bile acid synthesis, increased the biliary ratio of chenodeoxycholic to cholic acid (27) in a manner similar to the effects of Bt_2cAMP reported here. Botham et al. (11) showed that the addition of high concentrations of 7α-hydroxycholesterol to isolated rat hepatocytes stimulated the synthesis of chenodeoxycholate but inhibited cholate synthesis. They proposed that high concentrations of 7α-hydroxycholesterol inhibits 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one, the last intermediate common to both cholic and chenodeoxycholic acids. It is possible that the increased rate of bile acid synthesis produced by Bt_2cAMP leads to an accumulation of 7α-hydroxycholesterol which in-

hibits 12α-hydroxylase. Reduction of 12α-hydroxylase activity by either mechanism would decrease the synthesis of 5β-cholestan-3α,7α-diol, the precursor of chenodeoxycholic acid (28) accounting for the findings in the present study.

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Effects of Triarimol and Tridemorph on Sterol Biosynthesis in *Saprolegnia ferax*[1]

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ABSTRACT

The effects of two fungicides, triarimol and tridemorph, on sterol biosynthesis in *Saprolegnia ferax* were examined. Cultures grown in the presence of triarimol accumulated lanosterol. Tridemorph-treated cultures accumulated Δ^8-sterols including zymosterol, fecosterol and stigmasta-8-24(28)-dienol. The latter is a new sterol. A proposed scheme is given for sterol biosynthesis in *S. ferax* showing points of inhibition of triarimol and tridemorph. Results point to the intermediacy of lanosterol but not cycloartenol in sterol synthesis in *Saprolegnia*.

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INTRODUCTION

The Oomycetes are almost universally recognized as having characteristics which set them apart phylogenetically from other fungi. Their cell walls contain cellulose I instead of, or in addition to, chitin (1,2), they have a diploid vegetative thallus (3-7), and they have the α,ϵ-diaminopimelic (DAP) pathway for lysine biosynthesis (8).

The sterol composition of the Oomycetes is also unusual. Most fungi contain ergosterol (ergosta-5,7,22-trien-3β-ol) as the dominant sterol (9,10). Members of two orders of Oomycetes have been studied and their major sterols are cholesterol (cholest-5-en-3β-ol), 24-methylenecholesterol (ergosta-5,24(28)-dien-3β-ol), desmosterol (cholesta-5,24-dien-3β-ol) and fucosterol (stigmasta-5,E-24(28)-dien-3β-ol) (11). The only notable exception to this is *Zoophagus insidians*. It has been reported as having ergosterol as its dominant sterol (12).

In the biosynthesis of sterols, there is a bifurcation at the cyclization of epoxysqualene (13,14). Photosynthetic organisms produce cycloartenol (9β, 19-cyclo-5α-lanost-24-en-3β-ol) and nonphotosynthetic organisms, including fungi, produce lanosterol (5α-lanosta-8,24-dien-3β-ol) as the first cyclic intermediate (10,15). There are some notable exceptions to this generalization. Several nonphotosynthetic plants with close phylogenetic ties to photosynthetic plants do utilize the cycloartenol pathway (16,17).

Although cycloartenol has never been isolated from any fungus, there is some speculation regarding its role in Oomycete sterol biosynthesis. *Saprolegnia ferax* can incorporate tritium from either cycloartenol or lanosterol into its normal sterols (18). Inhibition of sterol biosynthesis soon after epoxysqualene cyclization should provide good evidence on the intermediates in this part of their sterol biosynthetic pathway. Triarimol [α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidine-methanol] was chosen because one of its sites of inhibition is 14α-demethylation (19-23). The other inhibitor used was tridemorph (2,6-dimethyl-N-tridecylmorpholine), which is known to inhibit opening of the 9β,19-cyclopropane ring in organisms utilizing the cyloartenol pathway (24).

MATERIALS AND METHODS

S. ferax ATCC. 36051 was obtained from American Type Culture Collection and maintained on PYG agar. Cultures were grown at 25 C on PYG (Cantino) broth, PYG broth plus 0.5% methanol, and PYG broth plus 0.5% ethanol for two weeks. Triarimol in methanol was added to flasks after autoclaving at a concentration of 10 mg per liter of media. Tridemorph in ethanol was added to the flasks after autoclaving at a concentration of 10 mg per liter of media. Each experiment was performed in triplicate.

Sterols were isolated by our usual methods, utilizing alumina and silica gel thin layer chromatography (TLC) (25). Identification was based on gas liquid chromatography (GLC) (26) and GLC-mass spectrometry (GLC-MS) (27). Authentic standards of lanosterol, fecosterol (5α-ergosta-8,24(28)-dien-3β-ol), campest-8(14)-en-3β-ol, and stigmast-8(14)-en-3β-ol were used for comparative purposes in GLC and GLC-MS analyses.

Electron impact (70 ev) mass spectra were obtained from a Finnigan-MAT model 4500 mass spectrometer which was operated at a source block temperature of 120 C. Sample introduction was via a split injection onto a 15 m by 0.32 mm i.d. fused silica column coated with a 0.25 μm film of DB-1 (bonded methyl silicone). The column was operated at a temperature of 255 C and a head pressure of 4.5 PSI helium. Data were collected and processed on an Incos data system.

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To confirm their structures, two of the sterols (from tridemorph-inhibited cultures) that were not separable by column chromatography were subjected to infrared analyses. They were also treated in acetic acid with hydrogen for 4 hr in the presence of 10% palladium on charcoal and the products obtained from the reaction were analyzed by GLC and GLC-MS.

RESULTS AND DISCUSSION

Control cultures of *S. ferax* grown in PYG, in PYG plus methanol, and in PYG plus ethanol were identical in their sterol composition. *S. ferax* produces 24-methylenecholesterol, desmosterol, fucosterol, cholesterol and lanosterol (Table 1) and differs from the previous report (11) only in the presence of lanosterol. This could be due to a difference in isolation methods. Lanosterol and fucosterol are not separated by GLC on an SE-30 column. Lanosterol was only identified in the control after TLC on silica gel which separated sterols into dimethyl, monomethyl and desmethyl fractions (25).

GLC-MS of sterols from control cultures verified the structures which have been previously reported (11). Lanosterol was also verified by GLC-MS by comparison with two authentic samples. Its molecular ion was at m/z 426 (30%). Other intense peaks were at m/z 411(50%), 393(21%), 341(4%), 297(4%), 283(4%), 273(7%), 259(15%), 241(11%), 229(9%), 215(10%), 203(7%) and 201(8%).

Triarimol greatly inhibited sterol biosynthesis in *S. ferax* (Table 1). Only 0.07 μg of sterol per mg dry weight was produced in triarimol-treated mycelia, whereas the control had 2.01 μg sterol per mg dry weight. Interestingly, over 90% of the total sterol in the inhibited cultures was lanosterol.

The amount of sterol produced in tridemorph-inhibited cultures was ca. 2.5 times the amount of sterol found in control cultures. This enhancing effect of tridemorph has been previously noted (24). The sterols that accumulated were all Δ^8-sterols (Table 1) and our results support previous work on the site of tridemorph inhibition in fungi (28,29).

Zymosterol (5α-cholesta-8,24-dien-3β-01) gave the molecular ion at m/z 384(65%). Other prominent ion peaks were at m/z 369(39%), 351(7%), 271(17%), 246(16%), 231(20%), and 213(29%). A computer search through the library of spectra matched the spectrum of the sample with authentic zymosterol.

The molecular ion peak of fecosterol was at m/z 398(50%). Other intense peaks were at m/z 383 (27%), 365(6%), 299(8%), 271(39%), 257(11%), 213(27%). This mass spectrum matched the mass spectrum of fecosterol in the computer library as well as the mass spectrum of an authentic sample of fecosterol from our laboratory.

The final sterol analyzed by GLC-MS from the tridemorph-treated cultures had a molecular ion at m/z 412(50%). Other prominent peaks were at m/z 397(27%), 314 (characteristic of a sterol with a $\Delta^{24(28)}$ bond but not as intense as is usually observed, 7%), 273(8%), 271(20%), 246(12%), 231 (17%), and 213(22%). This compound was tentatively identified as stigmasta-8,24(28)-dienol and verified by other procedures.

Both fecosterol and stigmasta-8(9),24(28)-dienol gave relatively weak peaks at m/z 314. An intense peak was expected there due to the presence of the $\Delta^{24(28)}$-bonds (27). Presumably, the presence of a $\Delta^{8(9)}$-bond alters the configuration in such a way that the molecule doesn't readily fragment at the C(22)-C(23) position. Because of the uncertainty concerning the expected MS fragmentations of fecosterol and stigmasta-8(9),24(28)-dienol, a meticulous GLC analysis was undertaken.

A comparison was made between the relative retention times of various sterol standards to determine the effects of position of double bond and alkyl substituents on relative retention time. Three different gas chromatographic columns were

TABLE 1

A Comparison of Sterols from Control, Triarimol-treated and Tridemorph-treated Cultures of *Saprolegnia ferax*

	Control		Triarimol-treated		Tridemorph-treated	
Sterol	% Sample	μg/mg dry wt	% Sample	μg/mg dry wt	% Sample	μg/mg dry wt
Cholesterol	1.3	0.03	-	-	-	-
Desmosterol	36.5	0.73	3.4	tr	-	-
24-Methylene cholesterol	43.3	0.87	-	-	-	-
Fucosterol	11.8	0.24	5.8	tr	-	-
Lanosterol	7.0	0.14	90.8	0.06	0.5	0.02
Zymosterol	-	-	-	-	18.0	0.92
Fecosterol	-	-	-	-	55.3	2.83
Stigmasta-8,E-24(28)-dienol	-	-	-	-	26.3	1.35
Total	99.9	2.01	100	0.07	100.1	5.12

employed, SE-30, OV-17, and QF-1. The separation factors are presented in Table 2, and Table 3 shows the calculated RRT's of possible sterol structures based on GLC-MS data, according to previously published methods (26,30). These calculated RRT's were then compared with the actual RRT of the sample and a positive identification was made in each case.

The IR spectrum of any sterol with a terminal methylene group, i.e. $\Delta^{24(28)}$-bond shows a strong peak in the 885-895 cm^{-1} region (31). Both fecosterol and fecosterol acetate from tridemorph-treated cultures showed a very strong peak in the 892 cm^{-1} region. Thus, IR data supports the presence of a 24-methylene group.

Fecosterol and stigmasta-8,24(28)-dienol were converted with hydrogen in the presence of acetic acid and 10% palladium on charcoal into $\Delta^{8(14)}$-sterols with saturated side chains. In this reaction, the $\Delta^{8(9)}$-bonds were isomerized to $\Delta^{8(14)}$ and the $\Delta^{24(28)}$-bonds were reduced. The products of this reaction, ergost-8(14)-enol and stigmast-8(14)-enol, were then compared by GLC and GLC-MS with authentic sterol standards, campest-8(14)-enol and stigmast-8(14)-enol. The mass spectra and GLC analyses were identical.

Tridemorph inhibits opening of the cyclopropane ring in all plants studied which utilize the cycloartenol pathway (24; G. Hosokawa, unpublished), but inhibits $\Delta^8 \rightarrow \Delta^7$ isomerase in fungi using the

TABLE 2

Double Bond and Alkyl Substituent Separation Factors of Sterols

Factor	Gas chromatographic system[a]		
	SE-30	OV-17	QF-1
$\Delta^{8(9)}/\Delta^5$	1.041	1.058	1.042
$\Delta^{8(14)}/\Delta^5$	0.992	0.999	1.007
$\Delta^{24(25)}$	1.081	1.194	1.068
$\Delta^{25(27)}$	0.955	1.007	0.953
24-Methyl	1.278	1.290	1.274
24-Methylene	1.251	1.330	1.265
24-Ethyl	1.594	1.593	1.520
24-Ethylidene (E)	1.594	1.681	1.486

[a]3% SE-30 on Gas Chrom Q, 245 C, 20 psi; 3% QF-1 on Gas Chrom P, 231 C, 20 psi; 3% OV-17 on Gas Chrom Q, 260 C, 20 psi.

TABLE 3

A Comparison of the RRT's of Three Unknown Sterols from Tridemorph-treated Cultures with the Calculated RRT's of Possible Sterol Structures

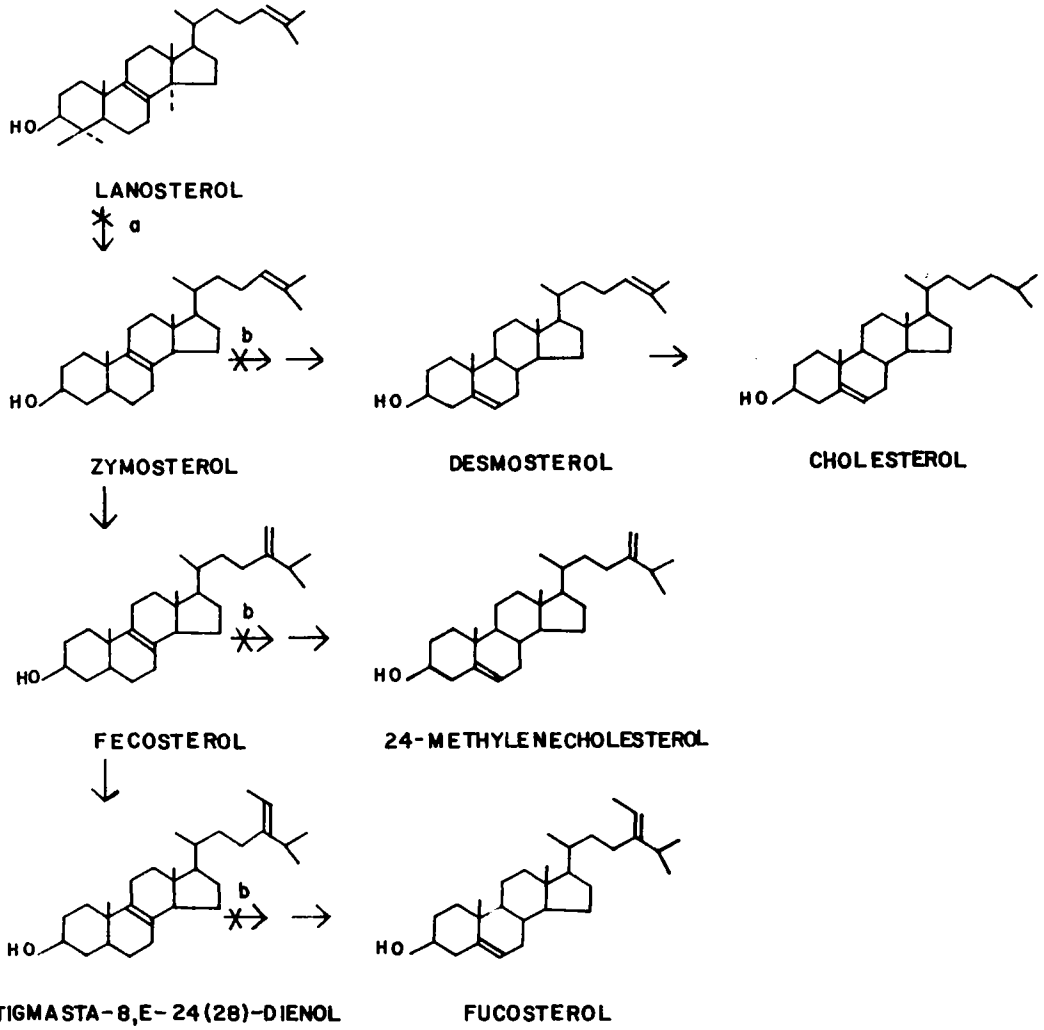
Free sterol	Gas chromatographic system		
	SE-30	OV-17	QF-1
C_{27} sterol	1.13	1.27	1.11
Zymosterol	(1.13)[a]	(1.26)	(1.11)
Cholesta-8,25-dienol	(0.99)	(1.07)	(0.99)
Cholesta-8(14),24(25)-dienol	(1.07)	(1.19)	(1.08)
Cholesta-8(14),25-dienol	(0.95)	(1.01)	(0.96)
C_{28} sterol	1.31	1.42	1.33
Fecosterol	(1.30)	(1.41)	(1.32)
Ergosta-8,25-dienol	(1.27)	(1.37)	(1.27)
Ergosta-8(14),24(28)-dienol	(1.24)	(1.33)	(1.27)
Ergosta-8(14),25-dienol	(1.21)	(1.30)	(1.22)
C_{29} sterol	1.65	1.79	1.55
Stigmasta-8,24(28)-dienol	(1.66)	(1.78)	(1.55)
Stigmasta-8,25-dienol	(1.59)	(1.70)	(1.51)
Stigmasta-8(14),24(28)-dienol	(1.58)	(1.68)	(1.50)
Stigmasta-8(14),25-dienol	(1.51)	(1.60)	(1.46)

[a]Values in parenthesis are calculated according to the methods of Patterson and Clayton (26,30).

lanosterol pathway (28,29). The fact that we were unable to show the presence of any 9,19-cyclopropyl sterols in *S. ferax* grown with tridemorph suggests that this fungus utilizes the lanosterol pathway. However, feeding experiments on *S. ferax* with labeled cycloartenol (18) and on other Oomycetes with unlabeled cycloartenol (32,33) have shown that cycloartenol can be metabolized by the Oomycetes.

Figure 1 shows a proposed scheme for sterol biosynthesis in *S. ferax*. If cycloartenol is the first cyclic precursor, it is probably converted to lanosterol by a cyclopropyl isomerase. The production of both lanosterol and cycloartenol has been reported in *Euphorbia pulcherimma* (34).

Lanosterol is the product of 2,3-oxidosqualene cyclase in all fungi thus far investigated. This work with *Saprolegnia* demonstrates the synthesis of lanosterol in control cultures, accumulation of large amounts of lanosterol but no cycloartenol in triarimol-treated cultures, and the presence of lanosterol but not cycloartenol in tridemorph-treated cultures where cycloartenol accumulation would be expected. These data strongly suggest that lanosterol is the product of 2,3-oxidosqualene cyclase in *Saprolegnia*. Although the tridemorph



a. SITE OF INHIBITION OF TRIARIMOL.

b. SITE OF INHIBITION OF TRIDEMORPH.

FIG. 1. Proposed sterol biosynthetic scheme in *Saprolegnia ferax*.

data make the possibility of a cycloartenol → lanosterol pathway seem remote, it cannot be totally ruled out at this point. However, a cycloartenol pathway can be proven only after the demonstration of cycloartenol synthesis by *Saprolegnia*. To date, this has not been demonstrated.

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METHODS

Systematic Protocol for the Accumulation of Fatty Acid Data from Multiple Tissue Samples: Tissue Handling, Lipid Extraction and Class Separation, and Capillary Gas Chromatographic Analysis

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ABSTRACT

A systematic procedure was developed for detailed fatty acid profiling of both neutral and polar lipid fractions isolated from hundreds of related bovine muscle and adipose tissue samples. A regimen was established for a nonbiased handling of tissue samples, which included their handling in a predetermined random order. Lipid class separation was accomplished concomitantly during the extraction of the tissues by a selective dry column method, which allowed a detailed analysis of minor but important polyunsaturated fatty acids associated with the polar fraction. Neutral lipids were derivatized to fatty acid methyl esters (FAME) by a literature procedure. However, to protect against lysis of plasmalogens in the polar fraction, a modified nonacidic esterification procedure was developed. FAME profiles were obtained on a programmable high resolution capillary gas chromatograph (GC). Run programs for unattended GC operation and data storage are described. By this overall procedure, the quantitation and peak identification were obtained for major and minor fatty acid constituents from bovine tissue in a manner that prepares for valid statistical interpretation of the resulting data.

Lipids 18:453-459, 1983.

This report describes a systematic approach used to gather fatty acid compositional data from a large set of tissue samples. The approach was developed to allow for an unbiased handling of the samples, proper record-keeping, and—most importantly—the timely completion of an otherwise unmanageable task.

This systematic approach was developed and used for 2 bovine dietary studies in conjunction with Oklahoma State University. One study investigated differences in beef quality of animals raised on forage (wheat pasture) compared to those on grain. The other determined the effect of the antibiotic supplement monensin on beef quality (1).

Three goals were considered during the development of the approach. First, fatty acid data needed to be accumulated in such a way as to allow later valid statistical analysis. Tissues were to be comminuted and extracted in random order. Data obtained by gas chromatography (GC) analysis were to be electronically stored for later retrieval and manipulation. Second, data needed to be detailed and accurate. This was to be achieved by the use of complete extraction and proper derivatization techniques, and by efficient GC separations and careful peak identification. Third, data needed to be accumulated in a timely way, despite the large number of tissue samples to be handled. This was to be accomplished by rapid extraction and derivatization techniques and the use of automated GC instrumentation. All the above mentioned criteria were met by the procedures described in this report.

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EXPERIMENTAL

Tissue Samples

Samples of bovine tissue were obtained from Oklahoma State University and consisted of the following: kidney knob fat (KD), *M. longissimus dorsi* (LD), *M. psoas* major (PM), *M. semi tendinosus* (ST), and subcutaneous fat (SQ), all of which were attained from the carcasses of animals used in 2 specific feeding studies. The first of these studies (Fig. 1) required 140 tissue samples and the second 116 samples.

Tissue Handling

Tissue samples were sealed in plastic pouches at Oklahoma State University, shipped to this laboratory in dry ice, and stored at -60 C until analyzed. Samples of ca. 100 g each were comminuted at 10 C in a food processor (Cuisinart CFP-5A, Cuisinarts, Inc., Stamford, CT) together with 30 μl of antioxidant (50% 1:1 BHA/BHT in ethanol, Tennox 5, Eastman Kodak Corp., Rochester, NY). Replicate samples were weighed (5 g $\pm$ 0.1 mg), sealed, and stored at -60 C. To eliminate experimental bias over the several weeks necessary for extraction of all the tissue samples, each sample was coded randomly before lipid extraction was undertaken. Frozen comminuted samples were then removed from storage and extracted on the basis of this sequence.

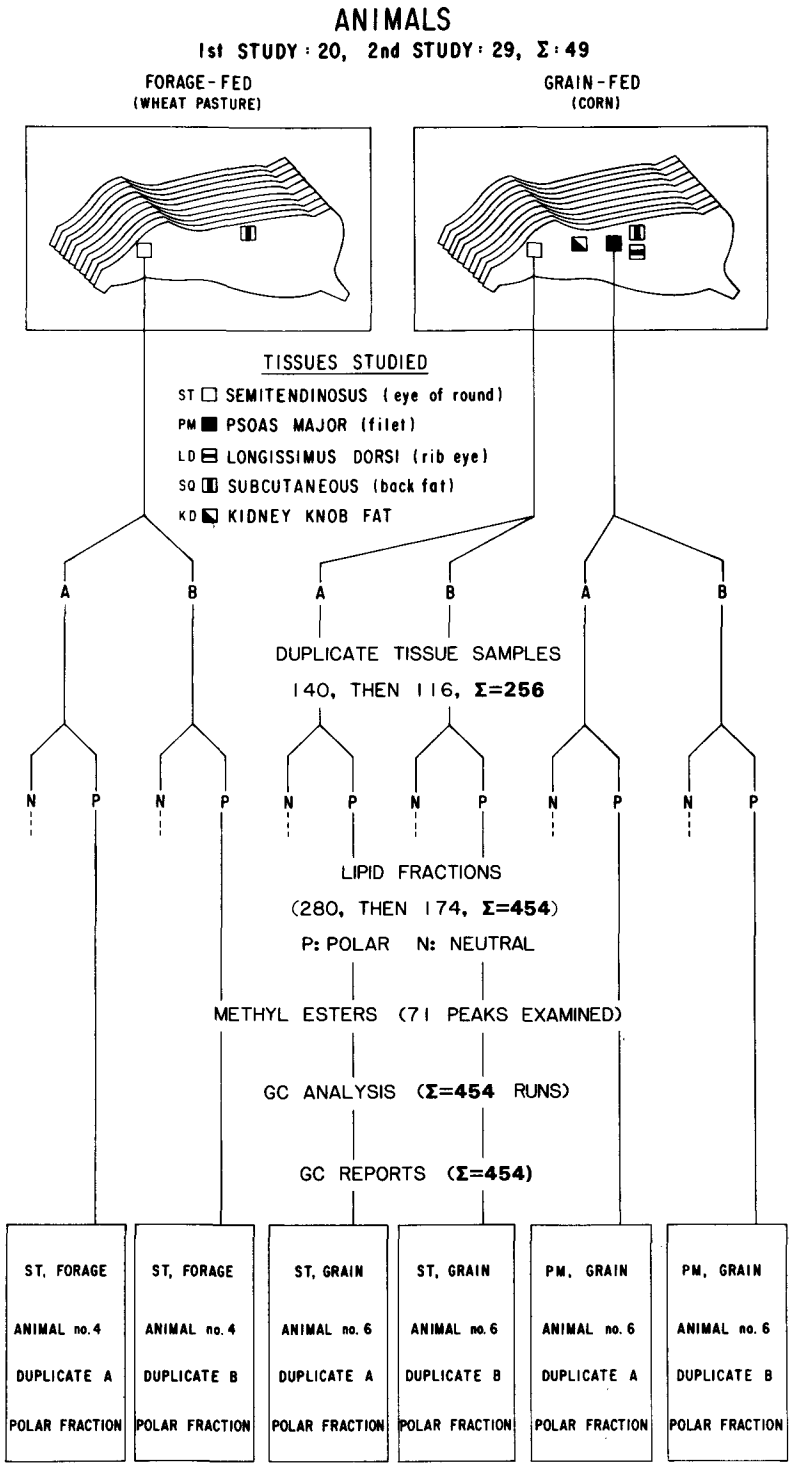

FIG. 1. Flow scheme for the analysis of multiple tissue samples.

Extraction of Lipids

Duplicates of each tissue sample (5 g ± 0.1 mg) were extracted sequentially by the dry column method (2). Before the sample was ground in the mortar, Tennox 5 (1 ml, 0.01 mg in methylene chloride) was added to the tissue. Eluates from each neutral and polar fraction were collected in 200 ml round bottom flasks. Solvent was removed on a rotary evaporator at room temperature, 1 ml of Tennox 5 added, and the contents transferred with hexane to a 100 ml volumetric flask and brought to volume with hexane. Aliquots were taken for thin layer chromatography (TLC), phosphorus analyses, and weight determination. A larger aliquot was reserved for derivatization to FAME's for subsequent GC analysis.

Derivatization of Glycerides to Methyl Esters: Neutral Lipid Fraction

The lipids were converted to their methyl esters by treatment with NaOH/methanol followed by BF_3/methanol (3).

Derivatization: Polar Lipid Fraction

A previously reported procedure (4) was modified in the following manner: an aliquot of the polar lipid fraction containing ca. 20 mg of lipid was first reduced in volume to about 5 ml on a rotary evaporator at room temperature. The contents of the flask were quantitatively transferred to a 15 ml centrifuge tube with a few ml of hexane, and the remaining solvent was removed under a stream of nitrogen. The residue was immediately dissolved in 1.0 ml of isooctane containing 4 mg of the internal standard methyl heneicosanoate followed by addition of 100 μl of 2 N KOH in MeOH (1.1 g/10 ml). The contents of the tube were mixed on a vortex mixer for 60 sec and then centrifuged to separate the layers. The lower methanol layer in the tube was removed with a microsyringe and discarded. A saturated ammonium acetate solution (0.5 ml) was added to the tube, the tube's contents stirred on the vortex mixer, centrifuged, and the aqueous layer removed and discarded. The above procedure was repeated with 0.5 ml of water instead of ammonium acetate. A small amount of anhydrous sodium sulfate was placed in the tube, the contents were stirred and allowed to stand for 30 min, and then the mixture was centrifuged. The upper isooctane layer (ca. 1 ml) of FAME was transferred to a 1 ml serum bottle (No. 223682, Wheaton Glass Co., Millville, NJ), which was then sealed with a cap lined with Viton A (Hewlett-Packard, No. 5080-8730, King of Prussia, PA). The vials were stored in a freezer until required for GC analysis. Completeness of esterification was established by TLC analysis of the reaction mixture.

Equipment

GC analyses were carried out on a Hewlett-Packard 5880A level 4 flame ionization capillary GC, equipped with magnetic tape storage capability and a Model 7672A automatic sampler. The column used for all analyses was a Quadrex 100 m 0.25 mm I.D. SP 2340 glass column (Quadrex, New Haven, CT). (Analyses may now be accomplished alternatively on either of two recently introduced flexible columns: a flexible glass column with the same stationary phase (50 m Quadrex Monarch series) or a fused silica column with a similar but bonded phase (50 m Quadrex 007-CPS 1). Both alternatives allow efficient separation of FAME's in substantially reduced analysis time, and are more easily installed than the fragile soft glass column used in the present work.) Carrier gas was helium at a flow of 1 ml/min and make-up gas was nitrogen at a flow of 30 ml/min. The temperature program employed was 150 C-170 C at 0.4°/min, then 1°/min to 200 C, at which temperature the oven was held for a maximum of 40 min until all FAMEs had been eluted.

Determination of FAME Identity and Reference Standards

Initial identification of FAMEs was made by injecting samples from bovine tissue into an HP 5840 GC (located in the laboratory of H. Slover, USDA, ARS, BARC, Beltsville, MD) that had been calibrated by GC/mass spectroscopy to identify major components in the mixture (3). Verification of other constituents was made by peak enhancement of the unknown using authentic compounds and by retention time analysis of the unsaturated FAMEs before and after hydrogenation.

A reference standard with 18 known FAMEs common to bovine muscle (Nu-Chek Prep, Inc., Elysian, MN) contained the relative amount of each component approximately found in a polar fraction of a typical muscle sample. This standard was chromatographed after each group of 10 bovine samples to ascertain if changes in retention times and peak shape, due to instrumental changes during the analysis of the preceding block of samples, had occurred.

RESULTS AND DISCUSSION

The methodology described in this study was developed to undertake a project in lipid analysis involving over 200 bovine tissue samples taken from 2 feeding studies at Oklahoma State University (1). Our independent investigations involved detailed analyses of the fatty acids of the tissue samples generated in these 2 studies. In all, 49 animals were used in the 2 studies. In the dietary study, samples were obtained from 5 tissue loca-

tions in each grain-fed animal and from 2 corresponding tissue locations in each forage-fed animal (5). In the monensin study, samples were taken from 2 locations per animal. The systematic protocol developed for examining the bovine tissues from the dietary study is shown in Figure 1. A similar protocol was used for the monensin study. To indicate the magnitude of both studies, the total numbers of tissue samples, lipid fractions, and GC reports are shown in Figure 1. This figure shows the sequence used to handle the samples employed in this study up to the generation of the reports of the GC analyses. The continuation of this diagram, which describes the data manipulation to the generation of the final report, can be found in Figure 2 of the following article (6). Although samples of muscle and adipose tissue from 5 locations were studied, only tissue from ST and PM are represented on the flow chart for simplification. The same sequence was used to examine all other tissue samples. Each tissue was ground to a uniform consistency (use of a food processor minimized sampling problems) in the presence of antioxidant, packaged as accurately weighed replicate portions, and quickly frozen until required. All samples studied were extracted in duplicate (total 256 samples) (Fig. 1) for lipid by the sequential dry column method (2). Since this method allows for the recovery of separate neutral and polar lipid fractions, no further separations of the lipid extracts were required prior to derivatization. Excellent agreement between duplicates was obtained for amounts of recovered lipid and for total phosphorus in these samples (5).

In most previous studies, FAME profiles of bovine muscle have been reported as those obtained from total lipid extracts. Such extracts generally contain only small amounts of polar lipid, and consequently their contribution is diluted in the GC trace by the overwhelming presence of the neutral lipids. Additionally, polar lipid of bovine muscle contains large amounts of plasmalogen (up to 30%), which produce dimethylacetals (DMA) of long chain aldehydes by acid-catalyzed esterification techniques. Although the DMAs would appear as only trace artifacts in the GC trace of the FAMEs of total lipid extracts, they would appear as major components in a GC trace of polar lipid FAMEs. Therefore, to prevent the acid-catalyzed lysis of plasmalogen lipids in the polar lipid extract, a published method for transesterification of total lipids (4) was modified to handle phospholipid concentrates. Room temperature alkaline transesterification converts all phospholipids to esters while generating no DMAs from the plasmalogens. The neutral lipid fractions, which contain no plasmalogens, did not present such problems, and were derivatized to their FAMEs by the technique of Slover and Lanza (3).

The 256 tissue samples (Fig. 1) after sequential extraction yielded only 454 neutral and polar lipid fractions, not the expected 512, because certain adipose samples were extracted for total lipid. These fractions were, in turn, derivatized to FAMEs by the methods described above. Each FAME sample contained a measured amount of internal standard according to the method of Slover and Lanza (3).

GC Analysis of FAMEs

Prior to analysis of the FAME samples on the HP 5880A GC, a sample run program was devised to run the instrument unattended during the actual analysis and to store the data when each run was completed. A profile of that run program is shown in Figure 2. Although that program was developed for a specific instrument, it illustrates the steps that are needed to carry out unattended profiling on any automated GC. This run program was written (in Hewlett Packard BASIC) for neutral lipid analysis, but the program for polar lipids was similar. The first command of the program (line 10) was to print "prgm for neutral lipid extracts." All commands in the program written within quotation marks are printed on the chromatogram and are informational only. List commands (lines 60, 360, and 400) serve similar purposes and instruct the instrument to print such current information on the chromatogram. Lines 30-50 are another informational set which is programmed to print the checklist (lines 450-510) prior to the start of the unattended automated analysis. The lines 70-280 describe the program used to start and advance the automatic sampler. This program instructs the sampler to inject the reference mixture (line 210) after 4 samples have been run, and then once after every next 10 samples have been run. Lines 290-330 describe a sequence designed to reject a report and prevent its tape storage if the total area found is less than 10 area counts (line 330). The feature is designed to shut down the instrument if, for example, no injection occurred due to a broken syringe. If the report is satisfactory, it is transferred to tape (line 340) and the program advances to the next step (line 350), which is a loop wherein the unit prepares itself for the next injection.

The utility of obtaining fatty acid profiles of separate neutral and polar lipid fractions of the same tissue, as opposed to profiles of a *total* lipid extract, is demonstrated by the 3 chromatograms (Fig. 3) that were generated from the above run program. Peaks are identified by manually inserted peak numbers (Chromatogram B, Fig. 3) to overcome run-to-run variations in retention times and to facilitate subsequent handling of data. Chromatogram C is derived from a total lipid extract and is representative of what would be seen from a traditional Folch extraction (7). Chromatograms

```
PROGRAM:     (ANNOTATION OFF)
10   PRINT " PRGM 5: PRGM FOR POLAR LIPID EXTRACTS."
15   REM  SAME AS PRGM 4, BUT WITHOUT STEPS 200,270.
20   FOR A=1 TO 11
30   READ P$
40   PRINT P$
50   NEXT A
60   LIST OVEN TEMP
70   INPUT "ENTER THE FIRST (N) AND LAST (M) BOTTLE # (N,M)",N,M
80   INPUT "ENTER THE DESIRED STOP #(1,2,OR 3) ",C
90   LET K=0
100  FOR I=N TO M STEP 2
110  LET K=K+1
120  IF K=5 THEN 150
130  IF K=10 THEN 430
140  GOTO 220
150  EDIT AUTO SEQ 4,1
160  EDIT AUTO SEQ 8,98
170  PRINT "-------------------------------------------------------------
180  PRINT "STOP# 1"
190  PRINT "REFERENCE VIAL #97"
210  START AUTO SEQ 97,97
220  EXECUTE X,"EDIT AUTO SEQ 4,"&VAL$(C)
230  EXECUTE X," EDIT AUTO SEQ 8, "&VAL$(I+1)
240  PRINT "-------------------------------------------------------------
250  PRINT "STOP# "&VAL$(C)
260  PRINT "SAMPLE VIAL #"&VAL$(I)
280  START AUTO SEQ I,I
290  LET S=0
300  FOR J=1 TO #PEAKS
310  LET S=S+AREA(J)
320  NEXT J
330  IF S<10 THEN 390
340  EXECUTE Y," SAVE REPORT "&VAL$(I)&" DEVICE# 16"
350  NEXT I
360  LIST CLOCK TIME
370  OVEN TEMP 70
380  STOP
390  PRINT "PROGRAM STOPPED BECAUSE NO PEAKS WERE FOUND IN LAST RUN"
400  LIST CLOCK TIME
410  OVEN TEMP 70
420  STOP
430  LET K=0
440  GOTO 220
450  DATA "() IS NEW TAPE CONDITIONED (DELETE DEVICE# 16)?"
460  DATA "() ARE GASES OK?","() IS PAPER SUPPLY OK?"
470  DATA "() ARE SAMPLES LOADED AND STIRRED?","() IS SAMPLE TBL OK?"
480  DATA "() ARE SOLVENT VIALS FULL?","() IS REFERENCE VIAL FULL?"
490  DATA "() ARE OTHER VIALS RETURNED TO FREEZER?","() IS SEPTUM OK?"
500  DATA "() IS THE FID IGNITOR CONTROL OFF?"
510  DATA "() IS THE OVEN TEMP NOW AT THE PROPER INITIAL VALUE?"
```

FIG. 2. Sample program for automated gas chromotograph.

A and B are derived from the neutral and polar lipid fractions, respectively, of the sequential extraction (2) of the same tissue. The advantage of examining separate chromatograms (A and B) is illustrated by the difference in fatty acid constituents associated with each lipid fraction. Specifically, the polyunsaturated fatty acids (peaks 380, 420, 460-660) are concentrated in the polar lipid fraction (B), but are overwhelmed by the contribution of the fatty acids of the neutral fraction when examined as a part of a total lipid extract (C). To achieve a fatty acid profile of the total lipid– with the content of the polyunsaturated acids obtained in detail--the data of the separate neutral and

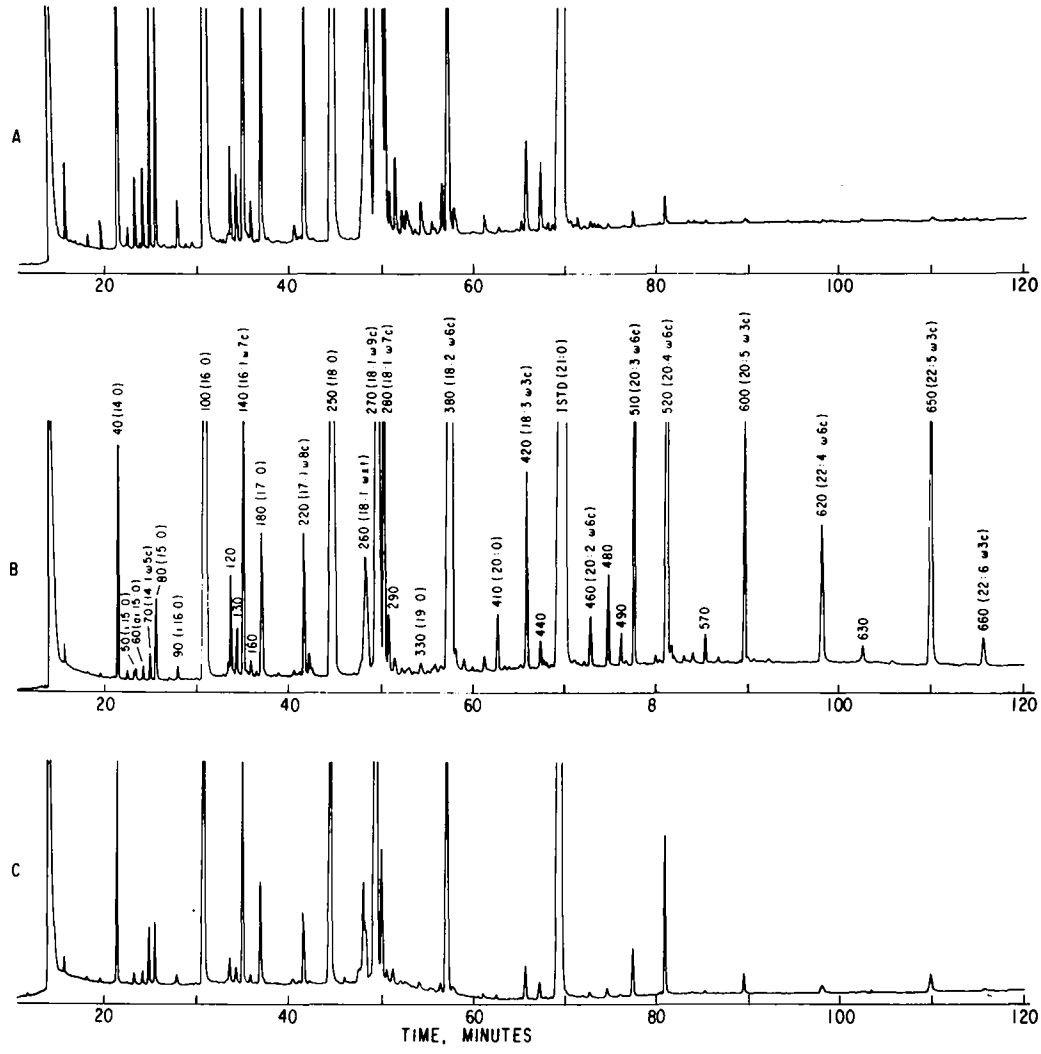

FIG. 3. Comparison of capillary GC chromatograms for neutral, polar, and total FAME extracts of bovine muscle tissue.
Trace A: Neutral FAMEs;
Trace B: Polar FAMEs (showing peak numbers and identities);
Trace C: Total FAMEs.

polar runs need only be consolidated.

At the end of each GC run, each trace was checked for completeness of integration of each component against the values in the sample report. Criteria used to judge the acceptability of each report have been described (3). Data for each run was, in turn, stored on magnetic tape. The stored data did not include the assigned peak numbers, which were entered manually as described in the next report (6).

We have demonstrated that fatty acid profiling on such a massive scale requires deliberate planning from tissue handling on through GC analysis. This profiling has been accomplished by use of a strict protocol for tissue comminution, by the dry-column method of lipid extraction and concomitant class separation, and by capillary column GC analysis that is automated for unattended operation and data storage.

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Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Systematic Management and Analysis of Fatty Acid Data from Multiple Tissue Samples

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ABSTRACT

A systematic approach has been developed for the collection and analysis of gas chromatographic (GC) data from multiple fatty acid profiles. The approach was applied to a series of polar and nonpolar tissue lipids generated in animal feeding studies to allow a comparison of mean fatty acid profiles as a function of either dietary regimen or tissue location. The magnitude of the studies, sufficiently large to minimize error from animal variabilities, mandated the use of computer assistance. Nevertheless, manual input was essential due to the complexity of the GC patterns, and was invoked for peak assignment and report editing. The approach discussed here allowed for the consolidation and statistical analysis of data from over 30,000 GC peaks, and generated results in both tabular and graphic formats. It should be extendable to other chromatographic studies of lipid components.

Lipids 18:460-466, 1983.

INTRODUCTION

The investigation of variations in tissue fatty acid profiles from experimental tissue studies is an enormous task, due to the large number of individual fatty acids that are separated by capillary column gas chromatography (GC) as well as the large number of GC runs mandated to eliminate animal variability. In our studies of the variation of bovine profiles as a function of dietary regimen or tissue location, data included over 30,000 assigned peak areas. It was evident that the data had to be handled systematically, beginning with the raw GC reports that were automatically stored on magnetic tape, through the statistical handling that allowed conclusions to be drawn from the studies. It was also evident that automation alone was no panacea; a substantial manual input would be required for editing during the entire process. This report presents a systematic approach for the management of voluminous and unwieldly data generated from large-scale animal studies.

EXPERIMENTAL

Computer Hardware

Initial data were processed first by the miniprocessor of the gas chromatograph (Hewlett-Packard 5880A GC, Level 4, with magnetic tape and ASCII keyboard accessories, Hewlett-Packard Corporation, Avondale, PA). Manually edited data were transferred to a minicomputer (Modcomp Classic 7861 with 10 million bytes of on-line (disk) storage, Modular Computer Systems Inc., Fort Lauderdale, FL). Final statistical analysis was accomplished by electronic transfer of data to the USDA's Washington Computer Center (WCC) (IBM 4341 and IBM 3033 attached processor with 31 billion bytes of on-line (disk) storage, International Business Machines Corp., White Plains, NY).

Computer Software

A program (Hewlett-Packard BASIC) was prepared for generation of a modified report by the GC's miniprocessor for each GC run. The program is designed to recall a stored GC report from magnetic tape, and then to list each peak's retention time, relative retention time (RRT), area, and area percent. Manual inputs are required to assign a time reference peak (RRT = 1; our peak #140, palmitoleate) and to eliminate the area of the internal standard peak (heneicosanoate). The output format also allows manual input of sample information (tissue source, dietary regimen, percent lipid, etc.) and most importantly, of peak identities. Identification numbers for the peaks were assigned manually, with the aid of RRT's and a regularly run reference mixture. An example of a manually annotated output is shown (Fig. 1). The 5880A GC did not permit editing or modification of a report for restorage, and did not contain an RS 232 interface for direct data transfer to the in-house minicomputer. Therefore, all data (peak numbers, their individual peak areas, and sample information) had to be transferred manually to the minicomputer for subsequent statistical analysis. To simplify this transfer, peaks with areas less than 0.10% of total peak area (not including the peak area of the internal standard) were disregarded. All

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*****************************************************************
(13)(ST)(B )(P )(F )(0.90)(0.70)(N)(Y)(15 -4 )(718 - 77 )
 A    B    C    D   E    F     G    H   I     J       K
ANIM TISS REPL FRAC DIET %LIP %PL MON? SICK? REPORT-  BOOK-
                                            TAPE     PAGE
*****************************************************************
[hp] 5880A  SAMPLER INJECTION @ 04:00  MAY 19, 1981
  SAMPLE # :  ID CODE   :
        15   77B/13ST/4
AREA %
   RT            AREA  TYPE    AREA %

 15.77          826.71   BB      20.328
 23.68          520.98   BB      12.810
 26.12          801.65   VV      19.712
 30.56          682.34   BV      16.778
 43.13         1235.22   BV      30.373
TOTAL AREA = 4066.91
MULTIPLIER = 1
ENTER RT OF PALMITOLEATE (OUR PK #140):  17.67
ENTER AREA OF 21:0 ISTD:  1235.22
  RT       REL RT   NAME  AREA      AREA %

  10.95     .620    40    14.39       .34
  11.93     .675           1.61       .04
  12.32     .697           2.00       .05
  12.48     .706           1.56       .04
  12.99     .735    80     8.03       .19
  14.30     .809    90     4.11       .10
  15.77     .893   100   826.71     19.37
  17.04     .965   110    49.97      1.17
  17.40     .984   130    24.83       .58
  17.67    1.000   140    35.23       .83
  18.03    1.020           3.52       .08
  19.14    1.083   180    20.06       .47
  21.17    1.198   210     6.24       .15
  21.36    1.209   220    19.09       .45
  23.68    1.340   250   520.98     12.21
  25.55    1.446   260    50.02      1.17
  26.12    1.478   270   801.65     18.79
  26.42    1.495   280    82.48      1.93
  26.74    1.513           1.57       .04
  30.56    1.729   330   682.34     15.99
  36.49    2.065   420   161.43      3.78
  38.09    2.156   440    16.04       .38
  43.13    2.441 <ISTD>  1235.22     28.94
  45.17    2.556   480    21.44       .50
  47.79    2.704   510   102.82      2.41
  50.90    2.880   520   379.06      8.88
  54.85    3.104   570    30.52       .72
  58.16    3.291   600   123.76      2.90
  66.08    3.740   620    28.55       .67
  73.88    4.181   650   229.17      5.37
  77.40    4.380   660    18.27       .43
*****************************************************************
********************* REPORT ANNOTATION ON *********************
```

FIG. 1. Sample modified report. Handwritten entries are shown. Top line entries are sample information for later classification of data. Upper abbreviated table shows only the largest peaks, and allows easy retrieval of the peak area of the internal standard. There are 2 pauses for input by keyboard; first, the retention time of the time standard peak, palmitoleate, must be entered to enable the program to list relative retention times of all peaks; then the area of the internal standard peak, heneicosanoate, must be entered to enable the program to renormalize the data after exclusion of this area. Finally, the data reappear with the newly calculated relative retention times and renormalized area percentages. Space is given for manual insertion of peak identification numbers. All peaks whose renormalized area percentages are under 0.10% are deleted. The manually entered data and the raw areas are then transferred to the minicomputer for further processing.

transferred data were processed on the minicomputer by a program that checked the validity of the manual input, renormalized the data, and finally output the results. The output was written to both a line printer for proofreading and a tape file for later transmittal to the WCC.

Data were processed at WCC by the Statistical Analysis System's (SAS Institute, Inc., Cary, NC) subroutines on statistical analysis and data management. SAS procedures MEANS and GLM were used to calculate mean values and other descriptive statistics and to perform analysis of variance (Bonferroni mean separation techniques (1)). SAS/GRAPH was used to produce histograms of the summarized data for illustrating various comparisons. Finally, SAS also was used to produce tabular summaries of the average peak values for various combinations of lipid fraction, tissue type, and dietary regimen.

Statistical Analysis

The goal of this research was to determine which fatty acids were present in statistically different amounts from tissue to tissue for animals in the same dietary regimen, and from regimen to regimen for the same tissues. The analysis of variance procedure was used to test these effects. Variation from animal to animal was partitioned from the tissue effect in those comparisons between tissues within a given diet. Analysis of variance produced probability levels (p) for each fatty acid comparison. For convenience, only those differences that were significant above the 95% confidence level ($p < 0.05$) were reported. In addition, those fatty acid comparisons with less than 3 degrees of freedom for error (4 degrees of freedom for the comparisons of 3 muscles of a single regimen) were considered invalid due to insufficient data. This situation occurred with peaks of very small area percentages that often were not reported for all 10 animals of a set; some of the missing values within a set resulted from the mandated 0.10% cut-off level.

Editing Procedures

Anomalies in data from over 30,000 peaks of these studies are to be expected because of errors in editing or peak assignment, deficiencies in electronic integration, or the appearance of spurious peaks. Consequently, human intervention is required for accurate results.

Deficiencies in electronic integration occurred most often with unsymmetrical peaks, particularly with the ill-defined group of peaks that comprises the signals from the *trans*-octadecenoate isomers. Because the GC runs were automated and unattended, such errors were noted long after the completion of the runs. Correction usually required the summation of several peak areas, but occasional adjustments had to be made by the archaic "cut-and-weigh" method, whereby the peak areas were related to the weights of the cut-out peaks. Spurious peaks generally were noted by inspection of the chromatogram, and were disregarded whenever they were not confirmed in the duplicate GC run. Errors from spurious peaks and from faulty peak assignments were also detected during the statistical analysis of the data. These errors were manifested by unusually high coefficients of variation (CV) for a particular peak in the composite report from the consolidation of individual runs. For example, peak 280 (*cis*-vaccenate) in one composite report (the mean value of peak 280 from 10 animals on the same diet, in which the value of peak 280 of each animal was itself the average of duplicate determinations) was 3.70% of the total peak area, but with a CV of 164%. To investigate the problem, the individual averages of duplicate determinations were examined for each of the 10 animals. One such average was suspect. Examination of the individual runs for that 1 animal then showed a misidentification of peak 280. Corrections were made, and the composite value for peak 280 diminished to 1.68% with an acceptable CV of 12%.

RESULTS AND DISCUSSION

The course of consolidation and analysis of the individual GC reports is outlined in Figure 2, a continuation of Figure 1 of the preceding article (2). Initially, each GC report was converted into a modified report (peak numbers and corresponding peak areas; Fig. 1). For each tissue, duplicate extractions were carried out (2) and eventually led to duplicate GC runs. Because the GC's miniprocessor was incapable of consolidating multiple GC reports, subsequent manipulations were accomplished on computers with greater capabilities. Initial data consolidation was the combination of duplicate modified GC reports. The middle 2 blocks in the upper part of Figure 2 represent duplicates A and B from 2 polar lipid extracts of *M. semitendinosus* tissue from animal no. 6, an animal that had been raised on a grain regimen. The computer-generated average of these 2 reports is represented as the middle block of the next row of blocks in Figure 2. All averages of A and B duplicates served as the raw material for analysis of variance. To generate a composite report—the next line of blocks in Figure 2—this average report from animal no. 6 was combined with the other 9 average reports from polar lipid extracts of *M. semitendinosus* tissue from the other 9 animals raised on a grain regimen. The resulting composite report—a mean of 10 average reports—is represented as 1 column of the computer-generated

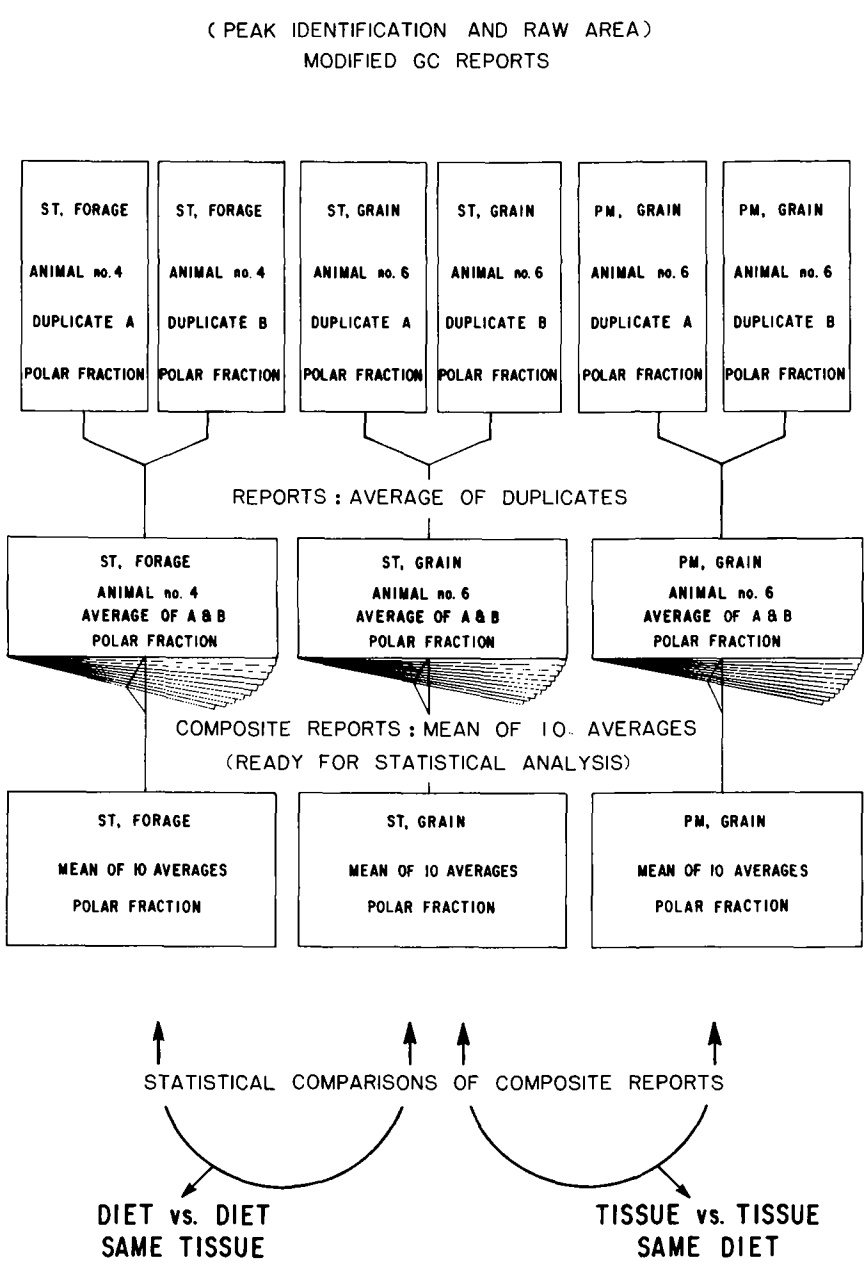

FIG. 2. Schematic for data analysis. Consolidation of data from 6 individual modified GC reports through the generation of composite reports is shown. In the actual studies, close to 500 individual reports were handled in this way. The 3 consolidated reports in the example illustrate the 2 types of comparisons that can be made a function of dietary regimen, using the same tissue (forage vs grain, using *M. semitendinosus* (ST) tissue), and a function of tissue location in the carcass, using the same dietary regimen (ST vs *M. psoas major* (PM), grain regimen). This schematic is a continuation of the schematic in Fig. 1 of the preceding article.

tabulation illustrated in Figure 3a, the column labeled "grain ST." (An analogous composite report was generated from data from corresponding neutral lipid extracts.) The column lists the mean normalized peak for each of the 60 most significant peaks, together with the number of observations (n). If the peak was seen for each animal of the set, n was 10 (9 for *M. longissimus dorsi* tissue). For example, in the mean report of the polar fraction of *M. semitendinosus* (ST) tissue of grain-fed animals, peak no. 40 (myristate) was seen for each animal (n = 10) and averaged 0.62% of the total peak area. Although the variance from animal to animal in this set of 10 is not presented in this figure, the variance information was used in the subsequent statistical analysis (done at the WCC because of the availability there of the appropriate software for Bonferroni mean separation techniques (1)). Such analysis served to determine whether any particular peak value differed significantly from its opposite, the corresponding value in another tissue (Fig. 2, right-hand blocks) or the corresponding value for the same tissue from animals raised on another dietary regimen (Fig. 2, left-hand blocks). Such

MUSCLE — FORAGE-GRAIN STUDY — POLAR FRACTION

PEAK	ID	GRAIN ST		PM		GRAIN PM		LD		FORAGE ST		LD		FORAGE VS. GRAIN ST		GRAIN ST	
20		0.10	1	.	0	.	0	.	0	0.10	1	.	0	.	0	0.10	1
40	14:0	0.62	10	0.40	10	0.40	10	0.55	9	0.62	10	0.55	9	0.72	10	0.62	10
50	i15:0	0.24	1	.	0	.	0	.	0	0.24	1	.	0	0.17	6	0.24	1
60	ai15:0	0.21	1	.	0	.	0	0.15	2	0.21	1	0.15	2	0.15	6	0.21	1
→ 70	14:1w5c	0.27	6	0.19	1	0.19	1	0.16	6	0.27	6	0.16	6	0.26	5	0.27	6
80	15:0	0.25	10	0.26	10	0.26	10	0.28	9	0.25	10	0.28	9	0.62	10	0.25	10
90	i16:0	0.16	2	0.11	2	0.11	2	0.10	1	0.16	2	0.10	1	0.14	8	0.16	2
100	16:0	19.46	10	16.15	10	16.15	10	19.18	9	19.46	10	19.18	9	20.82	10	19.46	10
110	16:1w7t	0.17	9	0.17	8	0.17	8	0.21	7	0.17	9	0.21	7	0.81	10	0.17	9
120		.	0	.	0	.	0	0.17	1	.	0	0.17	1	.	0	.	0
130		0.42	10	0.42	10	0.42	10	0.38	9	0.42	10	0.38	9	0.66	10	0.42	10
140	16:1w7c	1.29	10	1.25	10	1.25	10	1.26	9	1.29	10	1.26	9	1.40	10	1.29	10
150	ai17:0	0.18	5	0.13	6	0.13	6	0.12	7	0.18	5	0.12	7	0.28	10	0.18	5
160		0.16	4	0.12	3	0.12	3	0.10	4	0.16	4	0.10	4	0.10	1	0.16	4
170		0.14	1	.	0	.	0	.	0	0.14	1	.	0	0.16	1	0.14	1
180	17:0	0.76	10	0.68	10	0.68	10	0.72	9	0.76	10	0.72	9	0.89	10	0.76	10
190		0.10	1	.	0	.	0	.	0	0.10	1	.	0	0.11	4	0.10	1
200		0.17	3	0.43	2	0.43	2	.	0	0.17	3	.	0	0.22	1	0.17	3
210		0.22	5	0.48	2	0.48	2	.	0	0.22	5	.	0	0.30	10	0.22	5
220	17:1w8c	0.82	10	0.87	10	0.87	10	0.86	9	0.82	10	0.86	9	0.83	10	0.82	10
230		0.38	2	0.25	7	0.25	7	0.23	4	0.38	2	0.23	4	0.33	2	0.38	2
240		0.13	1	.	0	.	0	.	0	0.13	1	.	0	.	0	0.13	1
250	18:0	12.17	10	14.40	10	14.40	10	12.73	9	12.17	10	12.73	9	14.13	10	12.17	10
260	18:1w9t	1.09	10	0.95	10	0.95	10	1.02	9	1.09	10	1.02	9	1.19	10	1.09	10
270	18:1w9c	19.67	10	20.61	10	20.61	10	21.50	9	19.67	10	21.50	9	23.40	10	19.67	10

MUSCLE — FORAGE-GRAIN STUDY — POLAR FRACTION

PEAK	ID	GRAIN ST		PM		GRAIN PM		LD		FORAGE ST		LD		FORAGE VS. GRAIN ST		GRAIN ST	
20	U	0.70	1	.	0	.	0	.	0	0.70	1	.	0	.	0	0.70	1
40	14:0	2.31	10	2.58	10	2.58	10	2.29	9	2.31	10	2.29	9	3.48	10	2.31	10
50	i15:0	0.33	1	.	0	.	0	.	0	0.33	1	.	0	0.88	6	0.33	1
60	ai15:0	0.31	1	.	0	.	0	0.20	2	0.31	1	0.20	2	0.73	6	0.31	1
70	14:1w5c	0.70	6	0.91	1	0.91	1	0.54	6	0.70	6	0.54	6	1.56	5	0.70	6
80	15:0	1.21	10	1.58	10	1.58	10	1.09	9	1.21	10	1.09	9	1.77	10	1.21	10
90	i16:0	0.46	2	0.53	2	0.53	2	0.54	1	0.46	2	0.54	1	0.74	8	0.46	2
100	16:0	99.28	10	102.1	10	102.1	10	79.40	9	99.28	10	79.40	9	105.1	10	99.28	10
110	16:1w7t	0.97	9	1.08	8	1.08	8	0.91	7	0.97	9	0.91	7	4.18	10	0.97	9
120	U	.	0	.	0	.	0	0.62	1	.	0	0.62	1	.	0	.	0
130	U	2.24	10	2.78	10	2.78	10	1.62	9	2.24	10	1.62	9	3.35	10	2.24	10
140	16:1w7c	6.11	10	8.26	10	8.26	10	5.28	9	6.11	10	5.28	9	7.14	10	6.11	10
150	ai17:0	0.78	5	0.80	6	0.80	6	0.50	7	0.78	5	0.50	7	1.53	10	0.78	5
160	U	0.46	4	0.86	3	0.86	3	0.40	4	0.46	4	0.40	4	0.56	1	0.46	4
170	U	1.10	1	.	0	.	0	.	0	1.10	1	.	0	0.21	1	1.10	1
180	17:0	3.63	10	4.42	10	4.42	10	3.00	9	3.63	10	3.00	9	3.63	10	3.63	10
190	U	0.66	1	.	0	.	0	.	0	0.66	1	.	0	0.61	4	0.66	1
200	U	0.91	3	3.18	2	3.18	2	.	0	0.91	3	.	0	1.31	1	0.91	3
210	U	1.30	5	3.56	2	3.56	2	.	0	1.30	5	.	0	0.85	10	1.30	5
220	17:1w8c	4.27	10	5.79	10	5.79	10	3.86	9	4.27	10	3.86	9	3.16	10	4.27	10
230	U	0.72	2	1.48	7	1.48	7	0.80	4	0.72	2	0.80	4	0.78	2	0.72	2
240	U	0.16	1	.	0	.	0	.	0	0.16	1	.	0	.	0	0.16	1
250	18:0	65.00	10	94.84	10	94.84	10	52.16	9	65.00	10	52.16	9	70.58	10	65.00	10
260	18:1w9t	5.86	10	8.26	10	8.26	10	4.18	9	5.86	10	4.18	9	8.26	10	5.86	10
270	18:1w9c	101.5	10	136.1	10	136.1	10	90.15	9	101.5	10	90.15	9	118.8	10	101.5	10

FIG. 3a. (Upper report) Computer-generated composite report, normalized data. Abbreviations: ID, identification; ST, *M. semitendinosus;* PM, *M. psoas major;* LD, *M. longissimus dorsi;* i, iso; ai, anti-iso; ω, first double bond position from hydrophobic end; c, *cis;* t, *trans*. Integers next to compositional data specify the number of animals from which individual reports were consolidated into this composite report. Maximum n for ST and PM, 10; for LD, 9. Columns of data are repeated to allow convenient inspection for each comparison (3 tissue by tissue, 1 diet by diet).

FIG. 3b. (Lower report) Computer-generated composite report, gravimetric data (mg fatty acid/100 g tissue).

statistical analysis showed, for example (Fig. 3a, arrow), that the cited 0.27% value for grain-fed ST was identical ($p < 0.05$) to the corresponding value for grain-fed *M. psoas major* (PM, 0.19%) and also identical ($p < 0.05$) to the corresponding value for grain-fed *M. longissimus dorsi* (LD, 0.16%). To have concluded that these 3 values were different (i.e., without rigorous analysis) would therefore have been invalid.

By use of an internal standard, the aliquot size, and the percent lipid, sets of gravimetric data may be generated to show the results as mg fatty acid/100 g tissue (Fig. 3b) for use by nutritionists. Because the study was designed to determine those fatty acids whose normalized amounts differed significantly from their opposites, GC response factors were not included. To convert to a gravimetric tabulation (mg fatty acid/100 g tissue), the following algorithm was applied to individual GC reports:

$$\begin{aligned}
(\text{mg FAME}/100\text{ g tissue}) &= (\text{mg FAME/aliquot})(\text{scale-up factor}) \\
&= [(\text{area sum})/(\text{area 1 mg ISTD per aliquot})] \times [(\text{mg lipid}/100\text{ g tissue})/(\text{mg lipid/aliquot})] \\
&= [(\text{area sum})/(\text{area 1 mg ISTD per aliquot})] \times [(1000(\%\text{ lipid})/(\text{mg lipid/aliquot})]
\end{aligned}$$

$$\text{Since (mg fatty acid}/100\text{ g tissue}) = 0.95\ (\text{mg FAME}/100\text{ g tissue}),$$

$$\text{then (mg fatty acid}/100\text{ g tissue}) = \frac{950\ (\text{area sum})\ (\%\text{ lipid})}{(\text{area 1 mg ISTD per aliquot})\ (\text{mg lipid/aliquot})}$$

Definitions:

Aliquot = Portion of lipid extract set aside for derivatization to FAME.

(Scale-up factor) = Ratio of lipid weight of full sample to lipid weight in aliquot.

(area 1 mg ISTD per aliquot) = Peak area of internal standard (ISTD; here 21:0 FAME) that results from incorporation of 1 mg ISTD into the aliquot. (We used 4 mg 21:0/aliquot, and therefore divided our 21:0 peak area by 4.)

(area sum) = Sum of FAME peak areas, not including ISTD peak area.

0.95 = Factor to convert mg FAME TO mg fatty acid, valid (±1%) for FAME's C-14 through C-22.

To determine the weight contributions of each fatty acid, the (mg fatty acid/100 g tissue) figure is distributed according to the normalized report for each individual run. Thus, for any fatty acid P, (mg P/100 g tissue) = 0.01 (%P) (mg fatty acid/100 g tissue), where (%P) is taken from the normalized report. Then replicate runs are combined to generate an average report for each animal, and finally the set of average reports (usually 10) are consolidated into a mean report, such as shown in Figure 3b.

Tabular data (Fig. 3) were often unwieldy and overwhelming for purposes of study. More satisfactory was the use of graphics, including computer-generated graphics. By the introduction of field descriptors to classify each peak number, the final reports could be digested into conveniently read graphics. Although approximately half the peaks were not identified as particular fatty acids, the identified portion included over 95% of the total peak area. Unidentified peaks were classified as either saturated or unsaturated by hydrogenation experiments (3). All peaks were classified into 1 of 7 groups: (a) normal-chain saturated, (b) branched-chain saturated, (c) unidentified saturated, (d) unidentified unsaturated, (e) *trans*-monoenoic, (f) *cis*-monoenoic, (g) polyenoic. The horizontal bars at the bottom of Figure 4 illustrate one possible graphics output. Inspection of corresponding bars shows trends in fatty acid composition as a function of carcass location or dietary regimen, but does not show whether any differences are statistically significant. A breakdown of each segment of these bars into a set of histograms of constituent fatty acids, shaded to show significantly different pairs, allows a better interpretation of the trends in fatty acid patterns (Fig. 4, vertical bars). Figure 4 represents the polar fraction from *M. semitendinosus,* grain (G) regimen vs forage (F) regimen. Significant differences may be noted in 4 of the 9 identified polyunsaturated fatty acids. Although in this example the grain-fed animals generated more total polyunsaturated fatty acids than did the forage-fed (Fig. 4, bottom), this was mainly due to the (statistically significant) greater amounts of linoleate (18:2) (Fig. 4, top). Nevertheless, statistically significant greater amounts of polyenoic fatty acids, excluding the dienes, were seen in the forage-fed animals. (Such polyenoics may be responsible for off-flavors in forage-fed beef. The higher linolenate content of the samples from forage-fed animals (4,5) results from the ingestion of grasses whose fatty acids are comprised of predominantly linolenic acid. The full data sets and interpretation will be published elsewhere).

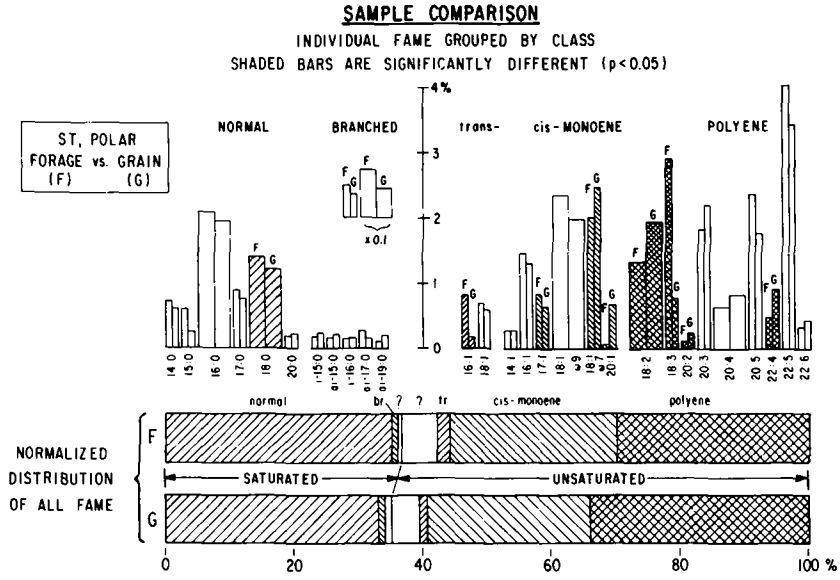

FIG. 4. Graphic representation of fatty acid distribution, expanded to show which peaks are statistically different from 1 data set (F) to another (G). Horizontal histograms are representative of computer graphics output. Vertical histograms show breakdown by fatty acid. Shaded pairs of vertical histograms are statistically different ($p < 0.05$). To allow a reasonable vertical scale, the largest vertical histograms are reduced to 1/10th their height and denoted by extra width. Abbreviations: ST, *M. semitendinosus;* FAME, fatty acid methyl ester; br, branched; ?, unidentified; tr, *trans*.

It should be evident that the processing of data sets of great magnitude requires computer assistance, not only for data management, but also to obtain statistically valid conclusions. We have demonstrated procedures that enable such processing and envision future studies that may be even more easily accomplished with increasingly sophisticated electronics.

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Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Dietary Lipid Modulation of Immune Responsiveness[1]

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ABSTRACT

The influence of dietary fat concentration and saturation on blastogenesis, cytotoxicity, antibody response and fatty acid composition of murine splenic lymphocytes was studied. Blastogenesis of lymphocytes from dietarily manipulated mice in response to alloantigens from control mice was significantly greater for those mice fed a diet containing minimal essential fatty acids (EFA) as the only fat source (EFA control) than those fed an EFA-deficient diet. When the dietary fat concentration was increased, blastogenic responses decreased compared to the EFA control diet. Lymphocyte-mediated cytotoxicity against allogeneic melanoma cells was greater for mice receiving diets with EFA only than for those deficient in EFA. However, cytotoxicity responses of mice fed additional polyunsaturated fat (PUF) decreased as concentration increased, whereas responses of mice fed the saturated fat (SF) diets decreased only when the dietary fat concentration was greater than 8%. As compared to diets with EFA control, direct plaque-forming cell (PFC) response was decreased for mice fed high levels of PUF and increased for mice fed high levels of SF; however, no difference in the percentage of IgM-positive cells was observed. These changes in PFC response were inversely related to the levels of linoleic acid in the lymphocyte. Thus, high levels of dietary fat, and particularly PUF, suppress lymphocyte functions when EFA requirements are met, whereas low levels (EFA control) intensify these responses. EFA deficiency, however, suppresses some lymphocyte responses. Thus, dietary lipids differentially modulate the levels of T- and B-cell responsiveness.
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INTRODUCTION

Fatty acids introduced into culture or injected subcutaneously into animals have been reported to influence immune function. Nevertheless, the exact effect of these fatty acids is a controversial issue; both enhancement and suppression of immunity have been reported. For example, PUFA dissolved in ethanol inhibited lymphocyte transformation in response to PHA (1); arachidonic acid dissolved in hexane, however, enhanced, whereas the same fatty acid dissolved in ethanol inhibited PHA-induced human lymphocyte blastogenesis (2,3). In contrast, others (4) have suggested that PUFA may not play an immunoregulatory role. Their hypothesis is based on observations that fatty acids bound to albumin did not inhibit lymphocyte transformation. However, changes of immune response after manipulation of fatty acids in vitro may not relate to changes of immune responsiveness in vivo because of the complexity of cellular and humoral interactions observed in vivo. Moreover, few studies have addressed the question of how dietary fat influences both T- and B-cell responsiveness. We (5) have previously reported that prenatal and postnatal dietary lipid manipulation can significantly influence several parameters of immune status in neonatal mice. For example, lymphocyte transformation induced by concanavalin A was significantly decreased as levels of PUF increased. In addition, the number of immunoglobulin-positive cells and serum IgG_1 and IgG_2 levels decreased with increasing fat concentration. Because of the controversial nature of fatty acid effects on immune response in vitro and the potential association between dietary fat and immune status, the purpose of the experiments reported herein were to determine the influence of dietary fat concentration and saturation on: lymphocyte blastogenesis, cytotoxicity, antibody response, and antibody response as related to the fatty acid composition of lymphocytes.

[1]Presented at the 73rd Annual Meeting of the American Oil Chemists' Society in Toronto, Canada, May 1982.

Abbreviations: BHT, butylated hydroxytoluene; EFA, essential fatty acid; FBS, heat-inactivated, fetal bovine serum; IP, intraperitoneally; MLC, mixed lymphocyte culture; PEC, peritoneal exudate cells; PFC, plaque-forming cells; PHA, phytohemagglutinin; PUF, polyunsaturated fat; PUFA, polyunsaturated fatty acids; SF, saturated fat; and SRBC, sheep red blood cells.

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MATERIALS AND METHODS

Animals and Diets

Six week old female C57BL/6J, BALB/cAnN, and C3H/HeJ mice previously fed a stock diet (Purina Rodent Chow, St. Louis, MO) were maintained as specifically pathogen-free in an air curtain isolator and fed at a level such that each mouse had 16 kcal of metabolizable gross energy available daily. At that level, weights increased at the same rate and there was no significant difference ($p > 0.05$) between groups fed the various diets. The semipurified diets consisted of a constant amount per kilocalorie of casein, salts, vitamins and fiber. Diets then had either 0% energy from corn oil (essential fatty acid deficient) or 1.4% energy from corn oil which provided minimal EFA for each diet (6) (Table 1). To test the influence of dietary fat concentration, 4 diets contained additional 20.3% or 40.6% energy from fat; 2 of those

TABLE 1

Composition of Experimental Diets

Ingredient	1 (0 Fat)	2 (EFA)	3 (8% Fat)	4 (8% Fat)	5 (20% Fat)	6 (20% Fat)
			g/100g diet			
Casein	18.2	18.4	21.0	21.0	24.8	24.8
Salt mix[a,b]	4.5	4.7	5.4	5.4	6.4	6.4
Vitamin mix[a,c]	1.2	1.2	1.4	1.4	1.7	1.7
Fiber[d]	4.0	4.1	4.7	4.7	5.5	5.5
Cerelose	72.0	71.1	59.0	59.0	41.6	41.6
Corn oil[e,f]	0	0.5	0.6	0.6	0.7	0.7
Safflower oil[g]	0	0	7.9	0	19.3	0
Coconut oil[g]	0	0	0	7.9	0	19.3
			Percent energy			
Protein	28.0	28.0	28.0	28.0	28.0	28.0
Fat	0	1.4	20.3	20.3	40.6	40.6
Carbohydrate	72.0	70.6	51.7	51.7	31.4	31.4

[a]See Erickson, et al. (7) for composition.
[b]Provides 1.3 g/kcal of gross energy.
[c]Provides 0.3 g/kcal of gross energy.
[d]Provides 1.1 g/kcal of gross energy.
[e]Provides 1.4% of the gross energy as an essential fatty acid source.
[f]See Ossmann et al. (8) for fatty acid analysis.
[g]See Erickson et al. (5) for fatty acid analysis.

diets contained coconut oil (8.4% unsaturated bonds) and 2 contained safflower oil (89.3% unsaturated bonds). Mice were fed the experimental diets for 4 weeks before immunization, and then continued on the same diets until they were killed. All dietary groups contained at least 6 mice per group in each experiment. Separate experiments were performed 2 or 3 times.

Mixed Lymphocyte Culture (MLC)

First, to test dietary fat influences on lymphocyte transformation in response to alloantigens, spleen cells from C57BL/6 dietarily manipulated mice acted as responder cells to stimulation by BALB/c spleen cells from mice fed the stock diet. Second, to test dietary fat influences on alloantigenicity, spleen cells from dietarily manipulated C57BL/6 mice acted as stimulator cells for BALB/c responder cells from mice fed the stock diet.

Single cell suspensions of spleen cells were prepared in RPMI-1640 with 25 mM HEPES buffer, 10% heat-inactivated FBS, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 3×10^6/ml. For the preparation of stimulator cells, spleen cells at a concentration of 10^7/ml were incubated with 50 μg/ml of mitomycin C in RPMI-1640 for 30 min at 37 C, washed and resuspended in complete medium at 3×10^6/ml. Quadruplicate cultures for each group were set up with 100 μl of stimulator and 100 μl of responder cells in a round bottom microtiter plate. Both one-way and two-way MLC were assayed. Controls included cultures of responder or stimulator cells alone as well as a coculture of syngeneic mitomycin C treated and untreated cells. All mice were treated individually. After 108 hr, 1 μCi of ^{3}H-thymidine (^{3}H-TdR; sp act 6.7 Ci/mmol) was added, and at 120 hr, the cells were collected onto glass fiber filters. The dried samples were placed into vials containing toluene and omnifluor (New England Nuclear, Boston, MA) and counted in a liquid scintillation spectrophotometer. The results are expressed as mean cpm $\pm$ SEM; the stimulation index (SI) was calculated:

$$SI = \frac{\text{CPM of allogeneic culture}}{\text{CPM of syngeneic culture}}$$

Cytotoxicity Assay

Cell-mediated cytotoxicity assays were performed in vitro as described by Benjamini et al. (9). The tumor target cell line, P51, was used for the assay. P51 was previously established from a transplantable B16 murine melanoma and propagated in culture (10-12). For the assay, P51 cells were harvested by a short trypsinization and washed; 5×10^6 cells were then injected IP into dietary-manipulated C3H mice. Ten days after injection, PEC were collected by lavage with Ca^{2+}–Mg^{2+} free Hank's balanced salt solution, centrifuged, and resuspended in RPMI-1640 with 25 mM HEPES, 10% FBS, and antibiotics. The resulting suspensions were greater than 90% lymphocytes. Tumor

target cells in their exponential growth phase were incubated at 37 C for 1 hr in RPMI-1640 containing 200 μCi of $Na_2{}^{51}CrO_4$ (sp act 128 mCi/mg). P51 cells were then washed, trypsinized, resuspended in complete medium, and 2.5×10^4 added per 38 mm^2 well (Microtest II, Falcon, Oxnard, CA). Quadruplicate cultures were set up for individual mice with an effector:target cell ratio of 50:1. Radiolabeled target cells were also plated alone. After 4 hr of coculture, aliquots of the supernatant were removed and samples counted in a gamma counter. Maximum release was determined by freeze-thawing the tumor cells 3 times. Spontaneous release was always less than 10%. The results were calculated by:

$$\% \text{ cytotoxicity} = \frac{\text{CPM released for test sample} - \text{CPM of spontaneous release}}{\text{total releasable CPM} - \text{CPM of spontaneous release}} \times 100$$

Hemolytic Plaque Assay

For measurement of primary responses, dietarily manipulated mice were immunized with 0.2 ml of 10% sheep red blood cells IP. Six days after immunization, spleen cells were assayed for antibody forming cells by the Cunningham and Szenberg modification of the Jerne plaque technique (13). Controls included samples without spleen cells and spleen cells from unimmunized mice. The former yielded no plaque-forming cells, whereas the latter yielded fewer than 5 plaque forming cells/10^6 nucleated spleen cells.

Extraction and Gas Chromatographic Analysis of Lipids

Lipid extractions were carried out by the method of Folch et al. (14), as modified by Johnson (15). For each sample, ca. 9×10^7 cells were lyophilized and 5.0 ml freshly distilled methanol containing 0.005% BHT was added. Samples were resuspended, flushed with nitrogen, and sealed with teflon tape before incubation at 55 C for 1 hr. After cooling, 10 ml freshly distilled chloroform with 0.005% BHT was added, the sample flushed with N_2, sealed, and the extraction continued at 25 C for 12 hr. Insoluble material was removed from each sample by filtration through a 0.45 μ size Millipore filter. The chloroform/methanol suspension was then washed twice with 2 M KCl, followed by one wash with 10 ml distilled water. The organic layer was passed through freshly packed anhydrous sodium sulfate columns and dried under N_2 and stored at −70 C until derivatized.

Dried lipid samples were resuspended in 3 ml petroleum ether and 300 μg of the stock C_{17} (100 μg/50 μl in isopropyl alcohol) was added to each. An aliquot was removed, placed into a round-bottomed flask, the solvent evaporated under vacuum, and 1.0 ml of 0.5 M NaOH in methanol added. The sample was heated over a steam bath for 5 min to achieve hydrolysis of the fatty acyl groups. After cooling, 1.0 ml methanolic BF_3 (14% w/v, Applied Science) was added, followed by heating at 100 C for 1 min. The solvent was evaporated under vacuum and the residue was extracted twice with 2.0 ml petroleum ether. Each extract was shaken with 1.0 ml ddH_2O, the ether layer removed carefully, combined in a round-bottomed flask, and the solvent evaporated under vacuum. The resultant methyl esters were resuspended in dichloromethane.

Gas chromatographic analyses were carried out on 1-5 μl samples of methyl esters on a Silar 10C column using hydrogen flame detection. Quantities were calculated on the basis of peaks relative to the internal standard of known fatty acid methyl esters.

Statistical Methods

The mean of replicate samples for individual mice were used for analysis. Data were initially subjected to one and two way analysis of variance (16), then to multiple-t pairwise comparisons of independent samples or (17) Scheffe's multiple range test (18). Correlation coefficients were determined for linear relationships (17).

RESULTS

Lymphocyte Blastogenesis in Response to Alloantigens

High levels of dietary fat, particularly PUF, have been shown to suppress lymphocyte responses to T-cell mitogens (5,8). We now wish to determine how dietary fats modulate lymphocyte responses to defined antigens. First, to test whether dietary fats directly influence the lymphocyte, transformation in response to alloantigens was measured. For this determination, spleen cells from dietarily manipulated mice acted as responders to stimulation by spleen cells from mice fed the stock diet (Fig. 1). Responses of $24{,}700 \pm 3{,}300$ cpm for lymphocytes from mice fed the EFA diet were significantly ($p < 0.05$) greater than responses of $9{,}200 \pm 1{,}000$ cpm for lymphocytes from mice fed a fat-free diet. With additional fat added to the diet, the levels of lymphocyte blastogenesis decreased such that at 20% concentration, responses of $14{,}500 \pm 2{,}400$ cpm for mice fed PUF diet and $14{,}200 \pm 1{,}000$ cpm for mice fed the SF diet were significantly ($p < 0.05$) less than mice fed the EFA.

Second, to test the dietary fat influences on alloantigenicity, spleen cells from dietarily manipulated C57BL/6 mice acted as stimulator cells for responding BALB/c splenic lymphocytes from mice fed the stock diet (Fig. 2). Responses of $52{,}500 \pm 1{,}740$ cpm for mice fed a fat-free diet were significantly greater than responses of lymphocytes

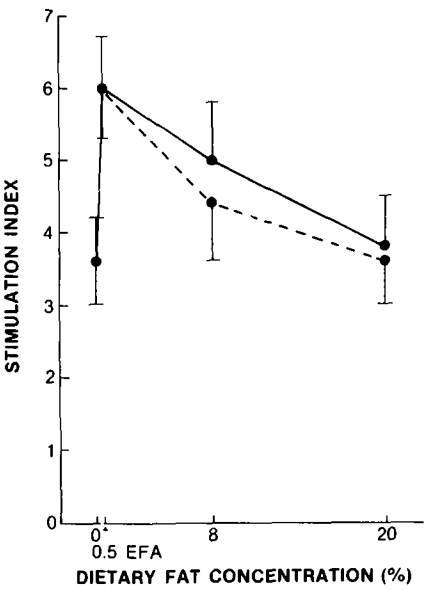

FIG. 1. Blastogenesis of lymphocytes from dietary-fat-manipulated mice in response to alloantigens from control mice. Diets contained either 0% fat (EFA-deficient) or 0.5% corn oil (minimum EFA). To test the influence of dietary fat situation, 4 diets contained additional safflower (PUF, ●— ●) or coconut (SF, ●- ●) oil. Data shown represents the mean and standard error of the mean for all mice. 36 mice were used in each individual experiment with 6 animals per diet group treated individually. All mice were assayed in quadruplicate. This was repeated 3 times.

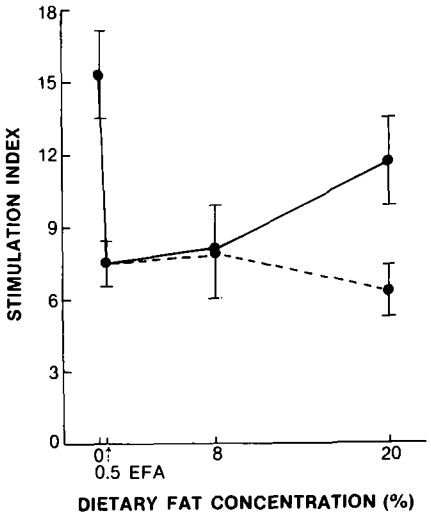

FIG. 2. Blastogenesis of lymphocytes from control mice in response to alloantigens from dietary-fat-manipulated mice. Diet contained either 0% fat or EFA. In addition to EFA four diets contained added PUF (●— ●) or SF (● ●). Data shown represents the mean and standard error of the mean for all mice. The number of animals used was the same as indicated for Figure 1.

from mice fed a diet containing fat except for those receiving a 20% PUF diet (42,000 ± 4,100 cpm). Responses of 26,500 ± 5,000; 23,600 ± 4,000; 28,000 ± 6,000; and 25,700 ± 3,000 cpm were observed for mice fed the EFA, 8% PUF, 8% SF, and 20% SF diets.

Peritoneal Exudate Cell Cytotoxicity Toward Melanoma Targets

The influence of dietary fat on PEC-mediated cytolysis was measured by radioisotope release from a standardized target cell taken from culture (Table 2). PEC from mice fed the EFA diet exhibited significantly higher levels ($p < 0.05$) of cytotoxicity than those mice receiving a fat-free diet. Cytotoxicity of PEC from mice fed additional PUF at either 8 or 20% concentration decreased compared to EFA controls. In contrast, the cytotoxic responses of mice fed the 8% SF diet were not significantly different ($p > 0.05$) than EFA diet controls but decreased in mice fed the 20% SF diet. Cytolysis mediated by PEC from mice fed the 20% PUF diet was not different as compared to cytolysis by PEC of mice fed the 20% SF diet.

TABLE 2

Cytotoxicity Mediated by Peritoneal Exudate Cells[a]

Dietary fat	% Cytotoxicity toward target cells[b]
0	20.4 ± 1.8[c]
0.5% (EFA)	27.5 ± 2.5
8% PUF	19.4 ± 2.7[c]
8% SF	25.2 ± 2.8
20% PUF	17.0 ± 1.8[c]
20% SF	20.5 ± 1.2[c]

[a]Cytotoxicity toward the P51 melanoma target mediated by allogeneic PEC from dietarily manipulated mice.
[b]Mean ± SEM for 6 mice in each dietary group.
[c]Significantly ($p < 0.05$) less than for mice fed the control (EFA) diet.

Antibody Formation by Single Cells

Dietary fat manipulation has been shown to influence serum immunoglobulin levels in neonatal mice (5). To determine whether similar lipids effect B-cell responses to specific antigens, direct plaque-forming responses to SRBC were measured (Fig. 3). The numbers of IgM plaque-forming cells/10^6 nucleated spleen cells were not significantly different ($p > 0.05$) for mice fed at either 0, 0.5, or 8% fat concentration. However, mice fed the 20% PUF diet had 276 ± 36 PFC/10^6 which was significantly

($p<0.05$) less than the number of PFC ($416\pm 40/10^6$) for mice fed the EFA diet; mice fed the 20% SF diet had 652 ± 46 PFC/10^6 which was significantly greater ($p<0.05$) than the EFA control.

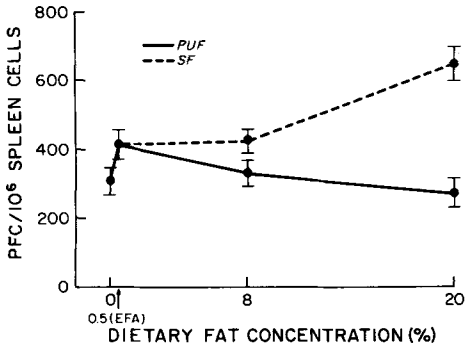

FIG. 3. Number of direct plaques from mice fed diets containing either no fat, EFA, or EFA plus additional PUF (●——●) or SF (●---●). Mice were fed various diets for four weeks before primary immunization with SRBC then maintained on the same diet until the assay on day 6. Data shown is the mean and standard error of the mean for all mice. In each experiment, 6 mice in each dietary group were treated individually; the experiment was repeated 2 times.

Fatty Acid Composition of Lymphocytes

To assess the fatty acid composition of lymphocytes after dietary manipulation and correlate potential changes with B-cell responses, fatty acid levels were determined by gas chromatography. With mice maintained on the stock diet, no significant change in the fatty acid profile of whole cell extracts was observed after immunization with SRBC as compared to control, nonimmunized mice (Fig. 4). However, by increasing either the PUF or SF concentration, the lymphocytes from mice immunized with SRBC had an increased level of palmitic and arachidonic acid as compared with the control (EFA) diet, whereas the concentration of stearic acid remained constant (Fig. 5). With increasing concentration of dietary SF, the levels of palmitoleic acid remained constant while levels of oleic and linoleic acid decreased in the lymphocyte. When the diets were supplemented with increasing amounts of PUF, the levels of linoleic acid increased with a concomitant decrease in both palmitoleic and oleic acid.

DISCUSSION

High concentrations of dietary fat and particularly PUF appear to suppress lymphocyte functions when EFA requirements are met. For example, with a T-cell function such as cytotoxicity, when mice were fed high levels of dietary fat, saturation

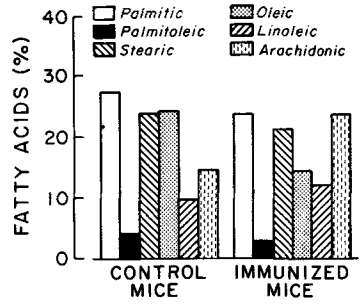

FIG. 4. Fatty acid composition of lymphocytes from immunized and control mice fed a stock diet. Values represent the means of all mice with triplicate samples for each dietary group of 6 mice. Standard errors of the mean remained <10% throughout. This experiment was repeated 2 times.

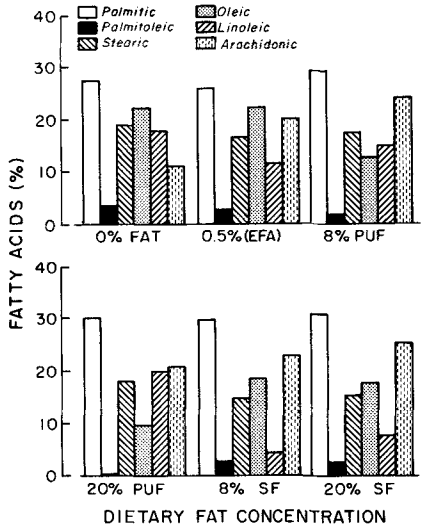

FIG. 5. Fatty acid composition of lymphocytes from dietarily manipulated mice. Animals were immunized with SRBC 6 days prior to assay. Values represent the means for all mice and at least triplicate samples for each group with 6 mice per dietary group; standard errors of the mean remained <6% throughout. This experiment was repeated 2 times.

of the fat had no influence. However, when the concentration was reduced, mice fed the PUF had suppressed responses when compared with mice fed the SF. In contrast, lymphocyte blastogenesis in response to alloantigens was not significantly influenced by saturation of the fat but by concentration only. These differences may reflect the time required for the assay. The cytotoxicity assay requires 4 hr of culture, whereas the MLC was measured after 120 hr of culture time. Thus, high levels of certain fatty acids contained in the plasma

membrane of the lymphocyte may flux into the culture medium when the cells are grown without high levels of the same exogenous fatty acid (19,20). Because it takes ca. 24 hr for cells to regain their characteristic fatty acid profiles after changing the surrounding lipid concentration, assessments of in vivo lipid modulation may be only reflected in the first 24 hr of the assay. Consequently, results of a 120 hr assay are not directly comparable with the results of a 4 hr assay and both assays may be influenced to varying degrees by the fatty acid content of the FBS. Based on the reports of other investigators (19,20), however, we would expect that the fatty acids in FBS would have little influence on a 1-4 hr assay compared to the 120 hr assay. Nevertheless, low levels of dietary fat such as EFA tend to increase the lymphocyte blastogenic responses whereas high levels tend to suppress this response. EFA deficiency, however, suppresses lymphocyte blastogenesis. Other investigators (21) have shown similar suppression of immune function; they demonstrated that subcutaneous injection of linoleic acid prolonged skin allograft survival and that a dietary deficiency of PUF resulted in immunopotentiation. In contrast, most levels of dietary fat suppressed the ability of alloantigens to stimulate blastogenesis in a mixed lymphocyte culture. Since changes in the levels of dietary fatty acids are reflected through changes in the fatty acid composition of the lymphocyte itself (22,23), the cell membrane of lymphocytes from mice fed diets with high levels of PUF or SF may have altered physical properties. A direct relationship between membrane lipid composition and surface protein mobility may exist such that the less fluid membrane lipids would result in reduced mobility of the surface proteins. This has been demonstrated in the case of patching and capping of H-2 antigens (20). Thus, changes in lateral mobility may influence the ability of H-2 antigens to stimulate lymphocyte blastogenesis in a MLC.

High levels (20%) of dietary PUF suppress direct PFC response to SRBC, whereas SF increase this response as compared to mice fed the EFA diet. This is in direct contrast to the work of other investigators (24) who have demonstrated that elevated levels of dietary PUF have no effect on the number of PFC. Since we observed no significant differences in the percentage of IgM-positive cells with dietary fat manipulation, we conclude that dietary fat influences are not due to changes in cell number but lead to a change in the frequency of B-cells responding to antigen. In addition to altering frequency, the secretory capacity of individual B-cells may be modified. Moreover, direct PFC response to SRBC appears to be inversely related to the levels of linoleic acid in the lymphocyte, i.e., as linoleic acid level increases, PFC response decreases. The correlation coefficients for all values were $r = -0.80$ ($p < 0.05$) and $r = -0.96$ ($p < 0.001$) for PUF diets only. The mechanism by which linoleic acid influences lymphocyte response is a matter of speculation. However, changes in the fatty acid composition of cell membranes can result in changes of membrane fluidity (25). These changes may adversely influence the necessary events in lymphocyte responsiveness, such as lymphocyte-antigen binding, resulting in few B-cells responding to antigen, a lower level of antibody production, or both. In view of this, we hypothesize that responses of lymphocytes to antigen may be modified depending upon the fatty acids available and that dietary fat manipulation will change the availability and thus the total fatty acid composition. Therefore, the concentration of fatty acids could affect the phase behavior of lipids, lipid-protein interaction, and conformation of glycoproteins within the membrane of the lymphocytes and immune responsiveness.

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Role of Membrane Lipids in the Immunological Killing of Tumor Cells: I. Target Cell Lipids[1]

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ABSTRACT

The metabolic and physical properties of tumor cells that are associated with their ability to resist or escape from immune attack have been investigated. The susceptibility of P815 murine mastocytoma cells to immune killing can be modulated. Culturing the cells with adriamycin or with hydrocortisone increases or decreases, respectively, the sensitivity of the cells to killing by antibody (Ab) plus complement (C); in addition, culturing the cells with mitomycin C or hydrocortisone increases or decreases, respectively, the sensitivity of the cells to killing by cytotoxic T lymphocytes (CTL). The susceptibility of the cells to Ab-C killing correlates with the ability of the cells to synthesize complex cellular lipids, but not DNA, RNA, protein, or carbohydrate. Further, tumor cells rendered sensitive to Ab-C killing by adriamycin are decreased in total lipid content and in their cholesterol/phospholipid mole ratio; hydrocortisone-treated resistant cells showed the opposite effects. The ability of tumor cells to resist CTL killing did not correlate with their total cellular lipid synthesis, but did correlate with the synthesis and composition of specific cellular phospholipids. In addition, tumor cells increased in sensitivity to Ab-C killing exhibited an increase in cell surface membrane fluidity, whereas cells increased in susceptibility to CTL attack showed an increase in their net negative cell surface charge density. These data show certain unique chemical and physical properties of tumor cells to be of fundamental importance for their ability to resist either humoral or cell-mediated immunologic attack; modulation of one or another of these cellular properties results in a change in the cells' susceptibility to immune killing by antibody plus C or by cytotoxic T lymphocytes.
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INTRODUCTION

A long-standing, and currently still accepted, dogma regarding the humoral or cellular immune killing of tumor cells is that the target cells are not actively involved in the lytic process, but merely serve to present the appropriate stimulating antigen to the attacker complex. However, recent evidence has suggested that tumor cells can play a role in influencing the outcome of immune attack. For example, it has been shown that line-1 and line-10 guinea pig hepatoma cells can bind C-fixing Ab and fix C without being killed (1). Similarly, these cells, when under Ab-C attack, demonstrate markedly higher levels of cell surface and intracellular lipid synthesis, presumably to compensate for C-induced release of lipid macromolecules from the cells (2,3). Moreover, it has been shown in a variety of cell systems that nucleated cells in different stages of cell growth show differences in their innate susceptibility to Ab-C killing (as reviewed in 1,4).

Parallel observations have been reported in systems where tumor cell killing is achieved with cytotoxic T lymphocytes (CTL). For example, it has been shown that the sensitivity of tumor cells to be killed by CTL correlates with the target cell's ability to retain the selective semipermeable properties of its membrane, not its ability to express antigen (5). Further, innate differences in tumor cell susceptibility to CTL killing during different phases of the cell cycle have been demonstrated (6).

Taken together, these observations suggest that certain metabolic activities in the target cell, or certain chemical or physical cellular characteristics that are under metabolic control, can affect the outcome of cellular and humoral immune attack. These properties of the target cell may enable the cell to "respond" to immune attack, thus preventing or repairing CTL- or C-induced damage. In addition, the chemical composition and/or physical characteristics of the target may affect its susceptibility to immune attack by affecting the efficiency or efficacy of the interaction between the tumor cell surface and the attacker principle. In the present report, we shall present our evidence that tumor cell lipid metabolism, lipid content and composition, and physical properties that are affected by lipid composition, play a significant role in the mechanism by which the cells influence the outcome of humoral and lymphocyte-mediated immune attack.

MATERIALS AND METHODS

Tumor Cells

P815 murine mastocytoma cells were maintained

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Abbreviations: Ab, antibody; C, complement; CTL, cytotoxic T-lymphocyte(s); HPLC, high pressure liquid chromatography; CHOL, cholesterol; CHL-E, cholesteryl ester(s); TG, triglyceride(s); FFA, free fatty acid(s); PA, phosphatidic acid; CL, cardiolipin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; LYPL, lysophospholipid(s).

in vitro in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 2% of a penicillin/Streptomycin/fungizone solution (Grand Island Biological Co.).

Drug or Hormone Treatment and Cytotoxicity Testing

P815 tumor cells were incubated for 1, 5, and 24 hr at 37 C in medium alone or in medium containing 20 μg adriamycin/ml, 50 μg mitomycin C/ml, 5×10^{-4} L-epinephrine methyl ether HCl, or 10^{-3}M hydrocortisone sodium succinate as described in refs. 7 and 8. The cells were then tested for their susceptibility to Ab-C killing by rabbit anti-P815 antibody plus guinea pig C in a trypan blue exclusion assay and to CTL killing by allogeneic (C57B1/6), P815-sensitized splenic T-lymphocytes in a 4 hr ^{51}Cr release assay. Details of these assays are described fully in refs. 7 and 8. In addition, after 24 hr of incubation with drug or hormone, the cells were washed thoroughly and reincubated for 1, 5 and 24 hr at 37 C in tissue culture medium alone. These reverted cells were then similarly tested for their susceptibility to Ab-C and CTL killing.

Measurement of Macromolecular Synthesis

Drug-treated, hormone-treated, or untreated P815 tumor cells were also tested for their ability to incorporate precursors of DNA, RNA protein, complex carbohydrate, and lipid synthesis as previously detailed (7). Briefly, 2.5×10^5 cells were suspended in 0.5 ml Eagle's medium containing 10 μCi [^{3}H]thymidine/ml (Amersham/Searle, Arlington Heights, IL; 24.6 Ci/mmol), 10 μCi [^{3}H] uridine/ml (Amersham; 46 Ci/mmol), 2.5 μCi [^{3}H] glucosamine HCl/ml (Amersham; 12 Ci/mmol), 5 μCi [^{14}C]palmitic acid/ml (New England Nuclear, Boston, MA; 7.06 mCi/mmol), or in 0.5 ml RPMI 1640 deficient in essential amino acids containing 10 μCi ^{14}C-labeled amino acids/ml (Schwarz/Mann, Orangeburg, NY, ca. 320 mCi/mmol of L-arginine, L-leucine, L-lysine, and L-valine). After incubation for 1 hr at 37 C, the cells were washed twice with 4 ml of ice-cold HBSS and twice with 4 ml of ice-cold 20% TCA. The TCA precipitates were solubilized in 10 ml Aquasol (New England Nuclear) and the radioactivity was quantified.

Measurement of Specific Lipid Synthesis and Lipid Composition

Drug-treated, drug-treated-recultured, hormone-treated, or untreated P815 cells were labeled with [^{14}C]palmitate as described above. The lipid fractions from these cells were extracted by using the Folch method outlined in ref. 9. Briefly, a cell pellet consisting of 5×10^6 treated or untreated cells was extracted in 25 ml of $CHCl_3/CH_3OH$(2:1, v/v) and reextracted in 8 ml of boiling $CHCl_3/CH_3OH$. The organic extract was washed with 6.5 ml of H_2O, the upper H_2O phase was removed, and CH_3OH was added until the $H_2O/CHCl_3$ interface disappeared. The final lipid extracts were concentrated to dryness by vacuum rotary evaporation and redissolved in 2 ml of $CHCl_3/CH_3OH$ (2:1).

The separation of cellular lipids and fatty acids was performed utilizing a modification of the methods outlined in ref. 10. All analyses were performed with a Waters Associates Model ALC/GPC 204 Liquid Chromatograph equipped with a Model 660 Solvent Programmer, two M6000A solvent delivery system pumps, and a Model U6K injector (Waters Associates, Milford, MA). The detection system utilized a Model 450 variable wavelength detector set at 206 nm (Waters). All data were quantified with a Waters Data Module. For the lipid separation, a μPorasil column (Waters), 3.9 mm$\times$30 cm, was used.

During the separation and quantitation of the cellular lipids, the fractions corresponding to each isolated lipid class were collected from the HPLC eluant; the fractions (1-2 ml) were placed into scintillation vials containing 10 ml Aquasol, and the radioactivity in each fraction was quantified.

Measurement of Physical Properties

The drug- or hormone-treated cells and the reverted cells were also tested for their membrane fluidity and cell surface charge properties. Isolated plasma membranes were prepared by the method of Rethy et al. (11) and tested for purity by enzymic analysis and distribution of ^{125}I-iodosulfanilic acid as described by Schlager and Ohanian (12). Fluorescence polarization measurements on the isolated membranes labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) were carried out as described by Shinitzky and Barenholz (13) and Van Hoeven et al. (14).

Cell surface charge measurements were carried out on whole unfractionated P815 cells using the method of cell partition into immiscible phases of critical mixtures of aqueous polymer solutions developed by Ballard et al. (15). This method correlates very well with whole cell electrophoresis in measuring net negative cell surface charge (16).

RESULTS

Effect of Drug Treatment on P815 Susceptibility to Immune Killing

P815 cells were increased in their susceptibility to cell-mediated killing, compared to controls, after 24 hr, but not after 1 or 5 hr, of incubation with mitomycin C (Fig. 1A). Cells washed free of drug reverted to control levels of susceptibility within 5 hr, but not within 1 hr, of reculture in

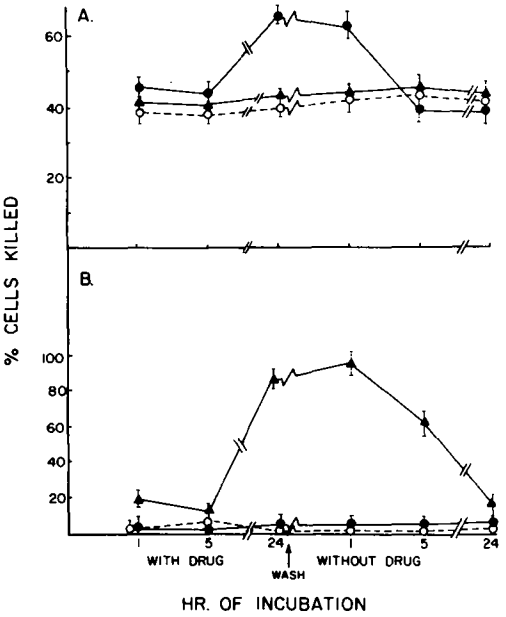

FIG. 1. Kinetics of drug effects on the susceptibility of P815 cells to killing by allogeneic P815-sensitized spleen cells (100:1, effector:target) (A) or by antibody (diluted 1:500) plus C (1:4) (B). Values represent mean ± SE of 4 experiments. Target cells were incubated with (●) mitomycin C; (▲) adriamycin; (○) tissue culture medium alone.

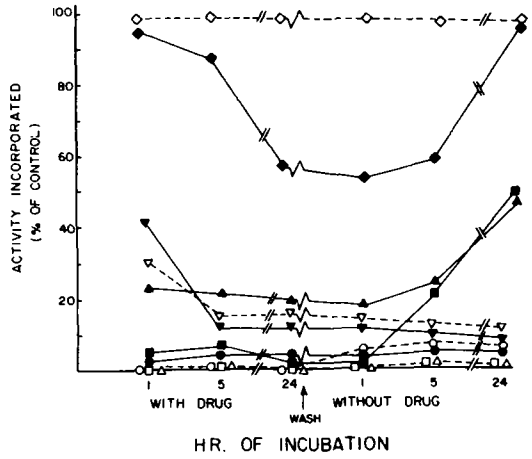

FIG. 2. Effect of treatment with adriamycin (closed symbols) or mitomycin C (open symbols) on the incorporation by P815 cells of thymidine (○, ●, control cells incorporated ca. 4100 cpm); uridine (□, ■, controls incorporated ca. 21,000 cpm); amino acids (△, ▲, controls incorporated ca. 52,000 cpm); glucosamine (▽, ▼, controls incorporated ca. 2600 cpm); or palmitic acid (◇, ◆, controls incorporated ca. 95,000 cpm). Values represent means of 4 experiments; SE omitted for clarity, remained <10% throughout.

drug-free medium (Fig. 1A). Adriamycin-treated cells remained as susceptible to cell-mediated killing as untreated controls at all time intervals tested (Fig. 1A).

As shown in Fig. 1B, P815 cells incubated with adriamycin were increased in their susceptibility to antibody-C killing after 24 hr, but not after 1 or 5 hr, of incubation with the drug. Cells washed free of the drug reverted to control levels of susceptibility within 24 hr, but not within 1 or 5 hr, of reculture in drug-free medium (Fig. 1B). Mitomycin C-treated cells were not increased in their susceptibility to C killing at any of the time intervals tested.

Macromolecular Synthesis of Drug-Treated P815 Cells

Adriamycin-treated and mitomycin C-treated P815 cells were maximally inhibited (80-99%), compared to untreated controls, in their DNA, RNA, protein, and carbohydrate synthesis within 5 hr of incubation with the drugs (Fig. 2). This was long before the cells showed an increase in susceptibility to antibody-C or cell-mediated killing (Fig. 1). In addition, mitomycin C-pretreated cells washed free of drug and reincubated in drug-free medium remained maximally inhibited in their DNA, RNA, protein, and carbohydrate synthesis 1, 5, and 24 hr after reculture (Fig. 2). Adriamycin-pretreated, -washed, and -recultured cells regained 40-50% of their RNA and protein synthesis, compared to controls, within 24 hr of reculture, but remained maximally inhibited in DNA and carbohydrate synthesis at this time (Fig. 2). In contrast, P815 cells were maximally inhibited (40%), compared to controls, in lipid synthesis after 24 hr, but not after 1 or 5 hr, of incubation with adriamycin; these cells returned to control levels of lipid synthesis after 24 hr, but not after 1 or 5 hr, of reculture in drug-free medium (Fig. 2). Cells treated with mitomycin C were not inhibited in lipid synthesis, compared to controls, at any time interval tested (Fig. 2). Total uptake by drug-treated and control cells of all radioisotopically labeled precursors used was similar (data not shown).

Effect of Hormone Treatment on P815 Susceptibility to Immune Killing

P815 cells were decreased in their sensitivity to cell-mediated killing, compared to controls, after 5 and 24 hr, but not after 1 hr, of incubation with hydrocortisone (Fig. 3A). Cells washed free of hormone reverted to control levels of sensitivity within 5 hr, but not within 1 hr, of reculture in hormone-free medium (Fig. 3A). Epinephrine-treated cells remained as susceptible to cell-

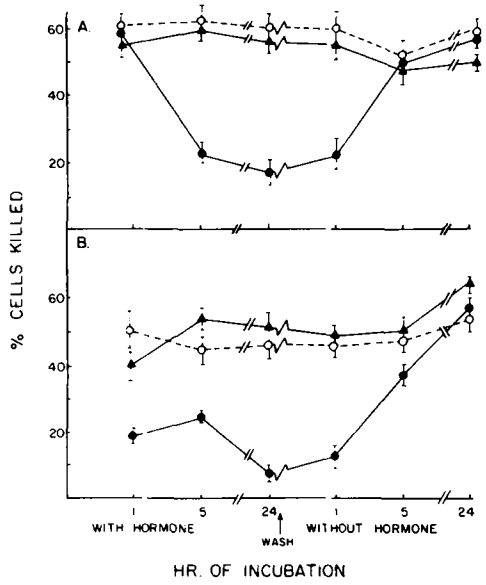

FIG. 3. Kinetics of hormone effects on the susceptibility of P815 cells to killing by allogeneic P815-sensitized spleen cells (200:1, effector:target) (A) or by antibody (diluted 1:200) plus C (1:4) (B). Values represent mean ± SE of 4 experiments. Target cells were incubated with (●) hydrocortisone; (▲) epinephrine; (○) tissue culture medium alone.

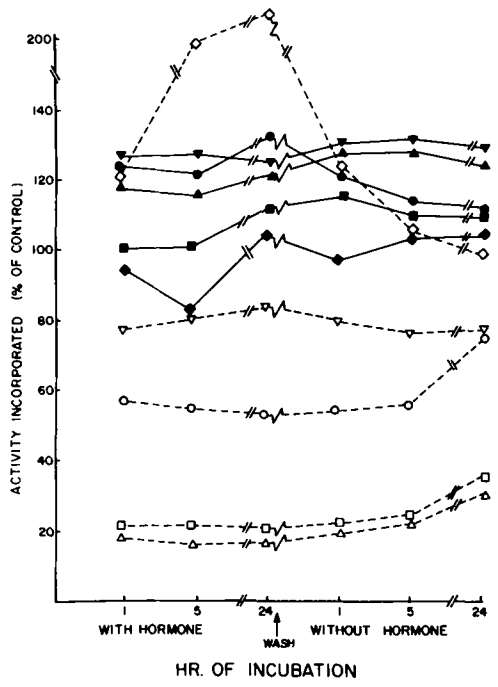

FIG. 4. Effect of treatment with epinephrine (closed symbols) or hydrocortisone (open symbols) on the incorporation by P815 cells of thymidine (○, ●, control cells incorporated ca. 3600 cpm); uridine (□, ■, controls incorporated ca. 18,500 cpm); amino acids (△, ▲, controls incorporated ca. 2450 cpm); or palmitic acid (◇, ◆, controls incorporated ca. 89,700 cpm). Values represent means of 4 experiments; SE omitted for clarity, remained <10% throughout.

mediated killing as untreated controls at all time intervals tested (Fig. 3A).

As shown in Fig. 3B, cells pretreated with hydrocortisone were increased in their resistance to antibody-C killing, compared to controls, within 1 hr of incubation with the hormones and remained more resistant after 5 and 24 hr in the continued presence of hormone. These cells washed free of hormone reverted within 5 hr, but not within 1 hr, to control levels of sensitivity to antibody-C killing (Fig. 3B). Epinephrine-treated cells remained as susceptible to antibody-C killing as untreated controls at all time intervals tested (Fig. 3B).

Macromolecular Synthesis of Hormone-Treated P815 Cells

Epinephrine-treated cells were enhanced between 1 and 36%, compared to untreated controls, in their DNA, RNA, protein, and complex carbohydrate synthesis at all time intervals tested (Fig. 4). In contrast, hydrocortisone-treated cells were depressed by 19 to 80%, compared to controls, in their synthesis of these macromolecules at all time intervals tested (Fig. 4). In addition, epinephrine treatment had no significant effect on lipid synthesis; palmitic acid incorporation into these cells was enhanced or depressed <20%, compared to controls, at any time interval tested (Fig. 4). In contrast, hydrocortisone-treated cells were enhanced 23, 194, and 222%, compared to controls, in their lipid synthesis after incubation for 1, 5, and 24-hr, respectively, with the hormone; these cells returned to control levels of lipid synthesis 5 hr after washing and reculture in hormone-free medium (Fig. 4). Neither of the hormones affected total uptake of any of the precursors of macromolecular synthesis used in these studies, compared to untreated controls.

Taken together, these data suggested that the ability of P815 tumor cells to resist antibody-C killing, but not CTL-mediated attack, depended upon the cells' ability to synthesize complex cellular lipids. In an effort to analyze further the possible role of lipids in influencing the susceptibility of tumor cells to antibody-C vs CTL attack, the specific lipid classes being synthesized by drug and hormone-treated cells, as well as the lipid composition of these cells, were examined.

HPLC Analysis of Lipid Synthesis and Composition of Drug- or Hormone-Treated P815 Cells

P815 cellular lipids were separable into neutral lipids (CHOL, CHL-E, TG), FFA, and phospholipids (PA, CL, PE, PS, PC, SPH, and LYPL) (Fig. 5). Cells that had been incubated for 24 hr with adriamycin (and were now more susceptible to antibody-C killing as shown in Fig. 1B) showed a marked decrease compared to control cells in their ability to synthesize CHL-E, PA, CL, PE, PS, and PC, with a concomitant increase in FFA accumulation (Fig. 5A). These changes were manifested in the lipid composition of the cells; adriamycin-treated cells showed a depressed content, compared to controls, of CHOL, CHL-E, PA, CL, PE, PS, and PC, with a concomitant increase in FFA content (Fig. 5B). When these cells were washed free of drug and reincubated for 24 hr in drug-free medium, they recovered control levels of susceptibility to C killing (Fig. 1B) and were indistinguishable from controls in their lipid synthesis (Fig. 5C) and composition (Fig. 5D).

In contrast, mitomycin C-treated cells (rendered susceptible to CTL killing as shown in Fig. 1A) showed a decrease, compared to controls, in their synthesis of PA, CL, PE, and PS, but a marked increase in synthesis of PC and SPH (Fig. 5A). These cells were decreased in their PA, CL, PE, and PS content, but markedly increased in their content of PC and SPH, compared to controls (Fig. 5B). When the cells were washed and reincubated for 24 hr in drug-free medium, they recovered control levels of susceptibility to CTL killing (Fig. 1A) and control levels of lipid synthesis (Fig. 5C) and composition (Fig. 5D).

As shown in Fig. 6A, cells treated with hydrocortisone for 24 hr were markedly inhibited in their synthesis of PC and SPH, whereas epinephrine-treated cells were indistinguishable in their lipid synthesis from control untreated cells. In addition, hydrocortison-treated cells showed a marked increase in their content of cellular CHOL, CHL-E, TG, and PS, and a marked decrease in PC and SPH compared to controls (Fig. 6B). Epinephrine-treated cells showed a similar cellular lipid composition to control untreated cells (Fig. 6B). Hydrocortisone-treated cells that were washed and recultured for 24 hr without hormone regained control levels of lipid synthesis (Fig. 6C) and a cellular lipid composition similar to control cells (Fig. 6D).

Effect of Drug or Hormone Treatment on P815 Cell Physical Properties

Drug- or hormone-treated P815 cells were also assessed for their plasma membrane fluidity and their cell surface charge density. Plasma membranes isolated from adriamycin-treated P815 tumor cells were nearly doubled in their fluidity compared to untreated controls; mitomycin C

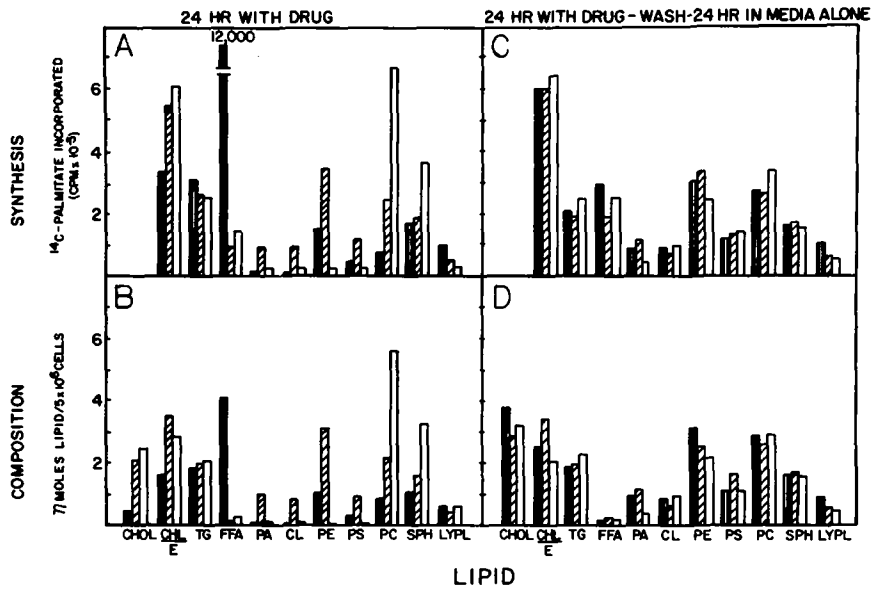

FIG. 5. Effect of incubating P815 tumor cells with adriamycin (▨) or mitomycin C (□) on cellular lipid synthesis (A) and composition (B), compared to control untreated cells (▨), and reversibility of these effects (C and D). See footnote for abbreviations.

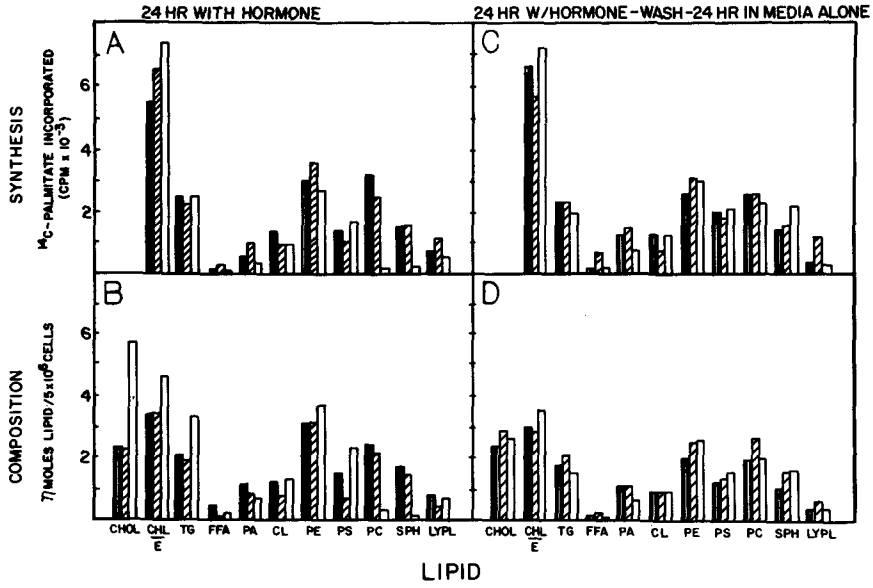

FIG. 6. Effect of incubating P815 tumor cells with epinephrine (▥) or hydrocortisone (□) on cellular lipid synthesis (A) and composition (B), compared to control untreated cells (▨), and reversibility of these effects (C and D). See footnote for abbreviations.

treatment had no such effect (Table 1A). In contrast, membranes isolated from hydrocortisone-treated cells were reduced by 55% in their fluidity compared to untreated cells; membranes from epinephrine-treated cells were the same as controls (Table 1A). Membranes isolated from drug- or hormone-treated reverted cells were indistinguishable from controls in their fluidity (Table 1A). Partition cell surface charge density measurements of normal P815 cells in culture alone for 24 hr at 37 C showed a σ value of 1.22; cells treated with mitomycin C were reduced by 30% in this value, whereas hydrocortisone-treated cells were increased by 20% in their σ value compared to the controls (Table 1B). Treatment of the P815 cells with adriamycin or epinephrine had no effect on the cells' surface charge density (Table 1B). Drug- or hormone-treated reverted cells showed surface charge density values indistinguishable from those of control, untreated cells (Table 1B).

DISCUSSION

The data presented in this report suggest that the ability of tumor cells to synthesize lipid, their cellular lipid content and composition, and their cellular physical properties controlled by lipid composition are of fundamental importance for the mechanism whereby the cells resist humoral and lymphocyte-mediated immune attack. In addition, the data show that the ability of tumor cells to resist C- or CTL-mediated killing does not depend on the cells' ability to synthesize DNA, RNA, protein, or complex carbohydrate; the drug- or hormone-induced modifications in the synthesis of these macromolecules were kinetically quite independent of their effects on the susceptibility of the cells to C or CTL killing. In contrast, the ability of the P815 cells to resist C-mediated killing correlated with their ability to synthesize lipids; the adriamycin-induced increase and the hydrocortisone-induced decrease in P815 susceptibility to antibody-C killing coincided with an inhibition and enhancement, respectively, of total cellular lipid synthesis. However, neither the mitomycin C- nor hydrocortisone-induced changes in P815 susceptibility to CTL correlated with the effects of these agents on total lipid synthesis; mitomycin C-treated cells were not inhibited in their lipid synthesis at any time interval, although the drug did render the cells more susceptible to cell-mediated killing after 24 hr in culture. Similarly, the effect on total lipid synthesis by hydrocortisone coincided with the hormone's effect on P815 susceptibility to C-mediated but not CTL-mediated killing. This suggests that the mechanism(s) whereby tumor cells can resist antibody-C and cell-mediated attack may be different. To gain insight into what these mechanistic differences might be, the metabolic activity, chemical composition, and physical properties of the cells were investigated further.

P815 tumor cells rendered sensitive to antibody-C attack are inhibited in their cholesterol synthesis

TABLE 1

Effect of Drug and Hormone Treatment on P815 Cell Membrane Physical Characteristics

Treatment	A. Fluidity (ϕ)		B. Surface charge (σ)	
	+24 hr[a]	+24 hr/−24 hr[b]	+24 hr	+24 hr/−24 hr
Adriamycin	0.50±.04	0.24±.05	1.16±0.29	1.34±0.29
Mitomycin C	0.25±.03	0.23±.04	0.86±0.19	1.42±0.22
Epinephrine	0.30±.05	0.26±.06	1.24±0.02	1.20±0.10
Hydrocortisone	0.12±.06	0.20±.04	1.47±0.19	1.21±0.13
Untreated	0.27±.06	0.21±.03	1.22±0.22	1.36±0.12

[a]P815 cells cultured with drug or hormone for 24 hr at 37 C.
[b]P815 cells cultured with drug or hormone for 24 hr at 37 C, washed, and recultured for 24 hr at 37 C in drug- and hormone-free media.

and reduced in their cholesterol content and show marginally reduced phospholipid synthesis and content (including phospholipids such as PA, CL, and PC), and a significant accumulation of FFA. P815 cells rendered more resistant to C-mediated attack show an increased synthesis and accumulation of cholesterol with little or no change in phospholipid metabolism or content. The end result of these changes is that tumor cells susceptible to C-mediated killing show a marked decrease in their cholesterol/phospholipid mole ratio, whereas resistant cells show a marked increase in this molecular relationship.

These results are also consistent with the concept that the fluidity of the tumor cell membrane may control the cell's susceptibility to C attack. The cholesterol/phospholipid mole ratio has been shown to be inversely related to cell membrane fluidity in a variety of cell systems (17,18); in addition, the efficiency of C in lysing erythrocytes, bacteria, liposomes, artificial lipid membranes, and nucleated cells has been shown to be directly related to the fluidity of the target membrane (1,4). Thus, when measured directly in these studies, the ability to modify the susceptibility of the P815 tumor cells to antibody-C killing appeared to be due to the drug- and hormone-induced modifications in cellular lipid synthesis and content with its concomitant effects on cell membrane fluidity.

In contrast, P815 cells that were modulated in their susceptibility to CTL killing were unchanged in their cholesterol/phospholipid mole ratios, but were instead markedly affected in their synthesis and content of phospholipids, especially PC and SPH. P815 cells sensitive to CTL killing showed elevated PC and SPH synthesis and content, whereas resistant cells were depressed below control levels in PC and SPH synthesis and content. Since these phospholipid molecules reside primarily in the cell surface membrane, the correlation between cell surface charge density and susceptibility to CTL killing was investigated. Using the cell partition method of Ballard et al. (15,16) to measure cell surface charge density directly, the cells rendered more susceptible to CTL killing than control cells were shown to have a lower σ value (surface charge density) than control cells, and cells resistant to CTL killing were shown to be increased in their surface charge density compared to controls.

Evidence has been presented in other systems to show that an intimate contact between a CTL and its target occurs and may or may not be antigen specific for target cell killing to occur (19,20). Despite this controversy, the present data indicate that the ability of the CTL to approach and interact with its target is as important in determining the outcome of CTL attack as antigen recognition. Lymphocytes have a relatively high σ value (15,16); thus, their ability to enter into the intimate contact with the target should depend on the σ of the target. A high-σ target cell (e.g. hydrocortisone-treated) will be more repulsive to the CTL than a low-σ (e.g. mitomycin C-treated) target; this correlates directly with the relative susceptibilities of these targets to CTL killing.

The overall significance of these results may be dramatic. Although all modifications reported here in tumor cell susceptibility to immune attack, lipid metabolism, lipid composition, and physical properties were accomplished with drug or hormone treatment, evidence has been accumulated to show that the tumor cells can accomplish such modifications themselves. In this regard, untreated P815 cells taken at different stages of their growth cycle have shown innate differences in susceptibility to C- and CTL-mediated attack; these differences occur at different times during the cells' growth cycle and appear to be correlated with the cells' intrinsic modification in lipid metabolism and physical properties (manuscript in preparation). This suggests that tumor cells have the innate capability to resist immunological attack by modifying their own lipid metabolism and physical

properties in much the same manner as has been done here with drugs and hormones. Thus, by understanding the mechanisms by which tumor cells may seek to subvert immunologic attack, we may be able to design regimens to overcome this subversion, and allow the immune system to play a more effective and potent role in tumor defense.

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Role of Membrane Lipids in the Immunological Killing of Tumor Cells: II. Effector Cell Lipids[1]

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ABSTRACT

Peritoneal macrophages (MΦ) from mice become cytotoxic after incubation in lymphokine (LK)-rich supernatants of antigen-stimulated spleen cell cultures. Tumoricidal activity is evident with MΦ treated with LK for 4 hr, becomes maximal after 8-12 hr incubation and decreases to control levels by 24-36 hr. To gain insight into LK-induced functional changes, the lipid composition of MΦ cultured with LK for 0-36 hr was analyzed by high pressure liquid chromatography. LK induced marked changes in MΦ lipid composition: cellular content of cholesterol (CHOL) and polyunsaturated fatty acids increased 2- to 3-fold after 8 hr when the cells showed maximal tumoricidal activity. Cellular lipid and fatty acid content returned to control levels by 24 hr when the MΦ had lost tumoricidal activity. These changes were not observed with equal numbers of MΦ cultured in control supernatants. To analyze further the role of CHOL and unsaturated fatty acids in MΦ tumor cytotoxicity, MΦ were enriched in CHOL or linolenic acid (18:3) and tested for their ability to kill 1023 tumor cells. Within 1 hr of culture, MΦ showed a 3- to 4-fold increase in CHOL or 18:3 content. 18:3-enriched cells were markedly tumoricidal, whereas controls cultured in delipidized medium alone or enriched with saturated fatty acid were not cytotoxic. CHOL-enriched MΦ were not tumoricidal; indeed, these cells were inhibited in their killing after treatment with LK compared to MΦ cultured in delipidized medium with LK alone. These results suggest that UFA aids, whereas CHOL negates, expression of MΦ tumor cytotoxicity.
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INTRODUCTION

Mouse peritoneal MΦ can be activated for nonspecific tumor cytotoxicity by any of several in vivo or in vitro treatments. MΦ treated with activation stimuli develop a spectrum of morphologic, functional and biochemical changes (1-4). However, only one of these changes has been shown to be a specific characteristic of the activated tumoricidal MΦ. For example, noncytotoxic cells from peritoneal exudates induced by sterile irritants (oil, starch, thioglycollate) share many properties with the activated MΦ. However, a clear functional link between metabolic modifications in MΦ and their tumoricidal activity has recently been demonstrated (4). In this regard, it was shown that there are several major modifications in MΦ lipid composition that correlate specifically with development of lymphokine-induced tumoricidal activity. Total lipid content of lymphokine-activated tumoricidal MΦ was markedly increased over that of noncytotoxic control cells; this increase was primarily due to an increase in free and esterified cholesterol. The subsequent loss of tumoricidal activity by MΦ with time coincided with a return to control levels of each of these lipid constituents (4). In addition, the UFA content of tumoricidal MΦ was markedly increased over inflammatory or unstimulated controls. The kinetics of percent UFA increase in MΦ coincided with their acquisition of tumoricidal activity; UFA content of MΦ returned to control levels as the cells lost their tumoricidal potency (4).

There have been recent reports suggesting a role for lipids, lipid-containing macromolecules, or lipid constituents, i.e. fatty acids, in the process of MΦ activation (5,6). In this regard, it has been suggested that the effect on MΦ cholesterol content by a high molecular weight serum lipoprotein is responsible for modulation of MΦ tumoricidal capacity (5). In addition, it has been suggested that alterations in the fatty acid composition of MΦ phospholipids affect the capacity of the cell for endocytosis (6,7). However, the effect of endogenous lipid and/or fatty acid composition on MΦ tumoricidal potential has not been explained fully. In this report, we have probed the roles that intracellular CHOL and UFA content play in regulating MΦ tumor cytotoxicity.

MATERIALS AND METHODS

C3H/HeN mice were injected intraperitoneally (IP) with 3 ml of 1.2% sodium caseinate. Four days later, peritoneal cells were collected from the mice as described in ref. 4 and differential cell counts were made on Wright-stained cell smears prepared by cytocentrifugation. Cell suspensions were prepared to contain 8×10^5 MΦ/ml of Dulbecco's

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Abbreviations: MΦ, macrophage(s); LK, lymphokine; CHOL, cholesterol; UFA, unsaturated fatty acid; 18:3, linolenic acid; 18.0, stearic acid.

modified Eagle's medium containing 10% fetal bovine serum delipidized by the method of Rothblat et al. (8) plus 2% antibiotics. One ml of the cell suspensions was added to 16 mm Cluster[24] culture wells (Costar) and incubated for 2 hr at 37 C in 5% CO_2-air. Nonadherent cells were removed by repeated washing. MΦ that were to be used for subsequent lipid extraction were plated onto sterile 15 mm diameter glass cover slips (Bellco Glass) that had been placed in the 16 mm Cluster[24] culture wells.

Adherent MΦ monolayers were incubated for 1 to 48 hr with LK prepared as described in refs. 4 and 9 or with varying concentrations of CHOL (from 20 to 600 nmoles/well), 18:3, or 18:0 (both at concentrations ranging from 40 nmoles to 1.0 μmole/well). The lipids used in these studies were HPLC-Standard grade (P-L Biochemicals). Lipids were dried under N_2 gas to free them of organic solvent; the lipids were then sonicated at the appropriate concentrations into medium supplemented with delipidized serum.

MΦ incubated with LK and/or CHOL, 18:3, or 18:0 were washed thoroughly and tested for their tumoricidal activity against 1023 methylcholanthrene-induced fibrosarcoma cells in a 48 hr CH_3-^{3}H-thymidine release assay described in detail in ref. 4. In addition, the cells were assessed for their lipid and fatty acid composition; MΦ lipids were extracted by subjecting the washed MΦ monolayer-containing glass cover slips to a chloroform-methanol Folch extraction (10). The lipid extracts were analyzed for lipid content and composition by HPLC as previously detailed (11). In addition, an aliquot of each lipid extract was saponified by the boiling ethanolic KOH method described in ref. 12. The resulting free fatty acids were derivatized to their p-bromophenacyl esters as described by Jordi (13); the derivatized fatty acids were separated and quantified by reverse-phase HPLC as previously described (13).

All HPLC equipment used for lipid and fatty acid analysis was from Waters Associates (Model ALC/GPC 204). Lipids were separated on a 3.9 mm × 30 cm μPorasil Column and detected at 206 nm. Derivatized fatty acids were separated on two 3.9 mm × 30 cm μBondapak C18 columns and detected at 254 nm. Quantitation was performed with a Waters Data Module/Integrator.

RESULTS

Mouse MΦ from peritoneal exudates induced by casein were activated for tumor cytotoxicity in vitro by treatment with lymphokine-rich supernatants. A typical lymphokine dose-response for induction of MΦ tumoricidal activity is shown in Figure 1A. MΦ treated with 1/3 or 1/12 dilutions of lymphokine, but not with the 1/48 dilution, showed significant cytotoxicity. In MΦ cultures treated with a 1/4 lymphokine dilution, tumoricidal activity was detected 4 hr after treatment, became maximal by 8 to 12 hr, and progressively decreased to control levels by 24 to 36 hr (Fig. 1B). Loss of MΦ cytotoxic activity with time was not due to cell

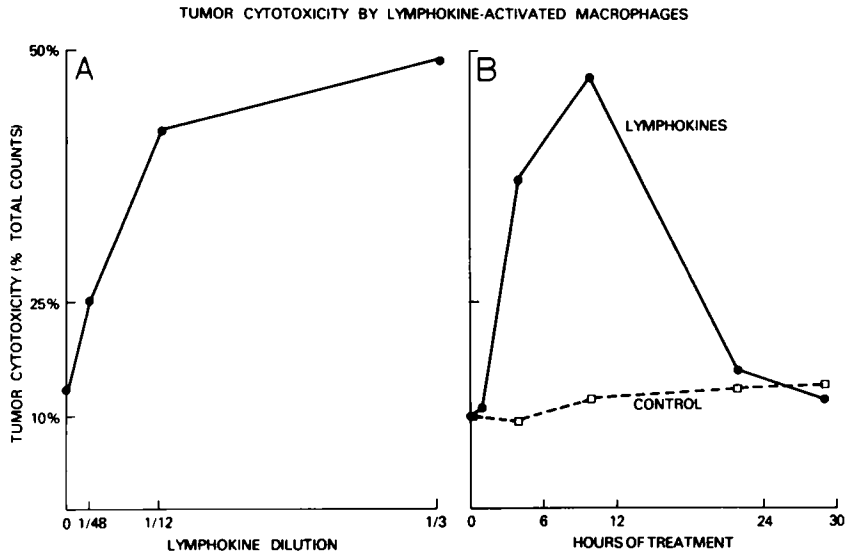

FIG. 1. Tumor cytotoxicity by lymphokine-activated macrophages. A. Effect of lymphokine dilution on induction of macrophage tumoricidal activity. Macrophages were treated with lymphokine for 9 hr. B. Kinetics of appearance and loss of macrophage tumoricidal activity in cells treated with a 1/4 lymphokine dilution.

death; no changes in vital dye uptake or in MΦ phagocytic capacity were detected. Loss of tumoricidal activity was also not due to depletion of lymphokine activity; replacement of lymphokines at 16 hr did not prolong MΦ cytotoxic activity, and supernatants from 16-hr lymphokine-treated MΦ cultures added to fresh MΦ induced strong cytotoxic activity. These results suggest that despite normal cell viability and otherwise effective lymphokine activity, tumoricidal response of lymphokine-activated MΦ in culture was gradually lost with time.

The effects of LK treatment on MΦ CHOL and fatty acid content are shown in Figure 2. As the MΦ acquired tumoricidal activity (at 8 hr incubation with LK), their CHOL content was increased 2- to 3-fold over control cells; as tumoricidal activity was lost (at 24 and 48 hr incubation in the LK), cellular CHOL content returned to control levels (Fig. 2A). With regard to UFA content, LK-activated MΦ exhibited a 2- to 3-fold increase in 18:3 with a concomitant decrease in 18:0 content as they acquired tumoricidal activity (at 8-12 hr with LK), and lost their 18:3 enrichment and 18:0 depletion as they returned to control level of tumor cytotoxicity (at 24-48 hr with LK) (Fig. 2B and C). The cells showed no major enrichment or depletion of any other identifiable fatty acid (including 16:0, 18:1, 18:2, and 20:4) during their acquisition and loss of tumor cytotoxicity (data not presented).

Taken together, these data suggest that the CHOL and 18:3 content of MΦ are of fundamental importance in their expression of tumor cytotoxicity. To gain insight into what roles these lipid molecules play in MΦ activation, experiments were undertaken to enrich the cells in these lipids artificially. As shown in Figure 3A, MΦ incubated with delipidized media enriched with 1 μmole/well of 18:3 showed a 2- to 3-fold increase in 18:3 content over controls after 8 to 24 hr incubation; after 48 hr incubation with this amount of 18:3, the intracellular 18:3 content had dropped but was still 2 times higher than the levels in control cells. Incubation of MΦ with 40 or 200 nmoles of 18:3 did not result in a significant enrichment of 18:3 (Fig. 3A).

In addition, incubation of MΦ with LK plus 18:3 caused an even greater increase in intracellular 18:3 accumulation between 8 and 48 hr compared to MΦ treated with 18:3 alone or LK alone (Fig. 3B). Interestingly, LK-treated MΦ incubated with the lower quantities of 18:3 showed an enhanced intracellular 18:3 accumulation only after 24 or 48 hr incubation (Fig. 3B).

MΦ enriched in 18:3 were markedly cytotoxic for 1023 tumor cells; MΦ cultured with 1 μmole of 18:3/well displayed marked tumoricidal activity after 8 to 24 hr of incubation with the fatty acid (Fig. 4A). Indeed, the 18:3-enriched cells exhibited

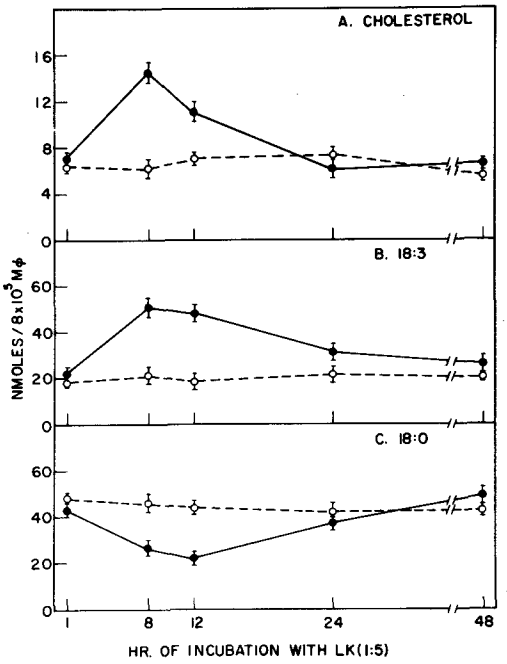

FIG. 2. CHOL, 18:3, and 18:0 content of macrophages incubated with lymphokine for 1 to 48 hr. Values represent means ± S.E. of 3 experiments. In each panel, open symbols represent lipid content of macrophages incubated in delipidized media alone.

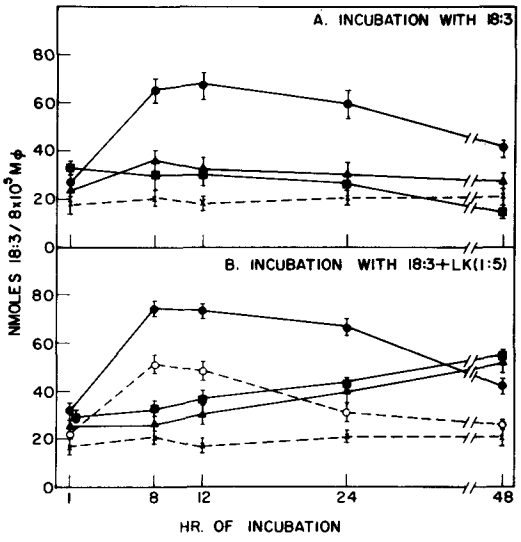

FIG. 3. 18:3 content of macrophages incubated with 18:3 (Panel A) or 18:3 plus lymphokine (Panel B) for 1 to 48 hr. (●) 1 μmole 18:3/well; (▲) 0.2 μmole 18:3/well; (■) 0.04 μmole 18:3/well; (○) lymphokine but no 18:3; (X) delipidized media alone. Values represent means ± S.E. of 3 experiments.

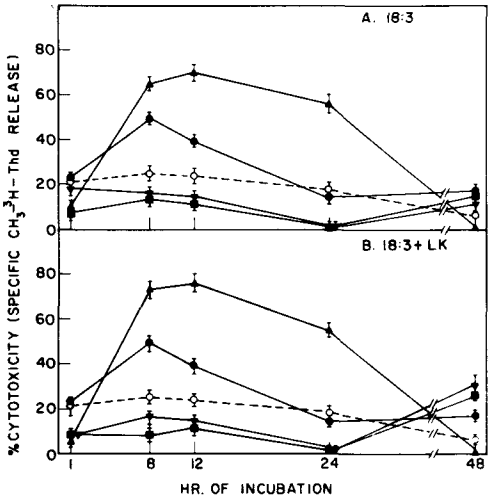

FIG. 4. Tumor cytotoxicity by macrophages incubated with 18:3 (Panel A) or 18:3 plus lymphokine (Panel B) for 1 to 48 hr. (●) lymphokine but no 18:3; (▲) 1 μmole 18:3/well; (■) 0.2 μmole 18:3/well; (▼) 0.04 μmole 18:3/well; (○) delipidized media alone. Values represent means ± S.E. of 3 experiments.

a higher percent cytotoxicity for 1023 tumor cells than MΦ treated with LK alone (70% vs. 50% peak cytotoxicity values), and were tumoricidal for longer periods of time than the LK-treated MΦ (24 hr vs. 12 hr) (Fig. 4A). MΦ that were enriched in 18:3 and treated with LK displayed the highest cytotoxic potential of all; these cells exhibited higher tumor cytotoxicity for a longer time period than MΦ treated with 18:3 alone or LK alone (Fig. 4B).

To test whether unsaturation is needed in a fatty acid for its activation of MΦ for tumoricidal activity, the experiments above were repeated with MΦ enriched in 18:0 by culturing the cells in delipidized medium supplemented with 1 μmole 18:0/well. As shown in Fig. 5, 18:0-enriched cells showed no innate tumoricidal activity compared to controls; these cells were also indistinguishable from controls in their ability to be activated to tumoricidal activity by LK (Fig. 5).

The role of CHOL in modulating MΦ tumoricidal activity was tested next. MΦ cultured in delipidized media containing from 20 to 600 nmoles CHOL/well were enriched in their CHOL content 2-fold over control cells within 1 hr of culture and remained elevated in their CHOL content through 12 hr of incubation (Fig. 6A). MΦ cultured with these quantities of CHOL and treated with LK were enhanced 3-fold in CHOL content over normal controls and even showed an increased CHOL content over MΦ treated with LK alone (Fig. 6B).

The enrichment of the MΦ with CHOL had no effect on the innate tumoricidal activity of the cells (Fig. 7A). However, MΦ enriched in CHOL were rendered refractory to activation by LK to tumoricidal activity at all time intervals tested (Fig. 7B).

DISCUSSION

Development of tumoricidal activity by mouse

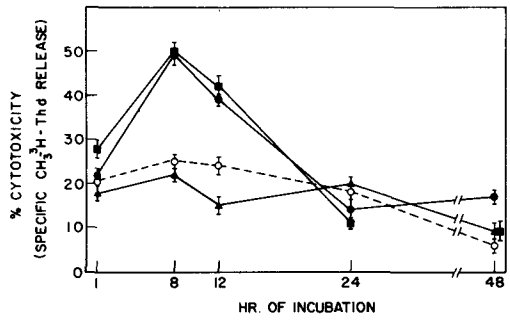

FIG. 5. Tumor cytotoxicity by macrophages incubated with 18:0 or 18:0 plus lymphokine for 1 to 48 hr. (●) lymphokine but no 18:0; (▲) 1 μmole 18:0/well; (■) 1 μmole 18:0/well plus lymphokine; (○) delipidized media alone. Values represent means ± S.E. of 3 experiments.

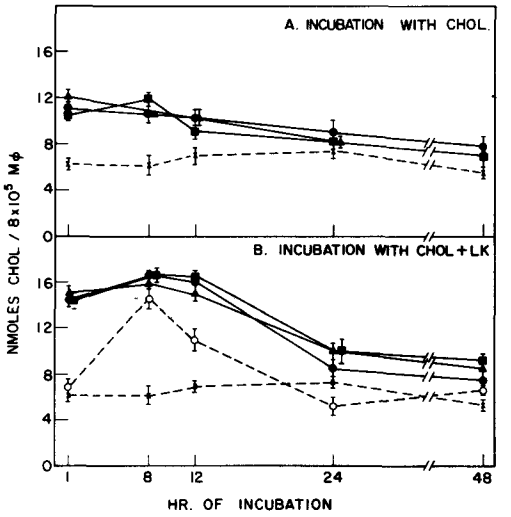

FIG. 6. CHOL content of macrophages incubated with CHOL (Panel A) or CHOL plus lymphokine (Panel B) for 1 to 48 hr. (○) 600 nmoles CHOL/well; (▲) 100 nmoles CHOL/well; (■) 20 nmoles CHOL/well; (○) lymphokine but no CHOL; (X) delipidized media alone. Values represent means ± S.E. of 3 experiments.

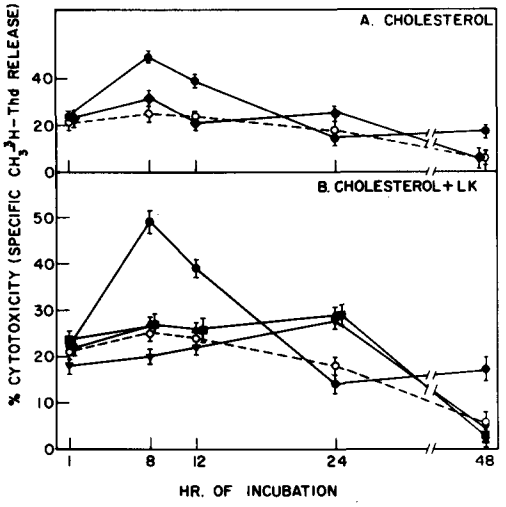

FIG. 7. Tumor cytotoxicity by macrophages incubated with CHOL (Panel A) or CHOL plus lymphokine (Panel B) for 1 to 48 hr. (●) lymphokine but no CHOL; (◆, ▲) 600 nmoles CHOL/well (with and without lymphokine, respectively); (■) 100 nmoles CHOL/well; (▼) 20 nmoles CHOL/well; (○) delipidized media alone. Values represent means ± S.E. of 3 experiments.

Mϕ treated in vitro with lymphokine-rich supernatants follows a reproducible time course: cytotoxic activity is maximal 6 to 12 hr after culture with lymphokines, it progressively decreases to control, and it reaches noncytotoxic levels by 24 to 36 hr. Such an experimental system lends itself well to a survey of metabolic properties of Mϕ that may be characteristic to acquisition and loss of tumoricidal activity. In this regard, we have shown previously (4) and have confirmed in this report that acquisition of tumor cytotoxic potential by Mϕ is accompanied by an accumulation in the cell of CHOL and UFA (primarily 18:3, at the expense primarily of 18:0).

We have now clarified these findings to provide insight into the roles that intracellular CHOL and 18:3 content of Mϕ play in their expression of tumoricidal activity. The data presented in this report show that increased Mϕ 18:3 content induces, whereas CHOL negates, expression of macrophage tumor cytotoxicity. This suggests that the LK-induced increase in these lipids in Mϕ during their acquisition of tumoricidal activity plays an opposite role. The increased intracellular 18:3 content may be providing a substrate for oxidizing intermediates in the formation by the Mϕ of toxic lipid hydroperoxides that could be exported and used as the cytotoxic principle against the tumor target cells (14-18). In contrast, the accumulation of CHOL appears to serve as a negative feedback inhibitor of Mϕ tumoricidal activity and may explain the loss of Mϕ tumor cytotoxicity with time in the continued presence of active LK molecules (see Fig. 1B). Indeed, the action of CHOL as a potential immunosuppressive agent (19) and as a modulator of membrane functional activity (20) has been described. These possibilities are presently under further investigation.

A further point of interest in these studies involves the relative amounts of 18:3 and CHOL needed to enrich Mϕ in these lipids. Compared to the baseline intracellular content of 18:3 in Mϕ (ca. 20 nmoles/8×10^5 Mϕ), a 50-fold excess of exogenous 18:3 (but not a 2- to 10-fold excess) was required to enhance the Mϕ 18:3 content. In contrast, as little as a 3-fold excess of CHOL over normal Mϕ CHOL content (ca. 5 to 6 nmoles/8×10^5 Mϕ) was sufficient to enhance Mϕ intracellular CHOL (see Figs. 2A vs. 6A). This disparity may be due to differences in the rate of 18:3 and CHOL uptake and excretion by the cells (21,22).

Taken together, these studies firmly establish the role of CHOL and 18:3 in mediating and regulating Mϕ tumoricidal activity in vitro. Further studies are needed to distinguish whether these lipids are acting during Mϕ activation or play a role in the expression of Mϕ tumoricidal activity. In addition, the role of these lipids in Mϕ action in vivo are also being considered.

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The Role of Neutral Glycolipids and Phospholipids in Myxovirus-Induced Membrane Fusion[1]

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ABSTRACT

Myxoviruses (influenza virus and paramyxovirus) enter host cells by two successive steps consisting of attachment and fusion between viral and cellular membranes. The initial attachment is known to occur through specific binding of the viruses with the neuraminic acid-containing receptors of cellular membranes. Evidence is presented here that, in the following step of membrane fusion, neutral glycolipids terminating in galactose and certain phospholipids (primarily lecithin and sphingomyelin) interact with the viral envelopes and that this interaction may be fundamental to the fusion process.
Lipids 18:489-492, 1983.

Myxoviruses contain an envelope which enwraps the ribonucleoproteins. Two types of glycoproteins are present as surface projections of the envelope (1,2); influenza viruses possess the hemagglutinin and the neuraminidase, whereas paramyxoviruses contain the hemagglutinin-neuraminidase complex and the fusion protein. During the initial phase of infection, the interactions of these glycoproteins with the host cells lead to attachment and fusion between viral and cellular membranes. The attachment occurs through a specific binding of neuraminic acid containing receptors of cellular membrane with the hemagglutinin of influenza virus or the hemagglutinin-neuraminidase complex of paramyxovirus (1,2). The fusion process seems to be mediated by lipophilic peptide segments of the hemagglutinin or the fusion proteins. But the cellular counterparts involved in this fusion step have not as yet been characterized. Evidence is given here that there is a specific interaction of the viral envelope with certain glycolipids and phospholipids preceding fusion. This protein-lipid interaction may be important for the initiation of membrane fusion and virus infection.

MATERIALS AND METHODS

Viruses

The viruses used were fowl plague virus (influenza virus) and Sendai virus (paramyxovirus). They were propagated in embryonated chicken eggs and purified according to Chuchulowius and Rott (3).

Lipids

All glycerophospholipids, cholesterol and oleic acid were pure compounds purchased from Sigma. Sphingomyelin was isolated from human brain according to Klenk and Huang (4). Ganglioside G_{M1} was obtained as described by Klenk and Gielen (5) from human brain. Gangliotetraose (Table 1, K) was prepared from ganglioside G_{M1} by repeated hydrolysis with dilute HCl (6). Ceramide lactoside (Table 1, J) was obtained from hematoside of butter milk (7) by treatment with the neuraminidase of Vibrio cholerae. Ceramide-triose (Table 1, L) was isolated from bovine spleen according to Tschöpe (8). Globoside (Table 1, M) was isolated from MDBK cells, where this glycolipid is a main component. Oleyl-lactose, phytyl-lactose and phytyl-melibiose (Table 1, N,O,P) were chemically synthesized by coupling lactose and melibiose to oleyl alcohol or phytol via the acetobromo derivatives (9). All natural and synthetic glycolipids listed above were purified to homogeniety by silicic acid column chromatography using chloroform/methanol in the ratios of 9:1, 8:2 and 1:1 as elution mixtures or by preparative silicic acid thin layer chromatography, using chloroform/methanol/water (65:25:4) as developing solvent.

Glycopeptide and Oleyl-Glycopeptide from Fetuin

Fetuin was isolated from bovine fetal serum by repeated precipitation with half-saturated ammonium sulfate. The procedure for the isolation of glycopeptide from fetuin and its derivatization into oleic acid conjugate are described elsewhere (10). Briefly, fetuin was exhaustively digested with pronase and the resulting glycopeptide (containing only a few amino acid residues) was purified by filtration through a Bio-gel P-2 column. The glycopeptide was derivatized to oleyl-glycopeptide by reacting with oleic acid anhydride in aqueous dimethylsulfoxide at 70 C for 1 hr. After this period, the excess oleic acid anhydride was hydrolyzed with 0.1 N sodium hydroxide and removed by extraction with diethyl ether after acidification with dilute HCl. The oleyl-glycopeptide was recovered from the aqueous dimethylsulfoxide phase after removal of dimethylsulfoxide by dialysis.

[1]Presented at the 73rd Annual Meeting of the American Oil Chemists' Society in Toronto, Canada, May 1982.

Virus-Induced Hemolysis

Hemolysis of chick erythrocytes with fowl plague virus and Sendai virus was carried out at pH 5.5 and pH 7, respectively, as described previously (11).

Inhibition of Hemolysis by Lipids

To a series of 10 ml conical centrifuge tubes were given 400 μl of a sodium acetate buffer (0.5 M, pH 5.5) or a sodium phosphate buffer (0.5 M, pH 7) containing increasing amounts (25 μg gradient) of various glycolipids or phospholipids. Lipids were added from stock suspensions in the form of micelles or vesicles after sonication in the respective buffer (2.5 mg/ml). In some cases (Table 1, C, H, N, O, P), to aid dispersion, some Tween 20 (about half the amount of lipid) was included during sonication. Then, 0.1 ml of fowl plague virus or Sendai virus containing ca. 100 μg of protein was added to each tube and mixed. Hemolysis was allowed to proceed for 15 min at 37 C after addition of 2 ml of a 1% suspension of chick erythrocytes. The tubes were centrifuged briefly and the supernatant was measured for hemoglobin using a Zeiss photometer (at 540 nm). The values in Table 1 indicate the concentration of lipids in the mixture which caused half-inhibition of hemolysis as compared to control tubes which contained no inhibitors. Hemolysis in control tubes gave an extinction of ca. 1.

Incorporation of Glycolipids into Erythrocyte Membrane

Glycolipids were incorporated into the membrane of erythrocytes by incubation at 37 C, in a similar manner to that described before by us and others (12,13). Incubation was carried out in a 1% erythrocyte suspension containing the indicated amounts of glycolipids or Tween 20 for 10 min at 37 C. Treated erythrocytes were washed twice with physiological saline, resuspended as 1% suspension in saline and used for hemolysis.

RESULTS AND DISCUSSION

Early studies showed that myxoviruses could specifically attach to receptors of cellular membranes containing neuraminic acid, and that such receptors could be identified in membrane components or biological fluids by their ability to inhibit hemagglutination competitively. In the present study, we used a similar approach to obtain evidence that neuraminic acid free lipids are involved in membrane fusion, a step needed for the penetration of myxoviruses into their host cells. As fusion between viral and erythrocyte membrane is linked to hemolysis (11), the lipids involved in fusion were conveniently identified from lipids of cellular membrane (erythrocyte ghost) by their ability to competitively inhibit hemolysis.

The results of such experiments are summarized in Table 1. Total lipid extract of erythrocyte ghost as such or after neuraminidase treatment showed

TABLE 1

Inhibition of Myxovirus-Induced Hemolysis by Phospholipids and Glycolipids

Lipids	Concentration (μg/ml) for 50% inhibition	
	Sendai virus	Fowl plague virus
A. Total erythrocyte lipid	160	120
B. Phosphatidylcholine	35	10
C. Phosphatidylethanolamine	>5000	>5000
D. Phosphatidylinositol	65	45
E. Phosphatidylserine	100	55
F. Cardiolipin	>5000	>5000
G. Sphingomyelin	20	10
H. Cholesterol	>5000	>5000
I. Oleic acid	>5000	>5000
J. Ceramide-glc β-gal	40	15
K. Ceramide-glc-gal-galNAc β-gal	55	25
L. Ceramide-glc-gal β-gal	30	20
M. Ceramide-glc-gal-gal β-galNAc	250	200
N. Oleyl-glc β-gal	3	6
O. Phytyl-glc-β-gal	1	5
P. Phytyl-glc α-gal	35	15
Q. Glycoprotein of erythrocyte	>5000	>5000

distinct inhibition of hemolysis. When the major lipids of erythrocyte membrane were examined separately, it was found that several pure phospholipids (phosphatidylcholine, sphingomyelin, phosphatidylinositol and phosphatidylserine) were more active than the total lipid. Conversely, other phospholipids (phosphatidylethanolamine and cardiolipin) were completely inactive. Cholesterol and oleic acid also failed completely to inhibit hemolysis. The varying hemolysis-inhibiting activity among these lipids might have arisen from the different physical states these lipids assumed after sonication. In general, it seems that liposome-forming phospholipids were active, and this is in accord with the observations of Haywood (14) and Maeda et al. (15) that liposomes containing phospholipids only could fuse directly with myxoviruses.

Glycolipids are minor components of cellular membranes. Interestingly, several glycolipids possess very potent hemolysis inhibiting activity. Brain gangliosides and hematoside had strong inhibiting activity (ca. 50 μg/ml for 50% inhibition) as expected since they contain neuraminic acid which can be involved in competition with the attachment site of myxoviruses. But several neuraminic acid free glycolipids also greatly inhibited hemolysis. These active neutral glycolipids shown in Table 1 contain galactose at terminal positions. In comparison, globoside (Table 1, M) terminating in N-acetylgalactosamine inhibited hemolysis only at much higher concentrations. Three synthetic analogues of glycolipids (Table 1, N, O, P) containing β- or α-terminal galactosides possessed high hemolysis inhibiting activity, indicating that both anomers can react with the viral envelopes.

Carbohydrate specificity was also observed with simple saccharides. Lactose was found to be active at high concentrations (3-4 mg/ml for 50% inhibition), but sugars like maltose and N,N'-diacetyl chitobiose were entirely inactive. In a further study, a long chain glycopeptide obtained from fetuin by exhaustive pronase digestion was isolated (10). This glycopeptide, which contained a long sugar chain and terminal galactose molecules, inhibited hemolysis at a high concentration (2 mg/ml for 50% inhibition). If this glycopeptide was converted to a "glycolipid" by covalently linking oleic acid to the terminal amino group, the hemolysis inhibiting activity was increased a thousandfold (2 μg/ml). This shows that, although carbohydrate may be determinant of specificity, glycolipid nature is important for effective interaction with myxoviruses. Neuraminic acid free glycoproteins did not seem to play a direct role in hemolysis as they failed to inhibit hemolysis (Table 1, Q).

None of the lipids tested impaired the adsorption process of myxoviruses, since the viruses retain their ability to agglutinate erythrocytes after interaction with these substances. The above hemolysis-inhibition results were interpreted to mean that phospholipids and glycolipids interfered with the fusion process by directly reacting with the viral glycoproteins responsible for fusion. If this is the case, then cellular membranes containing these lipids in elevated concentrations should be more readily fused by the viruses. We, therefore, performed experiments to enrich the erythrocyte membrane with several active lipids to see whether fusion by myxoviruses would now occur more extensively. All such experiments consistently resulted in great enhancement of hemolysis by myxoviruses after preincubation of erythrocytes with glycolipids terminating in galactose. Figure 1 illustrates the results obtained with oleyl lactoside. It can be seen that hemolysis steeply increased when elevating amounts of this glycolipid was used to pretreat erythrocytes (line 4). Preincubation of erythrocytes with the glycolipid alone did not lead to the same effect (line 1), nor did a detergent (Tween 20) cause hemolysis by itself in the presence of the virus (line 3). Another detergent (Triton X-100) was equally inactive under the same conditions (not shown). When globoside, an N-acetylgalactosamine-terminating glycolipid (Table 1, M), was

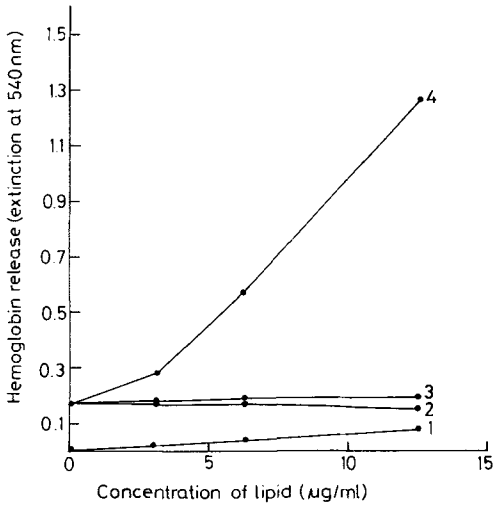

FIG. 1. Enhancement of influenza virus (fowl plague virus)-induced hemolysis by preincubation of erythrocytes with oleyl lactoside (line 4). (1) Control experiment. Erythrocytes were pretreated with oleyl lactoside and incubated in the absence of the virus. (2) Control experiment. Erythrocytes were pretreated with globoside (Table 1, M) and incubated in the presence of the virus. (3) Control experiment. Erythrocytes were pretreated with a detergent Tween 20 in the concentrations indicated and incubated in the presence of the virus. (4) Erythrocytes were pretreated with oleyl lactoside and incubated in the presence of the virus.

used to pretreat erythrocytes, there was even a slight supression of hemolysis with increasing doses of this glycolipid (line 2). This shows that the virus interacted with the glycolipids in a highly specific manner and that the effect of glycolipis observed was not due to an unspecific detergent action. These results are in accordance with the interpretation that glycolipids are reaction partners of the viral envelope which play a role in fusion. The same experiment was not performed with phospholipids, as phospholipids are the main components of cellular membrane and therefore could not be expected to be significantly enriched by incubation with exogenous phospholipids.

The relative significance of phospholipids and glycolipids for fusion remains to be established, but it is probably influenced by the accessibility of these lipids in the cellular membrane. It is possible that in natural membranes glycolipids may be more readily accessible to the virus than phospholipids because of their long extending carbohydrate chains. The dramatic enhancement of hemolysis which results when erythrocytes are preincubated with glycolipids suggests that exogenously supplied glycolipids are readily accessible to the virus and that they may have an important role in fusion. However, it remains to be determined in which way the exogenously applied glycolipids are incorporated so as to sensitize the red cells for hemolysis.

The reactivity of lipids may be determined by several factors. In the case of phospholipids, various head groups are differently charged depending on the pH. Glycolipids contain many types of carbohydrate chains subject to specific recognition in biological systems. This kind of recognition has already been described to be responsible for membrane interactions such as that occurring during intercellular adhesion (6).

From past investigations, it seems clear that neuraminic acid is the most probable attachment site of the myxovirus. The present study demonstrates that phospholipids and glycolipids, which are probably shielded from direct contact with the invading virus by masking proteins, can interact directly with the virus prior to membrane fusion. Based on a recent observation that neuraminidase is needed during myxovirus-induced membrane fusion, we postulated the existence of a second receptor in the cellular membrane, which is essential for membrane fusion but is exposed only after the action of neuraminidase (16). In this sense, the phospholipids or glycolipids described here may be such "receptors" which can be unmasked by the action of neuraminidase before they mediate fusion of membranes. It is possible that these lipids are the natural reaction partners of the F_1-fragment of paramyxovirus and the HA_2-fragment of influenza virus which contain amazingly similar hydrophobic N-terminal structures (17). The exposure of these hydrophobic peptides by a proteolytic cleavage during replication of myxoviruses is known to be essential for the fusion property and infectivity of these viruses (1,2). Lately, evidence is accumulating that not only myxoviruses but also several other enveloped viruses infect their host cells by membrane fusion. It remains to be established whether a similar mechanism of membrane fusion operates in all these cases.

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Intralipid Alters Macrophage Membrane Fatty Acid Composition and Inhibits Complement (C2) Synthesis[1]

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ABSTRACT

Intralipid (IL) is a soybean oil emulsion commonly used as a parenteral nutrient. IL is taken up by macrophages. These cells are a site of synthesis of several of the proteins in the complement system, a major mediator of the humoral system. These studies were undertaken to determine whether IL has an effect on the production of the second (C2) and the fourth (C4) components of complement by guinea pig and human macrophages in vitro. Guinea pig macrophages incubated with IL, in concentrations from 5 to 40 mg/dl, produced significantly decreased amounts of C2 and C4 when compared with controls (decreases from 40% at 5 mg/dl to 60% at 40 mg/dl). Human macrophages incubated in IL, 19 or 38 mg/dl, also produced significantly decreased amounts of C2 when compared to controls (decreases were 45 and 50% at 19 and 38 mg/dl, respectively). The maximum concentration of IL used in these studies did not significantly affect cell viability, or the production of lysozyme or β-glucosaminidase. For human macrophages, which were studied more thoroughly, the inhibition of C2 production was reversible. C2 levels returned to normal after removal of IL. Cells stimulated with opsonized zymosan produced levels of C2 comparable to stimulated control cells, despite the continued presence of IL within the cells. Human macrophages incubated with arachidonic acid, in addition to IL, produced C2 as well as control cells did. Thus, IL appears to have a selective, reversible effect on C2 production. It is possible that a general increase in fat metabolism, in response to the ingestion of IL, nonspecifically consumed arachidonic acid, decreasing its availability as a substrate for a cell product important in production of C2. Since the effects on C2 production were seen with concentrations of IL commonly seen in plasma of infants receiving IL intravenously, these studies have implications for the clinical use of oil emulsions in parenteral nutrition.
Lipids 18:493-500, 1983.

INTRODUCTION

Parenteral hyperalimentation has been used in the treatment of adults and children suffering from gastrointestinal abnormalities and malnutrition. Ten percent emulsions of oil stabilized with phospholipid are now being used as a major constituent of hyperalimentation fluids (1). At the present time, there are two oil emulsions commercially available for use in patients; IL (10% soybean oil, 1.2% egg yolk phospholipid, 2.25% glycerine in pyrogen-free water; also available as a 20% soybean oil emulsion) and Liposyn (same formulation as IL, except that the source of oil is safflower). These emulsions are commonly used in newborn infants, especially premature or small-for-gestational-age infants who frequently have feeding problems and require parenteral nutrition until adequate calories can be taken orally (2). Use of IV fat emulsions makes it possible to deliver adequate calories solely by peripheral vein, thus avoiding the use of central vein catheters and their associated complications.

Since the introduction of IL in the early 1960s, numerous animal studies have demonstrated deposition of a pigmented material containing unsaturated fatty acids and iron in macrophages, especially of the liver and the spleen (3). More recently, studies in humans have also demonstrated the presence of this fat pigment and also fat droplets in macrophages in the liver, spleen and lung (4). The fat pigment was seen in liver specimens obtained as long as 5 years after the last infusion of IL. Van Haelst and Sengers (5) noted large, solitary, or conglomerated fat droplets within lipophagosomes of macrophages on transmission electron microscopy of a liver biopsy specimen from a 5-week old child who had received IL for 12 days. They speculated that the accumulation of the fat might inhibit clearance of particulate elements by these cells.

More recently, Black et al. (6) have described a case of accidental intracranial IL infusion. Two months after the infusion, the inflammatory reaction in the brain was limited to fibrosis and an abundance of macrophages in association with the penetrating path of the needle, and macrophages in

[1]Presented at the 73rd Annual Meeting of the American Oil Chemist's Society in Toronto, Canada, May 1982.

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Abbreviations used: IL, Intralipid; C2 and C4, the second and fourth components of complement; LDH, lactate dehydrogenase; OpZ, opsonized zymosan; AA, arachidonic acid; PGE_2, Prostaglandin E_2.

the walls of the cavity. All the macrophages were lipid-laden. There was no inflammatory response to the extradural or subarachnoid deposits of the fatty material. It seems possible that the ingestion of fat by the macrophages interfered with the subsequent participation of the cells in the inflammatory process, thus reducing the usual influx of neutrophils.

Macrophages play a central role in the host defense system (7). These cells phagocytize and kill invading microorganisms, participate in surveillance against tumor cells, and synthesize and secrete numerous proteins that participate in the inflammatory process. Included in the proteins synthesized and secreted by macrophages are several proteins in the complement system (8), which is a major mediator of the inflammatory process (9). The effect of lipid emulsions on the capacity of macrophages to synthesize and secrete complement proteins had not been studied. These studies were designed to determine if macrophages that had ingested IL in vitro could synthesize normal amounts of the second component of complement, C2. C2 was chosen because it is one of the limiting components in the complement activation sequence.

METHODS

The methods of culture of guinea pig peritoneal macrophages (10), human peripheral blood monocytes (11), the functional assays of C2 (12), LDH, β-glucosaminidase and lysozyme (13), and the assays for cellular protein (14) and DNA (15) have been published. Intralipid was obtained commercially.

Cell Fatty Acid Analysis

Total lipids were extracted using the chloroform methanol (2:1) system of Bligh and Dyer (16). The crude lipid extract was then fractionated by silicic acid chromatography (17), separating neutral lipids from phospholipids. Recovery of lipids from the silicic acid chromatography was greater than 95%. The identity of these lipid fractions was confirmed by thin layer chromatography, as described by Skipski et al. (18). The lipids in the two fractions were hydrolyzed and the resulting fatty acids methylated, using the method of MacGee and Allen (19). The fatty acid esters were separated with gas-liquid chromatography containing a glass column, 4 mm i.d. x 6 ft., packed with 10% EGSS-X on gas-chrom P, 100-120 mesh. This column permitted good separation of the 16:0 through 20:4 fatty acid methyl esters.

RESULTS

The Effect of IL on C2 Production by Guinea Pig Peritoneal Macrophages

Initial studies of the effect of IL on macrophage function were done using guinea pig peritoneal macrophages because the morphology and function of these cells are well characterized and easily obtained for study in vitro (10,20). Ingestion of the IL by the macrophages after periods as short as 4 hr was confirmed by staining for neutral fat in the cells with oil red O and by transmission electron microscopy. Throughout the 48-hr culture period used for these studies, there was no evidence of lipid digestion within the lipophagosomes. However, there was evidence that the macrophages could partially digest and process the IL. Transmission electron microscopy demonstrated that 45% of the cells contained IL after a 4-hr culture period in the presence of IL. Parallel cultures of macrophages were incubated with IL for 4 hr and then washed and incubated for an additional 20 hr to allow the cells to digest the IL. When these cells were examined by transmission electron microscopy, only 10% of the cells contained IL, indicating that the macrophages had been able to digest some of the IL that had been ingested during the first 4 hr of culture.

Initial experiments indicated that cells incubated with IL, 40 mg/dl, produced decreased amounts of C2 and C4 when compared to control cells. In subsequent experiments, a concentration-response relationship for the effect of IL on C2 and C4 production was established. As shown in Figure 1, cells incubated in concentrations of IL as low as 2.3

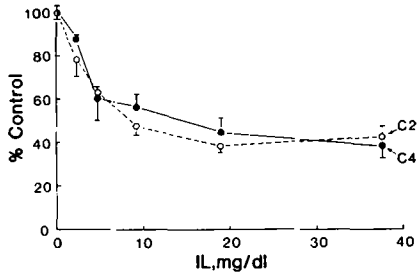

FIG. 1. Effect of IL on the capacity of guinea pig peritoneal macrophages to produce C2 and C4. Peritoneal exudate cells (80% large mononuclear cells) were harvested 4 days after intraperitoneal injection of sterile 3% acqueous solution of hydrolyzed starch in Hartley guinea pigs. After washing, the cells were incubated for 2 hr at 37 C to allow adherence of macrophages. The nonadherent cells were removed and the macrophages cultured in medium 199 containing 10% heat-inactivated fetal calf serum (56 C, 2 hr) alone or in medium 199 containing fetal calf serum and IL, in concentrations ranging from 2.3 to 37.5 mg/dl. After 48 hr in culture, the media were removed and titrated for C2 and C4 by using the appropriate hemolytic assay. The data are expressed as the mean ± SE for duplicate plates in 4 separate experiments. Production of both C2 and C4 by cells incubated in all concentrations of IL was significantly reduced when compared to the control, as determined by analysis of covariance ($p < 0.01$) (modified from Strunk et al. (10)).

mg/dl, produced decreased amounts of both C2 and C4 (10). Several lines of evidence indicated that the effect of IL on the C2 and C4 was due to decreased synthesis of the proteins (10). First, the IL did not interfere with detection of C2 or C4 in the medium. Second, the amount of C2 and C4 was decreased within the cells incubated with IL, indicating that the effect of IL on the production of C2 and C4 was not simply due to decreased secretion. Third, the IL did not affect secretion of other proteins by the cells. Fourth, the cells incubated with IL did not secrete proteases that cleaved the complement proteins. In addition, the effect of C2 and C4 synthesis was not the result of a generalized dysfunction of the cells, for cells remained viable.

The effect of the IL was due to the oil component of the emulsion because the phospholipid and glycerol had no effect on complement synthesis (10). Incubation of the cells with an emulsion made with castor oil also reduced C2 and C4 synthesis, suggesting that the particular fatty acid composition of the soybean oil was not responsible for the effect on C2 and C4 synthesis (10).

In the guinea pig macrophages, the effect on complement synthesis was seen with concentrations of IL commonly observed in blood of newborns receiving IL for hyperalimentation (21). Because of the possible clinical implications of these results, we have studied the effect of IL on human monocyte-derived macrophages, peripheral blood monocytes that have been allowed to mature into macrophages during extended periods in culture (13). Studies with human cells have the obvious advantage of being more relevant to the patient receiving IL, and these cells also remain in culture for longer periods of time, during which they can be manipulated.

The Effect of IL on C2 Production by Human Peripheral Blood Monocyte-derived Macrophages

Early in culture, peripheral blood monocytes are small, rounded, dense cells. After several days in culture, the cells spread and acquire morphologic characteristics of macrophages. The morphological changes are associated with changes in the functional capability of the cells, including the capacity to synthesize and secrete C2 (22). The effect of IL on C2 production was determined using cells that were producing C2 at a constant rate. Mononuclear phagocytes that had been in culture for 7 days were washed and then cultured in medium alone or in medium containing IL, in a final concentration of 9.5, 19, or 38 mg/dl, for 48 hr (23). The effect of IL on the production of C2 during this 48 hr interval is shown in Figure 2. The level of C2 produced by cells incubated in 19 or 38 mg IL/dl was significantly reduced when compared to C2

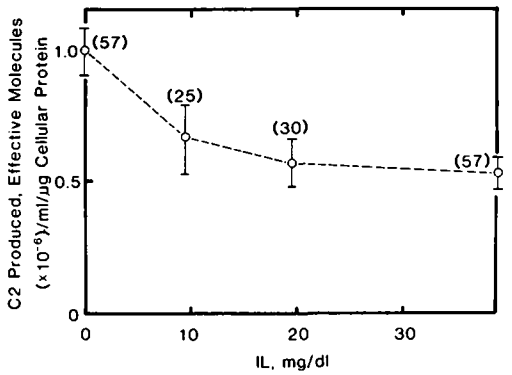

FIG 2. Effect of IL on capacity of human mononuclear phagocytes to produce C2. Mononuclear cells were separated from normal human peripheral blood by Ficoll-Hypaque; the monocytes were then adhered to plastic and cultured in medium containing heat-inactivated (56 C, 30 min) autologous serum (M199-HAS). After 7 days in culture, the conditioned medium was removed, the cells were washed and then cultured in fresh medium alone or in medium containing IL, 9.5, 19 or 38 mg/dl. After an additional 48 hr in culture, the media were removed and titrated for C2 by using the hemolytic assay. The data are expressed as the mean ± SE of the activity of C2 in the culture medium, corrected for amount of protein present in the cells adhered to the dishes at the end of the 48 hr incubation period. The number of plates for each condition is shown in parentheses. The amount of C2 produced by cells incubated in IL, 19 mg/dl or 38 mg/dl, was significantly reduced when compared to the control, as determined by analysis of covarience ($p < 0.05$ & $p < 0.01$, respectively) (Reproduced with permission from J.Immunol. 126; 2267-2271, 1981).

produced by the control cells. The decrease in the amount of C2 present in the medium was not secondary to an effect of IL on the hemolytic assay, since addition of IL to serum or "conditioned medium" from control cells did not affect C2 activity. In addition, the reduction in C2 production could not be explained by decreased secretion of C2 after normal synthesis, since intracellular C2 for both control and IL-treated cells was 1% or less of total secreted C2 (23). Ingestion of IL during the 48-hr incubation period was confirmed by transmission electron microscopy which showed an accumulation of lipid droplets in the cytoplasm.

Is the Effect of IL on C2 Production by Human Monocyte-derived Macrophages Reversible?

Two independent methods were used to determine whether the effect of IL on C2 production was reversible. First, the response of IL-treated human macrophages to a particulate stimulus, opsonized zymosan, was determined. Cells that were being incubated either in control or IL-containing medium

were allowed to phagocytize opsonized zymosan and the effect on C2 production was determined. Cells incubated in medium without IL produced significantly more C2 when exposed to opsonized zymosan ($p < 0.01$) (Fig. 3) (23). The amount of C2 produced by cells which were cultured with IL and opsonized zymosan was significantly increased ($p < 0.001$) when compared to the amount produced by cells in IL alone. The amount of C2 produced by the stimulated IL-treated cells was comparable to the amount produced by stimulated control cells, indicating that IL-treated cells could respond to a stimulus as well as control cells.

Second, reversibility of the effect of IL on the human macrophages was assessed by washing cells to remove IL and other components of the conditioned medium. The cells were then cultured in fresh medium without IL for an additional period before the medium was removed and titrated for C2. Cells were incubated with IL for either 12 or 48 hr before washing to remove IL. As shown in Figure 4, IL, 40 mg/dl, inhibited C2 production by 46% during the first 12 hr of culture (Fig. 4A, IL). When the cells were incubated with IL for 48 hr, the inhibition increased to 63% (Fig. 4B, IL). Cells were able to recover the capacity to produce C2 after being in culture with IL for either 12 or 48 hr. After incubation with IL for 12 hr, the cells were able to produce 90% as much C2 as the controls in the 36 hr after the IL had been washed away (Fig. 4A-Recovery). Cells that had been incubated with IL for 48 hr were able to recover most of the capacity to produce C2 in the 72 hr after IL had been removed (C2 production was inhibited by only 18%

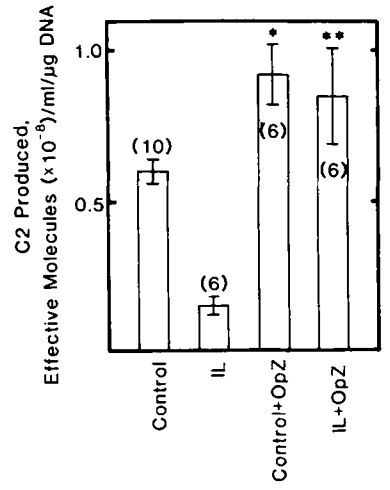

FIG. 3. Reversal of the effect of IL on the capacity of mononuclear phagocytes to produce C2 by incubation of the cells with opsonized zymosan (Op Z). Mononuclear phagocytes were cultured for 7 days before they were washed and incubated in M199-HAS alone or in medium containing IL, 38 mg/dl, as described in legend for Figure 1. During the second 24 hr of the incubation period with IL, opsonized zymosan, 10 particles/cell, was added to one-half of the control and IL plates. After a total of 48 hr incubation, the media were removed, centrifuged at 0C to remove particles that had not been ingested, and then titered for C2. Opsonized zymosan did not contribute directly to the amount of C2 in the medium, since fresh medium containing the particles had *no* C2 hemolytic activity and addition of the particles to "conditioned medium" from control cells did not affect the C2 titer. The data are expressed as the mean ± SE of the C2 in the culture medium, corrected for the amount of DNA present in the cell adherent to the dishes at the end of the 48 hr incubation period. The number of plates for each condition is shown in parentheses. The total amount of DNA was not significantly affected by any of the culture conditions; DNA were used to standardize the C2 instead of cellular protein because the zymosan interfered with the protein assay. *Significantly different from control ($p < 0.01$ for Student's 2-tailed t-test); **significantly different from IL ($p < 0.001$) (modified from Kolski and Strunk (23)).

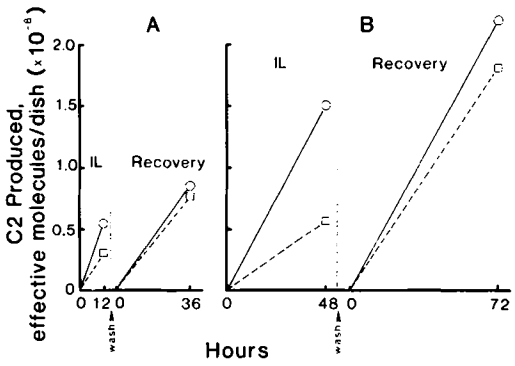

FIG. 4. Reversal of effect of IL on the capacity of human macrophages to produce C2 by removing the IL from the medium. Peripheral blood monocytes were cultured for 7 days to promote maturation of the cells into macrophages. The cells were washed and cultured in medium alone (O), or in medium containing IL, 38 mg/dl (□). After 12 hr (A-IL) or 48 hr (B-IL), the cells were washed 4 times to remove conditioned medium and IL, and then cultured for an additional 36 hr (A-Recovery) or 72 hr (B-Recovery) in medium without IL. The media were titered for C2 by using the hemolytic assay.

in this period - see Fig. 4B - Recovery). The human monocyte-derived macrophages were able to clear the IL as determined by transmission electron micrographs of the cells. After 48 hr incubation with IL, all cells contained large quantities of fat droplets. Cells initially incubated with IL for 48 hr, and then incubated for 48 hr in medium without IL were similar in appearance to the cells that had never been incubated in IL.

Selectivity of IL Effect

In order to assess whether the decreased C2 production by human monocyte-derived macro-

phages was associated with a general alteration of cell function, several additional functions were studied during the 48 hr period of incubation with IL (23). First, the release of the intracellular enzyme, LDH, into the extracellular medium was studied. This method is often used to assess cellular viability (24). LDH release was not affected by incubation of the cells with IL (percentage of LDH released in medium was 15 ± 5 for controls (mean ± S.D. for 12 plates) and 14 ± 10 for cells incubated with 38 mg/dl) (23). Second, production of lysozyme, an enzyme that is secreted and not stored intracellularly, and β-glucosaminidase, a lysosomal enzyme, were not significantly affected by incubation with IL (values for controls were 23 ± 12 μg lysozyme and 443 ± 65 μmol of β-glucosaminidase substrate reduced/μg cellular protein, compared to 23 ± 10 and 436 ± 70 for IL-treated cells, respectively) (23). Third, the capacity of cells to remain adherent to the plastic substratum and to produce total cellular protein, as measured by DNA and total protein of adherent cells, also were not altered by incubation with IL (values for controls were 2.2 ± 0.8 μg DNA/plate and 61 ± 30 μg total protein/plate, compared to 2.2 ± 0.7 and 63 ± 30 for IL-treated cells, respectively) (23).

Relationship of IL Effect to AA Metabolism

Revsin et al. (25) noted that the lipid profile of human fibroblasts in culture was altered after exposure to IL. They noted that cells incubated with IL had markedly increased levels of oleic (18:1) and linoleic (18:2) acids, the major constituents of IL, but that the levels of AA (20:4), were significantly reduced. Experiments were performed with guinea pig macrophages to determine if the effect of IL ingestion on these cells was similar to the effects observed on the fibroblasts. Incubation of the cells with IL significantly altered the fatty acid composition (Table 1). Similar to the results in the studies with fibroblasts, there was a decrease in the percentage of 20:4 in the phospholipid fraction and this change was not observed in the neutral lipid fraction. In addition to the changes in 20:4, percentages of 18:1 and 18:2, the major constituents of IL, increased significantly in both lipid fractions. Percentages of 16:0 increased significantly in the phospholipid fraction, but did not change in the neutral lipid fraction, whereas the percentage of 18:0 did not change significantly in the phospholipid fraction, but decreased significantly in the neutral lipid fraction. In contrast, during the 48 hr in culture with medium containing 10% heat-inactivated fetal calf serum alone, the only changes noted in the fatty acid composition of the control cells were a significant decrease in the percentage of 18:2 in the phospholipid fraction (from 22.4 ± 1.8% after the initial adherence to 14.2 ± 1.8% after the cells had been in culture for 48 hr) and an increase in the percentage of 20:4 in the phospholipid fraction (from 21.4 ± 4.4 to 30.6 ± 5.4).

Arachidonic acid is present in the membranes of all cells, including macrophages. Upon stimulation of macrophages, arachidonic acid is released from phospholipids and is then converted into metabolites that have potent regulatory effects on macrophage function (26-28). Since the cells incubated with IL had decreased levels of AA, specifically in the phospholipid fraction, we speculated that the decreased availability of AA might have been related to the effect of IL on C2 production. Therefore, we tested the effect on C2 production of

TABLE 1

Effect of IL on the Fatty Acid Composition of Guinea Pig Peritoneal Macrophages[a]

Individual fatty acids	Composition			
	Phospholipids		Neutral lipids	
	Control	IL	Control	IL
	%			
16:0	13.2 ± 2.2	22.3 ± 3.8	30.6 ± 5.0	24.0 ± 2.2[c]
18:0	26.9 ± 2.3	24.9 ± 2.2	22.3 ± 2.2	11.9 ± 1.2[c]
18:1	15.2 ± 1.7	19.8 ± 3.0[b]	21.4 ± 2.2	30.5 ± 2.7[b]
18:2	14.2 ± 1.8	17.6 ± 2.4[b]	14.0 ± 1.2	26.1 ± 3.9[b]
20:4	30.6 ± 5.4	15.5 ± 3.1[c]	11.8 ± 6.0	6.8 ± 0.9

[a]Guinea pig peritoneal macrophages were prepared as described in legend for Figure 1. After 48 hr in culture with medium containing 10% heat-inactivated (56 C, 2 hr) fetal calf serum alone (control) or medium with 38mg IL/dl (IL), the cells were lysed and the fatty acid composition determined as described in the methods. Results are mean ± S.D. for duplications in 5 separate experiments.

[b]Significantly greater than control, $p<0.01$, by the 2-tailed Student's t-test.

[c]Significantly less than control, $p<0.01$.

addition of AA to cells being cultured with IL. As shown in Figure 5, human monocyte-derived macrophages incubated with IL produced decreased amounts of C2 when compared to control cells. When the cells were incubated with both AA and IL, the cells were able to produce significantly more C2 than when incubated in the IL alone. AA alone in concentrations of up to 10 mg/dl had no effect on C2 production.

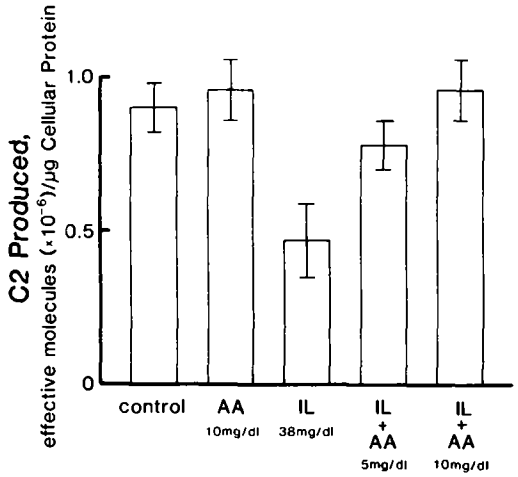

FIG. 5. Effect of arachidonic acid (AA) on the capacity of IL-treated and control human monocyte-derived macrophages to produce C2. Experiment was performed as described in the legend for Figure 2. The data are expressed as the mean ± S.D.; the number of plates for 7 for control, 8 each for AA and IL alone, and 10 each for IL and the 2 concentrations of AA. C2 production by cells incubated with IL was significantly decreased ($p < 0.01$) when compared to control. C2 production by cells incubated with IL and AA, both 5 mg/dl and 10 mg/dl, were significantly ($p < 0.01$) increased when compared to cells incubated in IL alone (modified from Kolski and Strunk (23)).

DISCUSSION

Following the observation that pigmented material is deposited in macrophages in the liver, spleen and lung during IL therapy, a number of studies have been done to determine if infusion of IL might result in impairment of the immune system. Older observations suggested that infusion of certain fatty acids (29) and dietary-induced chylomicronemia (30) produced marked inhibition of neutrophil chemotaxis in animals. Therefore, several authors have studied the effect of IL on neutrophil function. Nordenstrom et al. (31) and Fischer et al. (32) noted that incubation of normal human neutrophils with IL reduced the capacity of these cells to migrate both randomly and toward a chemotactic stimulus. However, extremely high concentrations of IL, 2500 mg/dl or higher, were required. Since concentrations in patients do not often exceed 1000 mg/dl, even in infants with decreased clearance of fat (21), the relevance of these observations was questioned. English et al. (33) subsequently demonstrated that the effect of these extremely high concentrations of IL on the neutrophil function was not due to the ingestion of IL by the neutrophil, but was related to the permeability of the neutrophil membrane to the glycerol in the IL. Two groups also studied the effect on neutrophil function of infusion of IL into normal adults. Even when amounts as high as 80 g IL were infused over a 2-hr period (equivalent of 12 g/kg/24 hr, with the usual maximal amounts being 4 g/kg/24 hr), there was either no effect on neutrophil chemotaxis (34) or a minimal effect that was completely reversed by 24 hr after the infusion was stopped (31). These studies suggest that IL infusions have no significant effect on neutrophil migration.

As mentioned earlier, numerous studies in animals and humans have demonstrated the presence of fat in macrophages in the liver, spleen and lung after infusion of IL. Van Haelst and Sengers (5) speculated that the accumulation of fat might inhibit clearance of particulate elements by these cells. In spite of these findings, several studies have demonstrated that the clearance function of the macrophage system apparently remains intact even after prolonged IL therapy. Rabbits given IL, 4 g/kg/24 hr for periods up to 6 days, had normal or increased capacity to clear radiolabeled endotoxin from the plasma, a function of macrophages of the liver, spleen, bone marrow and lung (35). Bradfield (36) demonstrated that infusion of a large amount of IL into mice, 1.6 g/kg as a single bolus, did not reduce clearance of ^{51}Cr-labeled sheep red blood cells by liver macrophages, whereas infusion of dextran sulfate, an agent known to block the uptake of particles by the macrophage system, reduced the clearance to less than 50% of control levels. Jarstrand et al. (37) demonstrated that patients receiving IL had a normal capacity to clear ^{125}I-microaggragated human serum albumin, a particle known to be cleared by the macrophage system.

In contrast to the studies on neutrophil chemotaxis and the capacity of macrophages in the liver and spleen to clear endotoxin or foreign particles, our studies with both guinea pig macrophages and human monocyte-derived macrophages indicate that IL significantly decreased the capacity of these cells to synthesize C2. The guinea pig macrophages appear to be more sensitive to the effects of IL, in that the levels of IL required to produce an effect

on the capacity of these cells to produce C2 were lower than in the human cells. In addition, the human cells were able to recover the capacity to produce C2 within 48 hr after the IL was removed from the medium, but the guinea pig cells did not recover by 48 hr. It is possible that the human cells can metabolize IL more efficiently than the guinea pig cells. Human monocyte-derived macrophages are known to contain lipoprotein lipase (38), an enzyme that is capable of metabolizing IL.

The ingestion of IL by these cells was also associated with an alteration of the fatty acid composition of the cells, with increased levels of palmitic, oleic, and linoleic acids and decreased levels of stearic and arachidonic acids. Because AA metabolites are known to be modulators of macrophage function, we speculated that the alteration of AA metabolism might be related to the reduction in C2 synthesis. Enrichment of the IL-containing medium with AA reversed the effect of IL on C2 production. It is possible that nonspecific utilization of AA during generalized increased metabolism of fat in response to the abnormally high fat content of cells might decrease the availability of AA as a precursor of a metabolite that is responsible for maintaining C2 production. The compound is not PGE_2, an AA metabolite known to be produced by macrophages, since the ingestion of IL by the human monocyte-derived macrophage did not alter production of PGE_2 (23). The findings of Spector et al. (39) support this hypothesis. They cultured human endothelial cells with linoleic acid alone, and noted a significantly reduced percentage of AA in the cells and a decreased capacity of the cells to produce prostacyclin. Addition of AA, 12 mg/dl, to the linoleic acid-containing medium reversed the effect of the linoleic acid on prostacyclin production.

The effect of IL on C2 synthesis was seen with concentrations of IL commonly observed in patients receiving IL for parenteral nutrition (21,40). A group of small-for-gestational-age infants had mean plasma levels of 222 mg IL/dl after 6 hr of continuous infusion at a rate of 3.6 g/kg/24 hr (40), rates commonly used. Even appropriate-for-gestational-age infants, who tolerate IL much better than small infants, had mean plasma levels of 86 mg IL/dl at this infusion rate (40). These levels are far above the 20 mg/dl that produced significant effects on C2 synthesis in human monocyte-derived macrophages. The function of the complement system is depressed in newborn infants (41), apparently because of an immaturity of the various cell types that synthesize the complement components. Theoretically, the ingestion of IL in vivo by macrophages, which are sites of synthesis for several of the complement components, could have an effect of the level of function of the complement system in newborn infants.

There have been no reports of levels of C2 or other complement components in infants receiving IL for parenteral nutrition. Our data suggest that potential effects of IL in C2, and perhaps other complement components, would most likely be avoided if plasma levels of fat were kept at low concentrations (less than 20 mg/dl), and furthermore, that an intermittent infusion schedule might be considered, allowing the macrophages an opportunity to clear the IL.

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Kinetic Analysis of Plasma VLDL-TG and VLDL-Remnant-TG Turnover in Anesthetized Rats

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ABSTRACT

We have estimated the turnover and relative pool sizes of nascent-VLDL-TG and VLDL-remnants-TG in anesthetized rats. [1-^{14}C]Palmitoyl- and [2-^{3}H]glyceryl-labeled "VLDL"-TG (including nascent VLDL-TG and VLDL-remnants-TG) were prepared by injecting labeled palmitate and glycerol into donor rats. Labeled serum from these rats was then injected intravenously into nembutalized male rats and serial blood samples taken for 30 min. Special care was taken to define any early components in the labeled "VLDL"-TG disappearance curves. In other experiments, the donors were rendered functionally hepatectomized 30 min after injection of ^{3}H-glycerol and the endogenous labeled VLDL-TG was allowed to circulate 30-60 min before collection of the TG-labeled VLDL-remnants-containing serum. The latter was injected into 4 recipient nembutalized rats and the remnant-TG-turnover measured by serial sampling as above. In two cases, ^{14}C-"VLDL" and ^{3}H-VLDL-remnants were injected as a single bolus into ether-anesthetized rats. Despite its complex composition, "VLDL"-TG in most cases disappeared in a single exponential fashion for 30 min with an average half-life of 5.9 min in nembutalized and 2.8 in ether-anesthetized rats. VLDL-remnants-TG showed a more complex behavior, but contained a major rapid component with a mean $t_{1/2}$ of ca. 1.5 min in both groups. The data, analyzed by multicompartmental analysis, were fitted to a simple model in which turnover of a larger nascent VLDL-TG pool with formation of a more rapidly turning over smaller pool of VLDL-remnant-TG is the rate-limiting step in overall TG removal from the $d < 1.006$ fraction of rat serum. The data are consistent with our theoretical prediction that under these conditions the kinetics of the VLDL-remnants cannot be resolved from analysis of the total composite "VLDL" (nascent plus remnant) pool.
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INTRODUCTION

Recent evidence presented by Mjøs et al. (1) and others (2-4) shows that the very low density lipoprotein (VLDL, $d < 1.006$) fraction of rats is a complex mixture of VLDL particles newly secreted by liver and intestine and the corresponding remnants of these particles (1). In the rat, plasma VLDL and their remnants probably each comprise 2 distinct types of particles: those containing apoB-PI(II), which are derived from the liver, and those containing apoB-PIII which also derive predominantly from liver, as well as from the intestine (2-5); moreover, both VLDL and their remnants each contain a wide spectrum of particles of differing TG content and sizes (1,6,7). For this reason, it is not surprising that labeled plasma TG or VLDL-TG may show a complex kinetic behavior when reinjected into rats (8). However, such complexity is not always seen. Several authors using different experimental conditions have reported that labeled VLDL-TG, when reinjected into rats, decays in a simple exponential fashion (9-11). In order to understand how the complex "VLDL"-TG ("VLDL"- is used throughout the paper to depict the mixture of apoC-rich and apoC-poor VLDL-particles (VLDL-remnants) in the complex $d < 1.006$ fraction) fraction could decay in a simple first-order manner, or to predict conditions under which a complex kinetic pattern might be expected, we sought published data regarding the relative pool size and turnover rate of VLDL-remnants-TG in rats. We have been unable to find this information.

The literature does contain several important references describing methods for preparing endogenously labeled VLDL remnants (1,6,7), the chemical composition of remnants (1) and the rates of hepatic uptake of remnant components both in vivo (12,13) and in vitro (7). Based upon these reports, we have designed 2 studies of "VLDL"-TG and of VLDL-remnants-TG turnover in nembutalized and in ether-anesthetized rats. We have used multicompartmental analysis of these data to approach the kinetic relationships between the nascent VLDL-TG and VLDL-remnants-TG compartments. This paper deals with these relationships and some of the problems encountered in trying to resolve the components of the complex "VLDL"-TG compartment by conventional tracer approaches.

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Abbreviations: SpA, specific activity; TG, triglycerides; TGFA, triglyceride fatty acids; VLDL, very low density lipoproteins; SAAM, systems analysis and modeling (a computer program).

EXPERIMENTAL PROCEDURES

Animals

Male Sprague-Dawley rats (Hilltop Labs., Los Angeles, CA) were housed in colony cages and fed Purina lab chow ad libitum until the day before the tracer experiment. Water was available at all times. The donors were either fasted overnight (experiment II) or fasted overnight with access to 20% glucose in their drinking water (experiment III). The recipients were all fasted overnight. The body weights of recipient rats averaged 365 g in experiments I and II and 300 g in experiment III. The recipient rats were all anesthetized (see Experimental Protocol).

Tracers

Labeled "VLDL" for experiment I was prepared as follows: a 374-g donor rat, fasted overnight, was anesthetized lightly with ethyl ether, and injected with 1.0 mCi [2-^{3}H]glycerol (SpA, 250 mCi/mmol) in the exposed jugular vein. The rat was reanesthetized with ether and exsanguinated from the aortic bifurcation at 30 min. The blood was allowed to clot at 4 C and centrifuged in a Sorvall RC2-B centrifuge (10,000 rpm × 10 min) using an SS34 rotor. Serum was aspirated and dialyzed at 4 C against 2 changes of one liter of saline for 1 hr each time. Each recipient rat received an intrajugular injection of 0.5-0.6 ml dialyzed serum (2.5×10^5 dpm of the $d < 1.006$ fraction 95% of which was in the TG-glyceryl moiety [see below]).

The same procedure was used to prepare labeled "VLDL" for experiment III except for the following: the donor rat (300 g) had access to 20% glucose in its drinking water the previous night. No other food was available. Instead of labeled glycerol, 410 μCi of [1-^{14}C]palmitate complexed to rat serum albumin (14) was injected into the exposed jugular vein. About 0.4 ml of the donor's dialyzed serum, labeled primarily in the TGFA moiety of the $d < 1.006$ fraction, was mixed with 2.0 ml of dialyzed serum that contained [2-^{3}H]glyceryl-labeled VLDL-remnants-TG (see below), and 0.55 ml was injected into the exposed jugular of each recipient rat. The injected dose (experiment III) contained a total of 6.1×10^4 cpm of ^{14}C-TGFA-labeled VLDL (and 3.3×10^4 cpm of ^{3}H-glyceryl-TG-labeled VLDL-remnants; see below).

Rat serum, containing VLDL-remnants labeled with [2-^{3}H]glycerol primarily in the TG-glyceryl moiety of the $d < 1.006$ fraction, was prepared as follows. In experiment II the donor rat (406 g) was fasted overnight, anesthetized lightly with ether, and injected in an exposed jugular vein with 2.0 mCi [2-^{3}H]glycerol. After 30 min (the estimated time for the VLDL-TG SpA to reach its maximum), the rat was lightly etherized and a functional hepatectomy performed by tying off the coeliac and superior mesenteric arteries and the portal vein. After allowing 30 min under anesthesia for the formation of labeled VLDL-remnants, the rat was bled from the aortic bifurcation. Serum was collected and dialyzed as described above. During the 30-min period post-hepatectomy, serial samples of blood were collected and the TG radioactivity analyzed. Based upon these measurements, we estimated that 65% of the labeled VLDL-TG had been hydrolyzed in the 30-min period. The dose contained 1.2×10^5 dpm of ^{3}H, 99% of which could still be recovered in the $d < 1.006$ fraction. We injected 0.50 ml iv into each recipient rat. In experiment III, the donor rat (300 g) was fasted overnight but with access to 20% glucose in its drinking water. The rat was injected with [2-^{3}H]-glycerol and functionally hepatectomized as described above; however, 60 min, instead of 30 min, was allowed following hepatectomy for the formation of labeled VLDL-remnants. Based upon analysis of serum TG-^{3}H in blood samples taken serially during the latter period, we estimated that 85% of the VLDL-TG was hydrolyzed; 84% of the remnants ^{3}H-TG could still be recovered in the $d < 1.006$ fraction. Serum, collected, dialyzed, and combined with the serum containing ^{14}C-TGFA-labeled VLDL, as described above, contained 3.3×10^5 cpm as ^{3}H in the 0.55 ml serum that was injected iv into each recipient rat.

Experimental Protocol

In experiments I and II, all recipient rats were deeply anesthetized with nembutal (50-60 mg/kg, ip with supplemental injections, ip, as needed). In experiment III, the rats were kept lightly anesthetized with ethyl ether. In experiments I and II, 2 groups of rats were set up. In experiment I, 6 rats were injected with serum labeled in the "VLDL"-TG-glyceryl moiety; in experiment II, 4 rats were injected with serum labeled in the VLDL-remnants-TG-glyceryl moiety. In experiment III, 2 recipient rats were injected with doubly labeled serum containing "VLDL"-TG labeled in the FA moiety and VLDL-remnants-TG labeled in the glyceryl moiety. The doses have been given above.

Recipient rats were treated in the same way, with the above exceptions. Thus, immediately following anesthesia both jugular veins were exposed, and the labeled serum injected into one of the veins at zero time. Serial blood samples (ca. 0.20 ml each) were drawn with a 1.0-ml tuberculin syringe, first from the contralateral vein and later from both veins. In some cases, additional blood samples were drawn from exposed femoral veins. The blood was allowed to clot and centrifuged. Clear serum (0.07-0.1 ml) was transferred with a Hamilton syringe to a 10 × 75 mm Kimax tube and the exact volume noted. To extract serum lipids (15), 0.45 ml of methanol/chloroform (2:1, v/v) was added

with vortexing, followed by 0.20 ml 0.9% NaCl, revortexing and spinning at 10,000 rpm for 5 min in the Sorvall RC-2B centrifuge in the HB-4 swing-out rotor. The blunted needle of a 100-μl Hamilton syringe was passed through the protein pellicle at the interface and the lower $HCCl_3$ phase quantitatively withdrawn and transferred to the origin of a TLC plate (Applied Science Laboratories, Inc., State College, PA). The chromatograms were developed with petroleum ether (bp 30-60 C)/ethyl ether/acetic acid (80:20:1, v/v/v). The TG-containing areas were scraped directly into counting vials, and 10 ml of Insta-Gel (Packard Instrument Co., Downers Grove, IL)/toluene (1:1, v/v) was added. Radioactivity was measured with a Beckman scintillation counter (model LS 330, Beckman Instruments, Palo Alto, CA). In some cases, after chromatography, the TG were eluted with chloroform and separate aliquots taken for both chemical analysis (16) and radioactivity measurement.

Plasma Volumes

Rat serum albumin (Sigma Chemical Co., St. Louis, MO) was iodinated with ^{125}I by the chloramine procedure (17); excess iodide was removed by dialysis (twice, 1 hr each time vs one liter saline at 0-4 C). The ^{125}I-labeled albumin was screened by iv injection into a rat that was exsanguinated 16 hr later (overnight). The screened albumin was again dialyzed as above and injected iv (1.0×10^5 cpm/rat) either into nembutalized or ether-anesthetized rats with exposed jugular veins, as in the previous procedures. Serial venous blood samples were drawn, and serum samples (50-100 μl) were counted in a gamma counter. Average values of cpm/ml serum based upon the samples obtained in the first 20 min were used to calculate the plasma volume (PV) in each rat. The mean PV is expressed as percent of body weight (ml × 100/g body wt).

Multicompartmental Analysis

We analyzed the data first by least squares fits either to a single exponential or to the sum of exponentials using the SAAM program of Berman and Weiss (18). Then we used the SAAM program to fit various models to the data. From these least squares fits, obtained with an IBM 3033 digital computer, we were able to estimate fractional rate constants and relative pool sizes of nascent VLDL-TG and their remnants. The models are shown and discussed in the text.

We made the following assumptions: (a) the volumes of distribution (% of body weight) for VLDL and VLDL-remnant particles were the same in all rats under any given experimental condition; (b) since serum from the donor rat was obtained at 30 min, close to the assumed t_{max} of its VLDL-TG SpA curve (19,20), the SpA of its small (see below) remnant-VLDL-TG pool was approximately equal to that of its precursor, i.e., the nascent VLDL-TG itself (21,22); (c) any kinetic differences between the TG of VLDL particles (and their remnants) containing apoB-PI/II and those containing apoB-PIII (2) would be too small to be resolved in this type of experiment (23); (d) with the possible exception of some IDL-like particles (see below), all of the radioactivity found in serum TG would float at $d < 1.006$ and was either part of a nascent (modified) VLDL or a VLDL-remnant particle (1).

We further assume that: (e) both nascent VLDL-TG and their remnants in the recipient rats were in a near-steady state (with respect to the tracee) during each experiment; (f) all plasma nascent (or modified nascent) VLDL-TG-glycerol or -FA that was not hydrolyzed to glycerol and FFA was converted to plasma VLDL-remnant-TG-glycerol or -FA; (g) on the average, 2/3 of the nascent VLDL-TG was hydrolyzed before a remnant was formed that had a high chance of being recognized by the liver and could be removed from the circulation. The basis for the latter assumption was earlier work by several authors who have reported that 30-40% of endogenously prepared VLDL-TG is found in liver within 5-10 min after iv injection of rats (19,24,25). Thus, assuming that all of the remnant VLDL-TG is taken up irreversibly by the liver, ca. 60-70% of the TG must have been hydrolyzed before the remnants returned to the liver. These assumptions regarding VLDL-remnants uptake are consistent with observations that only ca. 5% of VLDL-apoB is converted to IDL-apoB and LDL-apoB in rats (2), and that most of the labeled VLDL-apoB that disappears from the circulation can be recovered in the liver within the first 15 min after iv injection into rats (4,12,13). Some additional assumptions are listed in the text under the heading, "The Model." The sensitivity of our final model to errors in some of these assumptions has been discussed elsewhere (23).

RESULTS

Kinetic Behavior of "VLDL-TG" and VLDL-TG-Remnants

We carried out two studies in which either [2-^3H]glyceryl-labeled "VLDL-TG" or their remnants were injected into nembutalized rats. In a third study, [1-^{14}C]palmitoyl-labeled "VLDL-TG" and [2-^3H]glyceryl-labeled VLDL remnants were injected simultaneously into ether-anesthetized rats. The data, which will be shown below, were fitted to either single exponential functions (plus a small constant, if necessary) or to the sum of two exponentials for each rat. The intercepts and exponents of the exponential equations that describe the curves are summarized in Table 1. In the nembutalized rats, the complex "VLDL"-TG pool turned over in a simple exponential fashion with a

mean half-life of 5.9 min (range, 5.2-8.7 min). The corresponding remnants displayed a more complex behavior, with a major component (56% at t_0) showing a mean $t_{1/2}$ of 1.4 min and a minor component (44%) showing a mean $t_{1/2}$ of 7.1 min. In ether-anesthetized rats, in which we carried out double-labeled experiments in order to compare the "VLDL" and their corresponding remnants simultaneously in individual rats, both the "VLDL"-TG and their remnants could best be fit by the sum of two exponentials. (In one case, the second component of the "VLDL"-TG curve was not well defined.) The major (87% at t_0) rapid component of the "VLDL"-TG curves had a mean $t_{1/2}$ of 2.8 min, much shorter than that for the "VLDL"-TG curve in the nembutalized rats, with a second minor component (13% at t_0) showing a mean $t_{1/2}$ of 12 min. The remnant curves were similar, with a mean $t_{1/2}$ of 1.7 min for the major component (77%) and of 12 min for the minor component (23%).

The Model

Figure 1 shows the simplest model consistent with our data and the finding of Mjøs et al. that the VLDL fraction ($d < 1.006$) includes essentially all of the VLDL-remnants (1). We have assigned the more rapid, major component of the radioactive VLDL-remnants-TG curve to the physiological pool (compartment 2). A priori, the minor slower component in the VLDL-remnant-TG tracer could represent (a) an artifact of preparation, (b) a residual portion of particles still behaving as "VLDL" rather than VLDL remnants, (c) particles which have passed beyond the VLDL-remnant stage to form IDL, which are only slowly removed by the liver (2). These possibilities are discussed further below; since we have assumed that this minor component probably does not represent a physiological remnant and since its significance is unknown, we have not included it in the present kinetic analysis.

The composite "VLDL"-TG data are represented by the "summer compartment" 5. It includes the newly synthesized VLDL-TG (compartment 1), the VLDL-TG remnants (compartment 2, the kinetic behavior of which was defined above and then fixed accordingly in the "VLDL"-TG analysis), and a third, slowly turning over minor, undefined compartment 4 included for curve-fitting purposes in the ether-anesthetized rats (see below).

Computer Fits of the Model to the Data for Nembutalized Rats

The observed data for the 4 nembutalized rats that were injected with labeled VLDL-remnants-TG are shown in Figure 2 along with the computer-derived curve that represents the mean parameters (experiment II, Table 1) of the individual least

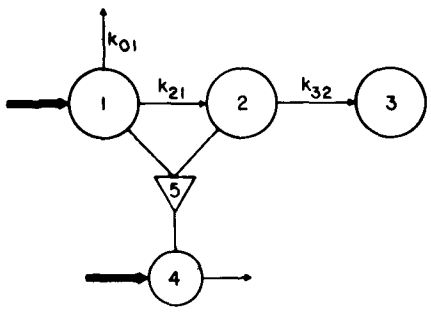

FIG. 1. Model of serum "VLDL"-TG kinetics in rats. Compartment 1 is nascent VLDL-TG (including modified, nascent particles that are not yet recognizable as remnants by the liver). Compartment 2 is VLDL-remnants-TG. Compartment 3 is the liver TG compartment into which remnants are taken up from the circulation; the further breakdown and mixing of this hepatic TG pool is not shown. Compartment 5 is a "summer" compartment (a mathematical concept used in the SAAM analysis and representing the sum of the radioactivity in compartments 1, 2 and 4 at any time). Compartment 4 is an undefined radioactive serum TG pool used for curve-fitting purposes. The fractional rate constants, k_{ij}, are the values to be determined as well as the relative pool sizes (Q_i) for compartments 1 and 2.

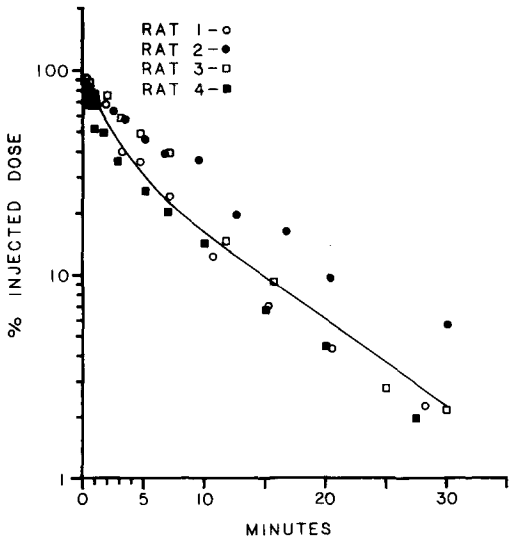

FIG. 2. Disappearance of [2-^{3}H]glyceryl-TG-labeled VLDL-remnants from the circulation of rats (experiment II). Each symbol represents the datum from a separate nembutalized rat. The dose (one preparation for all 4 rats) was injected iv at zero time as freshly obtained labeled serum from a functionally hepatectomized donor rat (see text). The curve is the least squares fit to the sum of two exponentials (see Table 1 for parameters) for the composite data.

TABLE 1

Parameters of the Exponential Equations Describing the Turnover of "VLDL-TG" and VLDL-Remnant-TG in Plasma of Anesthetized Rats

Experiment and rat no.	Anesthesia	Tracer injected	Parameters			
			A_1[a]	α_1[b]	A_2[a]	α_2[b]
I - 1	Nembutal	[2-^{3}H]Glyceryl-"VLDL-TG"	98	0.114	—	—
I - 2	Nembutal	[2-^{3}H]Glyceryl-"VLDL-TG"	98	0.134	—	—
I - 3	Nembutal	[2-^{3}H]Glyceryl-"VLDL-TG"	98	0.127	—	—
I - 4	Nembutal	[2-^{3}H]Glyceryl-"VLDL-TG"	98	0.080	—	—
I - 5[7]	Nembutal	[2-^{3}H]Glyceryl-"VLDL-TG"	98	0.124	—	—
I - 6[8]	Nembutal	[2-^{3}H]Glyceryl-"VLDL-TG"	98	0.121	—	—
Mean			98(100)[b]	0.117	—	—
II - 7	Nembutal	[2-^{3}H]Glyceryl-VLDL-remnant-TG	55	0.43	43	0.109
II - 8	Nembutal	[2-^{3}H]Glyceryl-VLDL-remnant-TG	55	0.24	43	0.064
II - 9	Nembutal	[2-^{3}H]Glyceryl-VLDL-remnant-TG	55	0.22	43	0.107
II - 10	Nembutal	[2-^{3}H]Glyceryl-VLDL-remnant-TG	55	1.09	43	0.113
Mean			55(56)	0.49	43(44)	0.098
III - 11	Ether	[1-^{14}C]Palmitoyl-"VLDL-TG"	96	0.29	14	0.057
III - 12	Ether	[1-^{14}C]Palmitoyl-"VLDL-TG"	96	0.20	14	0.060
Mean			96(87)	0.25	14(13)	0.059
III - 11	Ether	[2-^{3}H]Glyceryl-VLDL-remnant-TG	90	0.42	27	0.057
III - 12	Ether	[2-^{3}H]Glyceryl-VLDL-remnant-TG	90	0.38	27	0.060
Mean			90(77)	0.40	27(23)	0.059

[a]Percentage injected dose/total plasma volume.
[b]Min^{-1}. (Note: Extrapolated value for $A_1 + A_2 \neq 100\%$. Extrapolated intercept (in parentheses) normalized so that $A_1 + A_2 = 100\%$. See text for explanation.)

squares fits to the data (sum of 2 exponentials) for each rat. In each case, the fits were extremely good (SD values listed in Table 1); however, the range of values was large (0.22-1.09/min). From these data, a mean value of 0.49/min for nembutalized rats was assigned to k_{32} in the model shown in Figure 1.

The "VLDL"-TG data and the fits of the model (Fig. 1) to these data for each of 6 nembutalized rats (Experiment I) are shown in Figures 3A, B and C. No slowly turning over compartment (compartment 4, Fig. 1) was required. As can be seen in Figure 2, the model generates a curve for the 2-pool model (nascent VLDL-TG plus VLDL-remnant-TG) that closely approximates a simple exponential function such as one might expect from a one-compartment model. This kinetic behavior had been predicted in an earlier theoretical analysis (23).

The computer-derived parameters and a further description of the model for the nembutalized rats are shown in Table 2 and will be discussed below. However, it is noteworthy that the single slope defining the "VLDL"-TG data (Table 1) is almost identical to that of the value computed for $k_{1,1}$ (rate constant for the total efflux from compartment 1, Fig. 1). The faster fractional rate constant characteristic of VLDL-remnants in the nembutalized rats was not detectable in the analysis of the "VLDL"-TG curves, again as predicted on theoretical grounds (23).

Computer Fits of the Model to the Data for Ether-Anesthetized Rats

As shown in both Figures 4A and 4B, "VLDL"-TG and VLDL-remnants-TG turned over at similar rates in the ether-anesthetized rats. A minor slow compartment was present in at least one of the "VLDL"-TG curves and in both of the VLDL-remnant-TG curves. The remnant-TG data in each figure, as in the case of nembutalized rats, were fitted to the sum of 2 exponentials (Table 1) and only the faster, major component was used in the model to define k_{32} (Fig. 1). Then the "VLDL"-TG data (corresponding to summer compartment 5 and including compartment 4, in Fig. 1) were used to define the model's parameters. Again, excellent fits were obtained in which $k_{1,1}$ (the fractional turnover rate of the nascent VLDL component) was computed to be essentially the same value as that of the first component of the observed "VLDL"-TG curve (Table 1).

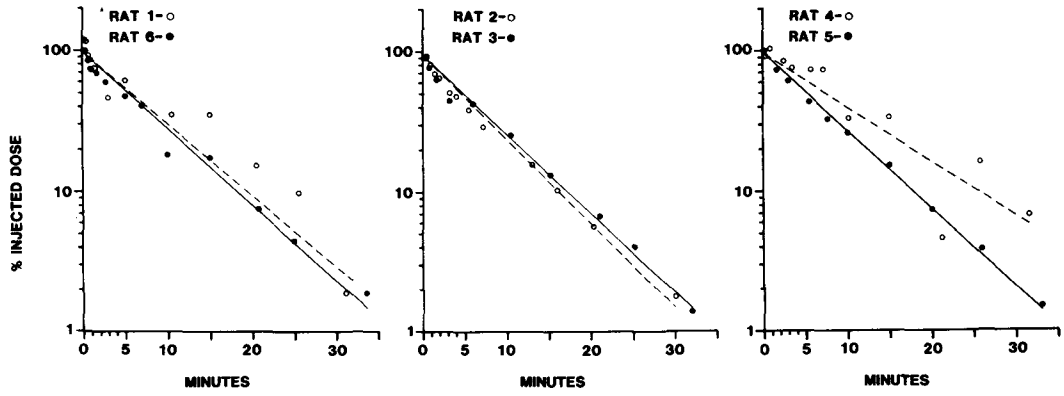

FIG. 3. Disappearance of [2-^{3}H]glyceryl-TG-labeled "VLDL" from the circulation of nembutalized rats (experiment I) and the fits of the model (compartment 5, Fig. 1) to the data. Each of the three panels (A-C) shows the data for two separate rats (n = 6). The dose (one preparation for all 6 rats) was injected iv at zero time as labeled serum freshly obtained from a normal donor rat. Each curve was obtained by fitting the data from each animal using the model shown in Fig. 1. See text for further details. Despite the complexity of the model, each predicted computer-derived curve (SAAM) closely approximates a single exponential function as do the data themselves. The model's parameters derived from these analyses are shown in Table 2.

TABLE 2

Parameters of the Model for "VLDL-TG" Turnover in Anesthetized Rats

Experiment and rat no.	Anesthesia	Q_2/Q_1 (%)[a]	k_{11}[b] ($k_{01}+k_{21}$)	k_{21}[b]	k_{32}[b]
I - 1	Nembutal	7.9	0.12	0.040	0.49
I - 2	Nembutal	9.5	0.14	0.047	0.49
I - 3	Nembutal	8.9	0.13	0.043	0.49
I - 4	Nembutal	8.3	0.087	0.029	0.49
I - 5	Nembutal	8.7	0.13	0.043	0.49
I - 6	Nembutal	8.5	0.13	0.043	0.49
Mean		8.6	0.12	0.041	0.49
III - 11	Ether	28	0.33	0.11	0.40
III - 12	Ether	18	0.21	0.071	0.40
Mean		23	0.27	0.090	0.40

[a]Q_2 and Q_1 are the pool sizes of VLDL-remnants-TG (compartment 2, Fig. 1) and of nascent (modified) VLDL-TG (compartment 1, Fig. 1), respectively.
[b]Fractional rate constants, min^{-1}; see model, Fig. 1.

Model Parameters

A summary of the above analyses, including the relevant fractional rate constants and the relative compartment sizes, is shown in Table 2. In the nembutalized rats, the nascent "VLDL"-TG pool turned over with a mean fractional rate constant of 0.12/min and was 12 times larger than the VLDL-remnant-TG pool. There was very little variation from rat to rat (see values of Q_2 as a percent of Q_1 in Table 2). In the ether-anesthetized rats, the nascent VLDL-TG pool was 4.3 times larger than the corresponding remnant-TG pool and turned over at a mean fractional rate of 0.27/min.

In the nembutalized rats (experiment I), the volume of distribution of "VLDL"-TG and their remnants was assumed to be equal to the plasma volume as determined with ^{125}I-albumin. This value averaged 2.8% of body weight. The extrapolated values at t_0 of the "VLDL"-TG and VLDL-remnants-TG radioactivity curves (Fig. 2) agreed exactly with this value. In ether-anesthetized rats, the plasma volume, based upon ^{125}I-albumin, was calculated to be ca. 4% of body weight. However,

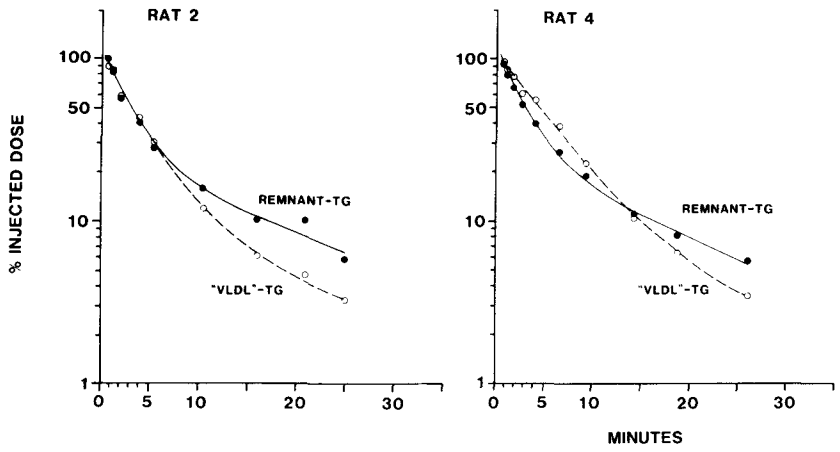

FIG. 4. Simultaneous studies of the disappearance of [1-^{14}C]palmitoyl-TG-labeled "VLDL" and [2-^{3}H]-glyceryl-TG-labeled VLDL-remnants from the circulation of rats. Data obtained from a separate recipient rat is shown in each panel (A,B); the rats were anesthetized with ether and injected with labeled serum. The latter was a mixture obtained from two separate donor rats (one, providing the ^{14}C-labeled "VLDL"-TG and the other, the ^{3}H-labeled VLDL-remnants-TG). The curves represent the least squares fits to the sum of two exponentials for the ^{3}H-labeled VLDL-remnants-TG data of each rat (●), and (b) the fit of the model (Fig. 1) to the ^{14}C-labeled "VLDL"-TG data (o) using SAAM.

the mean volume of distribution for the VLDL particles and their remnants was slightly lower (3.5% of body weight) as shown by extrapolation to t_0, and this value was used to evaluate the pool sizes and transport rates of the ether-anesthetized animals.

The serum VLDL-TG concentrations of rats fasted for 24 hr were found, in separate experiments, to be the same in anesthetized (ether or nembutal) and control rats. The grand mean (±SE) concentration was 0.53 ± 0.045 mg/ml (n = 16). This represented a mean (± SE) of 74 ± 3.0% of the total serum TG concentration. Based upon the volumes of distribution of "VLDL"-TG and their remnants and the calculated fraction of the total "VLDL"-TG present in the nascent VLDL particles in each group, we estimate that the nascent VLDL-TG pool sizes (Q_1) were 1.5 and 1.9 mg TG/100 g body weight in the nembutalized and etherized rats, respectively. Multiplying by the corresponding values for $k_{1,1}$ (Table 2), we calculate that the rates of nascent VLDL-TG oxidation and conversion to remnants (i.e., $Q_1 k_{1,1}$) were 0.15 and 0.46 mg/min/100 g body weight in the nembutalized and ether anesthetized rats, respectively. Since the pool size measurement upon which these calculations are based were made in a separate experiment than the tracer study and because we only studied 2 ether-anesthetized rats, we do not wish to draw any specific conclusions regarding the effects of one anesthetic compared to the other. Rather, we would emphasize that it is important to carry out further work along these lines in unanesthetized rats, as recommended by other workers (cf. Krause et al. (26) and references therein). The feasibility of such a quantitative study in vivo is established by the present work using anesthetized rats.

DISCUSSION

Despite the fact that the "VLDL"-TG fraction ($d < 1.006$) as isolated by conventional techniques (27) is a highly complex mixture of particles (1), including at least 2 different kinds of liver-derived nascent, and modified-nascent particles, one with apoB-PI/II and the other with apoB-PIII (2), as well as intestinal VLDL with apoB-PIII (3-5), and the corresponding remnants (1), the kinetic behavior of the composite $d < 1.006$ fraction can be quite simple. Thus, our nembutalized rats showed no clear evidence of either very rapidly or slowly turning over "VLDL"-TG components, although the 30-min-study duration probably was too short to rule out minor contributions of the latter kind. A minor slow component was more readily apparent in the ether-anesthetized rats, in part due to the fact that the disappearance of the early rapid component of their "VLDL"-TG curve was about twice as fast as that of the single slope seen in nembutalized animals. This difference may itself reflect different hemodynamic responses to the 2 anesthetics. The rate-limiting step in VLDL-TG disappearance is

catalyzed by capillary endothelial lipoprotein lipase, and both the slower disappearance of VLDL-TG and their lower distribution volume in the nembutalized animals indicated impaired peripheral circulation compared to the ether-anesthetized animals.

Thus, the initial disappearance of "VLDL"-TG may be highly dependent upon the experimental conditions used. Half-lives of 1-3 min have now been reported by several laboratories including ours, while much longer values have been found by others (8). The actual causes of these variations are still mostly unknown. Some of the rapid turnover rates may by artifacts. Thus, the $t_{1/2}$ of 1 min observed for glucose-fed (fat-free diet) rats (28) is at least 5 times faster than we would predict based upon studies using Triton WR-1339 (K.R. Bruckdorfer and N. Baker, unpublished observations; see also ref. 29). Moreover, it is difficult, if not impossible, to reconcile such rapid rates with VLDL-apoB kinetic studies reported by Elovson et al. (2).

Although there were some apparent differences in the kinetic behavior of "VLDL"-TG between our nembutalized and ether-anesthetized rats, the data from each animal in each group could be analyzed in a consistent way using a common simple model, once the fractional rate constant for VLDL-remnant-TG turnover had been independently determined. This value averaged 0.49/min and 0.36/min in the case of nembutalized and ether-anesthetized rats, respectively. By incorporating these values into our simplified model, we have been able to gain new insight into the kinetic relationship and relative pool sizes of the nascent (modified) VLDL-TG and VLDL-remnants-TG pools in fasted rats. We were unable to find such information in the literature. Our analyses indicate that the nascent (modified) VLDL-TG compartment is ca. 12 times and 4 times bigger than that of the VLDL-remnant-TG compartment in nembutalized and ether-anesthetized rats, respectively. Assuming, as we did in our multicompartmental analysis, that, on the average, 2/3 of the TG was hydrolyzed before a remnant particle was formed, each nascent VLDL particle would have 3 times more TG than a VLDL-remnant particle. Accordingly, we estimate that there was ca. $12/3=4$ and $4/3=1.3$ as many nascent VLDL particles as VLDL-remnant particles in the nembutalized and ether-anesthetized rats, respectively.

One of the interesting features of the particular model we used is the simplicity of the function predicted for VLDL-TG. We have already described the theoretical basis for this phenomenon (23). Such simple kinetic behavior is only expected when the SpA at t_0 of the nascent VLDL-TG compartment is about equal to that of the VLDL-remnants-TG. This should always be the case using exogenous labeling techniques, but can also occur using endogenously labeled "VLDL"-TG if the right time for harvesting the labeled "VLDL"-TG is selected. In this respect, it is theoretically possible that the more complex "VLDL"-TG curve observed in the 2 ether-anesthetized rats could reflect a difference between the donor's nascent VLDL- and remnants-TG specific activities, which we have assumed to be the same 30 min after injection of labeled glycerol or FFA. However, this is highly unlikely since we have shown earlier (23) that these specific activities should be essentially equal over a relatively broad time period near the time of the donor's maximum "VLDL"-TG specific activity.

A more likely explanation for the greater complexity of the "VLDL"-TG curve in the ether-anesthetized rats is that deep nembutal anesthesia, by decreasing the plasma volume, may have effectively excluded the VLDL-TG from an important site of LPL activity (or that nembutal inhibits and/or ether stimulates such activity). Further work is needed to validate this and other assumptions that we have made.

One of our findings that needs further study is the consistent presence of a slowly turning over VLDL-remnants-TG component. This was most prominent in the nembutal experiment, where the remnant tracer was generated during 30 min continued circulation of endogenously labeled VLDL in a functionally hepatectomized donor; a loss of 65% of the VLDL-TG occurred. While this equals the 2/3 hydrolysis assumed to trigger the VLDL to VLDL-remnant conversion, the nascent VLDL itself is highly polydisperse in size and it is possible that an average 65% hydrolysis could leave a substantial portion of particles which had not yet completed this conversion in the donor. The labeled TG in such particles would continue to be hydrolyzed by LPL in the recipients, giving rise to a slow apparent VLDL-remnant component with a slope similar to that for overall "VLDL"-TG disappearance.

Alternatively, a slower "remnant" component could be produced in the hepatectomized donor if TG hydrolysis in the absence of hepatic clearance proceeded beyond the normal remnant stage, to produce IDL-like particles, which are not rapidly removed by the liver, but rather are slowly further hydrolyzed to lower density lipoproteins (30). This is a more likely explanation in experiment III, where a longer (60-min) period of circulation in the hepatectomized donor gave an overall TG hydrolysis of 80%. In fact, 15% of the TG label in this remnant tracer was found in the $d < 1.006$ g/ml infranatant, identical to the extrapolated 15% slow component at t_0.

The question may, of course, also be asked whether the major, rapidly turning-over remnant components could be another artifact of extended

exposure to lipoprotein lipase in the hepatectomized donor. That is, rather than going all the way to IDL, could the majority of particles suffering a possibly larger than normal loss of triglycerides end up as "superremnants" with an abnormally rapid turnover not necessarily representative of "physiological" remnants? We find this unlikely, since in fact the remnants obtained at 30 min had lost only about two-thirds of their triglycerides, identical to the loss expected for remnant formation in normal animals, as discussed above. The 80% hydrolysis at 60 min in the hepatectomized donor was somewhat greater; however, since the 2 remnant preparations had very similar, rapid, removal rates in the non-hepatectomized recipients, there is no reason to assume that either of them represented some type of "superremnants" with abnormally rapid clearance from the circulation.

Returning to the more slowly turning-over remnant component, the possibility also exists that it could be the normal product of a special subclass of VLDL. Although nascent VLDL particles that contain apoB (PI/II) probably are converted to remnants more slowly than those that contain apoB (PIII) (2), we have shown that the difference in these rates would not be discernible as distinct fast and slow components in VLDL-TG decay curves such as those generated in our experiments (23). Cenedella et al. have presented indirect evidence that intestinally derived VLDL-TG labeled in the glycerol moiety turn over much more slowly than liver-derived VLDL-TG (31). It is conceivable that such particles could give rise to slowly turning over remnants. However, Windmueller et al. very recently showed directly that VLDL from intestine turn over more rapidly than those from liver even though both contain apoB-PIII (32). Although this latter finding relates to the rate of overall VLDL particle removal, it tends to argue against a relatively slower turnover rate for intestinal than hepatic VLDL-remnants. At this stage, the possible physiological significance of the slower VLDL-remnant component remains an open question.

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Lipid and Fatty Acid Composition of the Endogenous Energy Sources of Striped Bass *(Morone saxatilis)* Eggs

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ABSTRACT

The unique physiological flexibility of the early life stages of striped bass is attributed to the calorie-rich endogenous energy sources of the striped bass egg. Eggs of different aged striped bass from geographically separate populations were examined for lipid and fatty acid compositions and were found to be basically similar. Yolk components of the eggs contained significantly less total lipid than oil globules, were more diverse in lipid class composition and consisted mostly of polar lipids. Oil globules were entirely lipid material consisting predominantly of steryl/wax esters. Fatty acid compositions of yolk and oil globules differed according to their respective lipid compositions. The functional significance of these lipids is discussed in relation to the ecological context of the early life stages.
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INTRODUCTION

Studies on the feeding ecology and bioenergetics of striped bass embryos and larvae have indicated that early life stages are able to endure long periods of starvation and that they lack a critical period, defined as the sensitive and brief time when larvae begin active feeding and must convert from endogenous to exogenous energy sources (1,2). This physiological flexibility is largely attributed to the egg oil globule which persists for extended periods through the embryonic into the larval stage. At fertilization, the striped bass egg is characterized by a large single oil globule which comprises 55% of the total egg weight and 71% of the total caloric content. Proteinaceous yolk (5600 cal g^{-1}) and the oil globule (9500 cal g^{-1}) combine to an average 2.04 calories per egg. Lipid stores of the striped bass egg are of interest not only for their physiological and ecological functions, but also because they serve as reservoirs for lipophilic contaminants.

The following report presents information on the lipid and fatty acid compositions of the yolk and oil globule components of fully mature striped bass eggs. The object of our study was to further understanding of the physiology of the early life stages of striped bass by providing a detailed description of their initial lipid stores.

MATERIALS AND METHODS

Specimen Collection and Preparation

Five sexually mature striped bass were collected from the San Francisco Bay/Delta estuary and 2 females from the Coos River in Oregon. California fish were younger (5 and 6 yr) and smaller (62.4 cm mean standard length) than those from Oregon (8 and 14 yr; 78.5 cm standard length) such that fecundities also differed (0.6 vs 1.8 million eggs per female for California and Oregon, respectively). Subsamples of the left ovary (~150 g wet weight) were quick-frozen and stored at −40 C until analyzed. Eggs were removed from the center of each ovary, homogenized and centrifuged to separate yolk and oil globule components. The yolk and oil layers were aspirated into separate containers and lipids extracted from each fraction by the chloroform/methanol extraction procedure of Bligh and Dyer (3).

Prior to lipid analysis samples were stripped of solvent and ca. 50 mg of yolk or oil lipid was weighed and dissolved in 1 ml $CHCl_3$. This solution, or appropriate dilutions, was used for thin layer chromatography (TLC), quantitative analysis of lipid classes by TLC with flame ionization detection (FID) and gas liquid chromatography (GLC).

Thin Layer Chromatography/Flame Ionization Detection

Lipid classes present in the yolk and oil samples were separated and identified by comparison with a standard mixture on Silica Gel G plates (Supelco, Bellefonte, PA) developed in hexane/diethyl ether/acetic acid (80:20:1) and visualized with iodine and by spraying with 5% molybdophosphoric acid in 95% ethanol. A second plate, spotted and developed in the same manner, was sprayed with Liebermann-Burchard reagent after development, to confirm the presence of steryl esters.

Quantitation of individual lipid classes was achieved by TLC/FID. A comprehensive description of this relatively new technique is given by Ackman (4). An Iatroscan TH-10 analyzer was used with an Omniscribe B-5000 dual pen strip

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chart recorder. The FID was operated with a hydrogen flow rate of 160 ml/min and air flow rate of 2000 ml/min. Chromarods (type SII) were spotted with ca. 20 μg of lipid in $CHCl_3$ solution and developed in hexane/diethyl ether/formic acid (85:15:0.02). Following development, the rods were dried at 110 C for 4 min and scanned at a rate of 0.42 cm/sec. Peak areas were measured with a stepping pen integrator and were converted to weight percent lipid class by the use of correction factors (5,6) derived from authentic lipid standards. Steryl esters (SE) and wax esters (WE) could not be separated by the solvent system described above nor by any of the several other solvent mixtures attempted and are therefore reported as a sum.

Gas Liquid Chromatography

Methyl esters of fatty acids of all yolk and oil samples were prepared using 14% boron trifluoride in methanol, following saponification with 0.5 N potassium hydroxide. Esters were extracted with hexane and purified by TLC prior to GLC analysis. Fatty acid methyl esters were separated isothermally at 210 C on a Quadrex fused silica wall coated open tubular column (50 m×0.21 mm id) coated with Carbowax 20-M operated in a Hewlett-Packard 5830 gas chromatograph equipped with an FID. Helium was used as the carrier gas at a flow of 1.5 ml/min (60 psig). Individual methyl ester peaks were provisionally identified using pure standards, secondary standards and equivalent chain length calculations. Relative composition was obtained by area normalization directly from the Hewlett-Packard 18850A terminal. Aliquots of the methyl esters were hydrogenated and rechromatographed to ensure the accuracy of identification of major components and to confirm that there was no significant loss of polyunsaturates during GLC.

RESULTS AND DISCUSSION

Lipid Composition

The high total lipid content of the egg was largely attributable to the oil globule. Yolk, which comprised an average 38% of the egg dry weight, contained 3.8% total lipids, while the oil globule was 100% extractable lipids. Eggs of other fishes, even those with distinct oil globules, have been found to have significantly less total lipid contents than eggs from striped bass (7-10). For example, a survey of eggs from 6 species by Kaitaranta and Ackman (10) showed total lipids to vary 2.4-9.2%.

Lipid classes differed sharply between egg yolk and oil globule components (Table 1), thus confirming that our technique successfully separated yolk and oil globule lipids. Only wax and steryl esters and triacylglycerols were present in the oil globules with steryl and wax esters averaging 90.4% of the total lipids. This is also the major lipid found in some marine zooplankton, particularly arctic upper-water crustacea (11,12). The presence of wax esters in fish is documented mostly in somatic tissues (muscle and liver) of deep-water forms (13-15). The occurrence of steryl/wax esters in eggs is ecologically more diverse (10), including marine and freshwater species: *Mugil cephalus* (16), *Merluccius capensis, Theragra chalcogramma,* and *Scomber japonicus* (17), *Trichogaster cosby* (18), *Perca fluviatilis, Lota lota* (19), and *Sciaenops ocellati* (7). We suspected wax esters were in striped bass eggs because Dergaleva and Shatunovskiy (20) had found declining concentrations of this lipid class in larvae and juveniles of striped bass from 8 to 40 days after hatching. An important morphological feature common to eggs of all these species is the presence of discrete oil globules.

Although yolk lipids comprised a small portion of the yolk weight, it was more diverse in its composition than oil globule lipids. Phospholipids were an average 78.9% of yolk total lipids. This is not unusual since phospholipids are documented as the major lipid of egg yolks in a variety of fishes (10,19,21-23). Yolk is the endogenous energy source first consumed by the embryo and larvae (1), and is totally absorbed within 5 days after hatching (at 18 C), a period characterized by extensive tissue differentiation and growth. Since phospholipids are the major lipid constituents of biomembranes, it seems logical they were preferentially transferred

TABLE 1

Lipid Class Composition (Percent) of Oil Globules and Yolk Lipids of Striped Bass Eggs[a]

Egg component	Steryl and wax esters	Triacylglycerols	Free fatty acids	Sterols	Phospholipids
Yolk	5.3±1.7	6.5±1.9	6.6±1.8	2.8±0.9	78.9±2.9
Oil globule	90.4±2.8	9.6±2.8	---	---	---

[a]Values presented as means±standard errors for all fish combined, 4 determinations per fish.

from the yolk during this period. Free fatty acids and sterols were also present in small quantities and, like phospholipids, were present only in the yolk. The varying amounts of free fatty acids were probably due to hydrolysis of the samples during storage or laboratory work-up. Sterols were most likely cholesterol, which is common to most animal tissues.

Fatty Acid Composition

Fish lipids are generally characterized by high contents of long-chain polyunsaturated fatty acids. Such was the case with both striped bass yolk and oil globule lipids (Table 2). The dominant isomers of the C_{18} to C_{20} monoethylenic fatty acids were the 18:1ω9 and 20:1ω9 reported elsewhere for fish lipids (24). The respective fatty acid composition of yolk and oil globule lipids basically reflected differences in their lipid classes. The fatty acid composition of the yolk lipids was typical of marine phospholipids being dominated by palmitic (16:0), eicosapentaenoic (20:5ω3) and docosahexaenoic (22:6ω3) acids. Oil globule lipids were made up of fatty acids very similar to those found in somatic tissues of meso- and bathypelagic fishes, animals known to often have large amounts of wax esters (14,25). The principal acids were oleic (18:1ω9), palmitoleic (16:1ω7) and docosahexaenoic (22:6ω3). Egg lipids of striped bass from this study had a mixed fatty acid composition which is indicative of overlapping freshwater and marine environments (26). Total C_{16} and C_{18} fatty acids were high; palmitic acid was high in the yolk and the tetraenoic acids low. The lack of a clear pattern agrees with the life history of the species, namely, that it inhabits the estuarine zone.

The fatty acid composition was also consistent among individual females regardless of location, age, condition or pollutant burden. Lasker and Theilacker (27) also found remarkably similar fatty acid patterns among sardine eggs of different females at different stages of maturation.

The functional roles of high lipid contents and steryl/wax esters in fish embryos and larvae have not been determined, but it may be proposed that they generally serve in marine organisms for metabolic energy or buoyancy (28). Their most clearly identified purpose is in providing energy, especially in situations of food deprivation (15,25,29), emergencies of delayed hatch (30), or when large reserves of energy are needed for rapid embryonic development (7). In the context of the early life stage ecology of striped bass, the unique lipid characteristics serve to provide: (a) abundant energy for rapid embryonic development (incubation period = 48 hr at 18 C); (b) necessary flotation of pelagic eggs and early larvae until formation of functional swim bladders; and (c) long-term energy for feeding larvae because of uncertainty of food availability (e.g., dietary zooplankton patchiness).

TABLE 2

Fatty Acid Composition of Yolk and Oil Globule Components of Striped Bass Eggs[a]

Fish fatty acids	Yolk	Oil globule
Saturates		
14:0	1.2±0.1	1.6±0.4
16:0	17.5±1.9	6.2±0.6
18:0	9.9±0.9	0.7±0.2
Monoenes		
16:1ω9	1.3±0.2	2.2±0.4
16:1ω7	4.6±0.5	13.8±1.6
18:1ω9	15.5±1.8	35.7±2.7
18:1ω7	4.2±0.4	5.4±0.6
20:1ω11	trace	0.3±0.4
20:1ω9	1.2±0.3	1.1±0.2
20:1ω7	0.1±0.0	0.1±0.1
Dienes		
18:2ω6	0.8±0.3	1.8±0.6
20:2ω6	0.2±0.1	0.2±0.0
Trienes		
18:3ω3	0.2±0.1	0.7±0.2
20:3ω6	0.1±0.0	0.1±0.0
Tetraenes		
18:4ω3	0.3±0.1	0.8±0.3
20:4ω3	0.2±0.1	0.6±0.1
20:4ω6	3.3±0.4	1.3±0.3
22:4ω6	0.3±0.3	trace
Pentaenes		
20:5ω3	8.6±3.8	6.4±1.6
22:5ω6	0.2±0.1	0.3±0.1
22:5ω3	2.4±0.7	2.9±0.7
Hexaenes		
22:6ω3	19.9±2.2	10.0±1.5

[a]Percent composition by weight presented as means ± standard error.

The occurrence of discrete oil globules in fish eggs and larvae is more common than one might think. In a worldwide review of fish egg surveys, Ahlstrom and Moser (31) found that most pelagic marine fish eggs have discrete oil globules. As the oil globules of these and inshore and estuarine species are analyzed, we believe that steryl/wax esters will be found to be the principal form of endogenous energy in the eggs.

Recent findings show that striped bass eggs from geographically distant populations contained high levels of petrochemicals, pesticides and chlorinated hydrocarbons (32) which correlated with reduced reproductive capacity and lowered early life stage survival. The advantages of lipid to the embryo and larva are then offset by the facility with which

lipophilic and toxic chemicals are accumulated from the environment.

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Modulation of Prostaglandin Synthesis in Rat Peritoneal Macrophages with ω-3 Fatty Acids[1,2]

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ABSTRACT

In view of the findings that ω3 fatty acids inhibit the synthesis of prostaglandins (PG) from arachidonic acid (20:4ω6) and that among immunologically active cells, the macrophage is a major producer of PG, we undertook a study of the effect of dietary α-linolenic acid (18:3ω3) on PG synthesis in the macrophage. Rats were fed purified diets containing either 10% corn oil (CO) or linseed oil (LO), providing either a low (1/32) or high (3.5/1) ratio of 18:3ω3 to 18:2ω6, respectively, for 6 weeks. Fatty acid analysis of macrophage phospholipids showed that there was an appreciable increase in the percentage of ω3 fatty acids and a decrease in the ω6 fatty acids in macrophages from rats fed the LO diet. The changes in fatty acid composition were associated with a significant decrease in the synthesis of prostaglandin E (PGE) by macrophages from rats fed the LO diet. Macrophages from rats fed the 2 dietary oils did not differ in their ability to degrade PG, thus the difference in PG production appeared to be a consequence of decreased synthesis only. The dietarily induced changes in PGE synthesis were readily overcome in vitro by culturing macrophages with complexes of fat-free bovine serum albumin and either 20:4ω6 or 20:5ω3.
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Fatty acids of the ω3 series have been shown to inhibit the synthesis of prostaglandins (PG) from arachidonic acid (20:4ω6) both in vitro and in vivo. Eicosatrienoic (20:3ω3) and eicosapentaenoic acids (20:5ω3) are poorly oxidized by purified PG cyclooxygenase and 20:5ω3 can act as either a reversible competitive inhibitor or an irreversible inactivator of this enzyme (1). Results of dietary experiments are in agreement with these findings. The addition of increasing amounts of α-linolenic acid (18:3ω3) to the diets of rats is associated with decreased concentration of PG in serum (2) and decreased PG synthesis by homogenates of liver and spleen (3).

PGE_1 and E_2, both derived from ω6 fatty acids, have been associated with the inhibition of various aspects of the immune response, including lymphocyte proliferation (4), lymphokine secretion (5), macrophage collagenase synthesis (6), granulocyte-monocyte stem cell proliferation and differentiation (7), natural killer cell activity (8), and the tumoricidal activity of activated macrophages (9). It has been proposed that PG may serve as the mediators of a negative feedback mechanism for regulating the extent and duration of the cell mediated immune response (5).

Among immunologically active cells, the macrophage is considered to be a major source of PG. In view of this and of the proposed role of prostaglandins as mediators of immunoregulation, it was of interest to investigate the effect of ω3 fatty acids on the synthesis of prostaglandins in the macrophage.

[1]Part of a dissertation submitted by Linda J. Magrum in partial fulfillment of the requirements for the Ph.D. degree in Nutritional Sciences.

[2]Honored Student Presentation at the AOCS 74th Annual Meeting, Chicago, 1983.

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MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley weanling rats of 60-80 g in weight (Holtzman Co., Madison, WI) were housed individually in polypropylene cages with Sanicel[R] bedding (Paxton Processing Co., Inc., Paxton, IL) and provided with a diurnal light cycle of 12 hr. The animals were fed ad libitum one of two purified diets and provided with tap water. The diets were adequate in all nutrients and differed only in the source of fat, one containing 10% by weight corn oil (CO) and the other linseed oil (LO). The composition of the diets and the fatty acid analysis of the oils used in the diets are shown in Table 1. Because of the relatively high content of fat in the diet and the high degree of unsaturation of the linseed oil, the vitamin E content of the diets was increased 10-fold over the amount recommended for rats by the National Academy of Sciences-National Research Council (10). Rats were maintained on the diets for 6 weeks.

Isolation and Purification of Peritoneal Macrophages

Peritoneal cells were collected by injecting anesthetized rats intraperitonially with 50 ml sterile phosphate-buffered saline (PBS), pH 7.4. After massaging of the abdomen, PBS containing peritoneal cells was withdrawn with a syringe. Cells were centrifuged (400×g, 10 min), then resus-

TABLE I

Composition of Purified Diets

	CO	LO
	(g/100 g)	
Corn oil[a]	10	–
Linseed oil[b]	–	10
Casein[c]	20	20
Dextrose[d]	18.5	18.5
Cornstarch[e]	30.5	30.5
Cellulose[f]	5	5
Mineral mixture[g]	3.5	3.5
Vitamin mixture[h]	12.5	12.5

[a]Fatty acid analysis: 16:0, 8.7%; 18:0, 1.4%; 18:1ω9, 16.5%; 18:2ω6, 71.0%; 18:3ω3, 2.2%. Courtesy of Best Foods, CPC International, Inc., Englewood Cliffs, NJ.

[b]Fatty acid analysis: 16:0, 5.5%; 18:0, 2.6%; 18:1ω9, 12.5%, 18:2ω6, 17.5%; 18:3ω3, 61.8%. Courtesy of Cargill, Inc., Minneapolis, MN.

[c]Shamrock Brand, Erie Casein Co., Erie, IL. Supplemented with 0.3 g DL-methionine/100 g diet.

[d]Staleydex 333, A.E. Staley Co., Decatur, IL.

[e]A.E. Staley Co., Decatur, IL.

[f]Solka-Floc, Brown Co., Berlin, NH.

[g]AIN-76[IM] Mineral Mixture, ICN Nutritional Biochemicals, Cleveland, OH.

[h]Vitamins (mg/kg diet): thiamin HCl, 30; riboflavin, 30; pyridoxine HCl, 8; nicotinic acid, 100; pantothenic acid, 100; folic acid, 2; biotin, 0.2; cyanocobalamin, 0.05; retinol acetate, 8; calciferol, 4; DL-α-tocopherol, 735; menadione, 2; myo-inositol, 220; choline chloride, 1000; brought to 125 g with dextrose.

pended in RPMI Medium 1640 containing L-glutamine (GIBCO Laboratories, Grand Island, NY), 2 g sodium bicarbonate per liter, 25 mM 4(2-hydroxy-ethyl)-1-piperazine-ethanesulfonic acid (HEPES) and 5% rat serum (previously treated by heating to 56 C for 30 min), pH 7.4. Serum from rats fed the 10% CO diet was used in the medium provided to cells from rats on this diet, while serum from LO-fed rats was used in the culture of cells from animals on the LO diet. Cells were counted with a hemocytometer and adjusted to a concentration of 1×10^6 cells/ml. Cell viability, as determined with the use of a phase contrast microscope, was routinely between 95 and 99%. Peritoneal cells were plated in plastic culture dishes and were incubated in a 5% CO_2 atmosphere at 37 C for 3 hr to allow macrophages to adhere. Nonadherent cells and medium were then removed by aspiration and culture dishes were rinsed at least twice with warm PBS (37 C). The rinsing procedure removed ca. 50% of the total cell number, but provided a macrophage population that was greater than 90% pure, as indicated by morphology and staining with α-naphthyl acetate esterase (Sigma Chemical Co., St. Louis, MO).

Lipid Extraction and Fatty Acid Analysis of Peritoneal Macrophages

In order to induce macrophage migration to the peritoneal cavity and thereby increase the number of cells obtained, rats (20 per dietary treatment) were injected intraperitoneally with 1 ml per 50 g body weight of 5% corn starch solution in PBS 3 days prior to the harvesting of peritoneal cells. Instead of being plated at a concentration of 1×10^6 cells/ml, the total cell population from one rat ($4\text{-}8 \times 10^7$ cells) was resuspended in 20 ml culture medium. Ten ml of the cell suspension was pipetted into each of two 100×15 mm sterile polystyrene Petri dishes (Lab-Tek Division, Miles Laboratories, Inc., Naperville, IL).

Three ml ice cold 0.1 mM EDTA was added to the adhered macrophages to inhibit phospholipase activity and cells were loosened with a plastic scraper. Macrophages from 2 rats were combined at this point to provide one sample. The partially lysed cell suspension was placed in a 30 ml centrifuge tube, to which 18 ml chloroform and methanol (2:1, v/v) containing 0.005% BHT was added. Cells were further lysed by sonication for 2 min over ice with a cell disrupter (Heat Systems—Ultrasonics, Inc., Plainview, NY) Model W-225R equipped with a microtip. The organic phase was collected and reduced in volume under vacuum in a rotary evaporator (Buchler Instruments, Fort Lee, NJ).

Lipid extracts were spotted on Silica Gel H thin layer plates and subjected to 2-dimensional thin layer chromatography (11). Plates were washed prior to use in chloroform/methanol/water (65:35:4, v/v/v) and activated at 100 C for 30 min. The thin layer plates were developed in the first dimension in chloroform/methanol/28% aqueous ammonia (65:35:5, v/v/v), dried under nitrogen, then developed in the second dimension in chloroform/

acetone/methanol/acetic acid/water (10:4:2:2:1, by volume). Phospholipids were visualized by minimal exposure to iodine vapors, and identified by comparison to chromatograms of authentic standards (Supelco, Inc., Bellefonte, PA, and Applied Science Laboratories, State College, PA). Phospholipid fractions were removed from plates by aspiration and placed directly into glass tubes containing 4% H_2SO_4 in methanol. Methyl esters of fatty acids were formed by heating samples for 1 hr at 100 C. Samples were then cooled, neutralized with one vol of 5% $NaHCO_3$ and extracted with one vol hexane. Fatty acid methyl esters were identified using a Hewlett-Packard gas liquid chromatograph, Model 7610A (Packard Instrument Co., Downers Grove, IL) with a 180×0.4 cm glass column packed with 10% SP 2330 on 100/120 Chromosorb W AW 1-1851 (Supelco, Inc., Bellefonte, PA) operated isothermally at 190 C. Results, expressed as percentage of total fatty acids, were determined using a Hewlett-Packard 3380A Integrator.

Prostaglandin Assays

Prostaglandins were determined by radioimmunoassay using rabbit antiprostaglandin E-BSA serum (Miles Laboratories, Elkhart, IN) according to the directions specified by the manufacturer and described in detail by Weston and Johnston (12). This antiserum is reported by the manufacturer to have minimal cross-reactivity (less than 5%) with PGA_1, A_2, $F_{1\alpha}$, $F_{2\alpha}$, B_1 and B_2, and 70% cross-reactivity with PGE_1. Cross-reactivity with PGE_3 was found in our laboratory to be 10%. No attempt was made to correct for cross-reactivity. Tritiated PGE_2, sp act 165.0 Ci/mmol, was purchased from New England Nuclear, Boston, MA. Standard curves were prepared using serial dilutions of PGE_2 (Upjohn, Inc., Kalamazoo, MI) from 10 ng/ml to 0.15 ng/ml. Free PG were removed by the addition of dextran-coated charcoal, 1% w/v Norit A charcoal and 0.1% Dextran T-70 (Pharmacia Fine Chemicals, Uppsala, Sweden). Quantitation of the PG was performed using logit transformation and linear regression of the standard curve. Only standard dilutions in the most linear portion of the standard curve were used to form the equation of the line. Furthermore, PG determinations were made only for those sample dilutions which fell within the straight line portion of the standard curve. Serial dilutions of selected samples demonstrated curves parallel to the standard curve and recovery of standard PGE_2 added to samples was routinely determined to be ca. 95%. Data are expressed as pg PGE/ml culture medium, the amount obtained from the culture of ca. 5×10^5 adherent macrophages.

Determination of PGE Synthesis and Degradation

Much of the literature on PG synthesis and function in the macrophage deals with PGE_1 and PGE_2. We therefore chose to follow changes in the synthesis of these PG in macrophages from rats receiving the LO and CO diets. Peritoneal macrophages from 8 rats per dietary treatment were collected and purified as described using 2 ml cell suspension in 35×10 mm plastic culture dishes (Becton, Dickinson and Co., Oxnard, CA). Two ml medium containing 5% of the appropriate rat serum, and 50 μg/ml Zymosan A (Sigma Chemical Co., St. Louis, MO) was added to adhered macrophages to stimulate PG synthesis (13). PG synthesis was terminated after the desired incubation period by the addition of indomethacin (Sigma Chemical Co., St. Louis, MO) at a final concentration of 28 μM (14). Macrophage viability was not affected by indomethacin at this concentration. The culture medium was collected, centrifuged (600×g, 10 min) to remove zymosan and any loosened cells and frozen until PG assays were carried out. PG concentration in the medium was determined at a zero time point, 1 hr and 4 hr. The zero time point represents the concentration of PG present in the supernatant of cultures that were provided with zymosan and indomethacin directly after macrophage adherence. These samples were immediately chilled in ice, then centrifuged and frozen. The PG concentrations reported for the 1 hr and 4 hr time points represent the difference between the total amount of PG assayed at these time points and the zero time point.

To investigate the possibility that the difference in PG produced by macrophages from rats receiving the 2 dietary treatments might be due to differences in the rate of PG degradation, 2 additional experiments were done. An investigation of degradation of PG over time in culture was carried out by adding 28 μM indomethacin after a 1 hr incubation to 3 additional cultures per rat. These were further incubated for 1, 2 or 3 hr. Rates of PG degradation were also investigated by determining the recovery of exogenous PGE_2 from macrophage cultures. Indomethacin (28 μM), 50 μg/ml zymosan and 12.5 ng/ml PGE_2 were added to one culture per rat. These materials were also added to culture dishes in the absence of cells to serve as a control and both were incubated for 1 hr.

Addition of Fatty Acids to Macrophages in Culture

In order to investigate the extent to which dietarily induced changes in PG synthesis were related to availability of substrate, complexes of fat-free bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) and either 20:4ω6 (NuChek Prep, Elysian, MN) or 20:5ω3 (Sigma

Chemical Co., St. Louis, MO) were incubated with macrophages from rats receiving the LO or CO diets. The complexes were formed by incubating the sodium salt of the fatty acids with BSA at a 3:1 molar ratio in a 37 C shaking water bath for 24 hr. This procedure is an adaptation of the method of Mahoney et al. (15). Medium without serum, but with antibiotic and antimycotic (Penicillin, 100 units/ml; Fungizone, .25 μg/ml; Streptomycin, 100 μg/ml) (GIBCO Laboratories, Grand Island, NY) and either BSA alone, BSA-20:4ω6 or BSA-20:5ω3 at a final concentration of 2.5 mg/ml BSA was added to adhered macrophage cultures. Cultures were incubated for 24 hr at 37 C, then washed several times with warm PBS. Two ml medium (without serum) containing 50 μg/ml zymosan was added to each culture and they were further incubated for 1 hr.

Statistical Analysis

Statistical analysis was performed using Statistical Analysis System (SAS) (Cary, NC) computer programs. These included the ANOVA procedure (analysis of variance for balanced data), the GLM procedure (general linear models, analysis of variance for unbalanced data), and Student-Newman-Keuls test ($\alpha = 0.01$) for variability. Data are expressed as mean $\pm$ standard error of the mean (SEM).

RESULTS

The body weights of the rats fed the two diets for 6 weeks, 394 $\pm$ 7 g (CO) and 390 $\pm$ 8 g (LO), were not significantly different.

Fatty Acid Composition

Tables 2, 3 and 4 show the fatty acid composition of the major macrophage phospholipids, ethanolamine phosphoglyceride (PE), choline phosphoglyceride (PC), and serine phosphoglyceride (PS). There was a significant increase in the ω3 fatty acids and a decrease in the ω6 fatty acids in each of these phospholipids from rats receiving the LO diet. In PE, the percentage of ω3 fatty acids increased from 0.5 to 20.9%, in PC from 0.8 to 12.0%, and in PS from 0.6 to 7.6% of total fatty acids. The ω6/ω3 ratio in these phospholipids changed from 107.4 (CO) to 1.5 (LO) in PE, 63.0 (CO) to 2.9 (LO) in PC and 62.5 (CO) to 2.5 (LO) in PS.

In PE, there were striking differences in the amounts of 20:4ω6, 22:4ω6 and 22:5ω3 present in macrophages from rats receiving the 2 diets. The percentage of 20:4ω6 decreased from 31.5% when the CO diet was fed to 19.3% of total fatty acid when the LO diet was fed; the percentage of 22:4ω6 decreased from 12.8% to 1.8% and 22:5ω3 increased from 0.2% to 11.0% of total fatty acids. There was also a considerable rise in the 20:5ω3 content of macrophage PE upon feeding rats the LO diet. This fatty acid increased from less than 0.1% when rats were fed the CO diet to 7.4% of total fatty acids when rats were fed the LO diet.

In PC, the most marked change in fatty acid

TABLE 2

Effect of Increased Dietary α-Linolenic Acid on the Fatty Acid Composition of Rat Peritoneal Macrophage Ethanolamine Phosphoglyceride

Diet[a]	CO (n = 10)	LO (n = 10)	P Value[b]
Fatty acid			
16:0	7.2 $\pm$ 0.8[c]	7.3 $\pm$ 0.5	NS[d]
16:1ω7	0.4 $\pm$ 0.1	0.5 $\pm$ 0.1	NS
18:0	25.7 $\pm$ 0.6	25.5 $\pm$ 0.8	NS
18:1ω9	10.8 $\pm$ 0.3	12.9 $\pm$ 0.4	.0007
18:2ω6	6.1 $\pm$ 0.6	7.7 $\pm$ 0.4	0.0536
18:3ω3	-[e] -	0.8 $\pm$ 0.1	0.0001
20:0	- -	- -	NS
20:1ω6	0.7 $\pm$ 0.1	0.3 $\pm$ 0.3	NS
20:2ω6	0.6 $\pm$ 0.02	0.6 $\pm$ 0.1	NS
20:3ω6	2.0 $\pm$ 0.3	2.5 $\pm$ 0.3	0.09
20:4ω6	31.5 $\pm$ 1.0	19.3 $\pm$ 0.5	0.0001
20:5ω3	0.1 $\pm$ 0.04	7.4 $\pm$ 0.4	0.0001
22:4ω6	12.8 $\pm$ 0.4	1.8 $\pm$ 0.1	0.0001
22:5ω3	0.2 $\pm$ 0.1	11.0 $\pm$ 0.4	0.0001
22:6ω3	0.2 $\pm$ 0.1	1.7 $\pm$ 0.5	0.0083

[a]CO = 10% corn oil, LO = 10% linseed oil.
[b]Determined by analysis of variance.
[c]Mean $\pm$ SEM, percent of total fatty acids.
[d]Not significantly different at $p > 0.10$.
[e]Not detectable.

TABLE 3

Effect of Increased Dietary α-Linolenic Acid on the Fatty Acid Composition of Rat Peritoneal Macrophage Choline Phosphoglyceride

Diet[a]	CO (n = 10)	LO (n = 10)	P Value[b]
Fatty acid			
16:0	22.3 ± 1.5[c]	17.5 ± 1.3	0.0342
16:1ω7	1.7 ± 0.2	3.2 ± 0.2	0.0001
18:0	13.3 ± 0.3	16.2 ± 0.2	0.0001
18:1ω9	11.2 ± 0.3	15.5 ± 0.2	0.0001
18:2ω6	14.7 ± 0.4	18.8 ± 0.5	0.0001
18:3ω3	-[c] -	2.2 ± 0.1	0.0001
20:0	0.1 ± 0.05	0.2 ± 0.1	NS[d]
20:1ω6	0.7 ± 0.1	- -	0.0001
20:2ω6	1.4 ± 0.1	0.6 ± 0.05	0.0001
20:3ω6	1.0 ± 0.1	1.5 ± 0.05	0.0001
20:4ω6	28.0 ± 0.7	13.9 ± 0.7	0.0001
20:5ω3	0.2 ± 0.03	5.5 ± 0.4	0.0001
22:4ω6	4.6 ± 0.4	0.5 ± 0.03	0.0001
22:5ω3	0.2 ± 0.05	3.5 ± 0.2	0.0001
22:6ω3	0.4 ± 0.1	0.8 ± 0.1	0.0007

[a]CO = 10% corn oil, LO = 10% linseed oil.
[b]Determined by analysis of variance.
[c]Mean ± SEM, percent of total fatty acids.
[d]Not significantly different at $p > 0.10$.
[e]Not detectable.

TABLE 4

Effect of Increased Dietary α-Linolenic Acid on the Fatty Acid Composition of Rat Peritoneal Macrophage Serine Phosphoglyceride

Diet[a]	CO (n = 6)	LO (n = 6)	P Value[b]
Fatty acid			
16:0	6.8 ± 0.8[c]	5.0 ± 0.6	NS[d]
16:1ω7	0.6 ± 0.3	0.6 ± 0.3	NS
18:0	39.7 ± 1.4	42.4 ± 0.8	NS
18:1ω9	21.5 ± 1.2	24.8 ± 0.7	0.0510
18:2ω6	5.3 ± 0.7	6.8 ± 0.4	NS
18:3ω3	-[e] -	0.3 ± 0.3	NS
20:0	0.2 ± 0.1	0.05 ± 0.05	NS
20:1ω6	1.0 ± 0.4	0.8 ± 0.4	NS
20:2ω6	0.5 ± 0.05	0.4 ± 0.2	NS
20:3ω6	1.5 ± 0.3	1.9 ± 0.2	NS
20:4ω6	12.9 ± 0.8	7.9 ± 0.5	0.0030
20:5ω3	0.6 ± 0.6	1.4 ± 0.05	NS
22:4ω6	8.7 ± 1.5	1.1 ± 0.2	0.0001
22:5ω3	- -	5.7 ± 0.4	0.0001
22:6ω3	- -	0.2 ± 0.2	NS

[a]CO = 10% corn oil, LO = 10% linseed oil.
[b]Determined by analysis of variance.
[c]Mean ± SEM, percent of total fatty acids.
[d]Not significantly different at $p > 0.10$.
[e]Not detectable.

composition of macrophages from rats fed the 2 diets was found in the percentage of 20:4ω6. This fatty acid represented 28.0% of total fatty acids when CO was fed and 13.9% when LO was fed. The percentages of 20:5ω3, 22:5ω3 and 22:4ω6 also differed significantly ($p < 0.0001$), but the changes were not as great as those seen in PE.

In PS, the greatest changes in fatty acid composition were found in 22:4ω6, which represented 8.7% of total fatty acids when rats were fed CO and 1.1% when rats were fed LO, and in 22:5ω3, which was not detectable when CO was fed but represented

5.7% of total fatty acids when LO was fed.

In addition to the major changes seen in the content of 20:4ω6, 20:5ω3, 22:4ω6 and 22:5ω3 of macrophage phospholipids, there was either a trend towards or a significant increase in 18:2ω6, an increase from nondetectable to trace levels of 18:3ω3, and a trend towards increased, but still trace amounts of 22:6ω3 in all macrophage phospholipids from rats fed LO as compared to CO diet.

Macrophage PG Synthesis and Degradation

Figure 1 illustrates the synthesis of PGE (PGE_1 + PGE_2) by macrophages that were stimulated with zymosan. At both 1 hr and 4 hr, the PGE present in the culture medium of macrophages from rats fed the LO diet was significantly less than the present in the medium of macrophages from rats fed the CO diet ($p > 0.0001$). At both time points, the mean PGE present in macrophage cultures from LO-fed rats was approximately one-third that present in macrophage cultures from CO-fed rats (445 ± 61 (LO), 1451 ± 107 (CO) at 1 hr and 1020 ± 160 (LO), 3554 ± 431 (CO) at 4 hr). From 1 hr to 4 hr, the mean PGE synthesized by macrophages from rats fed the LO diet increased by a factor of 2.3, while that synthesized by macrophages from rats fed the CO diet increased by a factor of 2.4.

Figure 2 shows the results of the first experiment designed to test whether the decreased amount of PGE found in culture medium of macrophages from rats receiving the LO diet might be due to increased degradation of PG rather than decreased synthesis. In neither group of macrophages did further incubation after termination of PG synthesis result in decreased amounts of PGE in the culture medium as compared to that present at 1 hr. The amounts of PGE present at 1, 2 and 3 hr incubations after termination of PG synthesis are neither significantly different from the 1 hr time point nor from one another.

Analysis of PGE present in the culture medium 1 hr after the addition of exogenous PGE_2 to cultures of macrophages showed that not only were there no differences in the rate of degradation of PG by macrophages from rats fed the 2 diets, but that no degradation occurred. In neither the macrophages from rats receiving the CO diet nor those receiving the LO diet was the amount of PGE_2 found in the culture medium different from the control value (2129 ± 121, 2201 ± 86, 2029 ± 130 pg/ml, respectively).

Differences in the synthesis of PGE by macrophages from rats receiving the 2 dietary oils were completely overcome by incubating macrophages with a complex of fatty acid-free BSA and either 20:4ω6 or 20:5ω3. When cultured with BSA-20:4ω6 for 24 hr, macrophages from both LO and CO-fed rats synthesized comparable amounts of

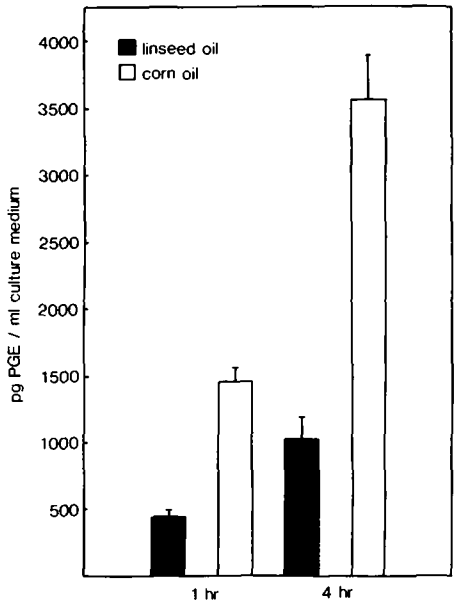

FIG. 1. Prostaglandin E synthesis by peritoneal macrophages from rats fed a purified diet containing 10% by weight of either corn oil or linseed oil. Approximately 1×10^6 adherent macrophages were stimulated with 100 μg zymosan in a final volume of 2 ml culture medium for either 1 hr or 4 hr. PG concentrations represent the difference between a zero time point and the time point indicated. Results are expressed as mean ± SEM, $n = 6$ (linseed oil diet), $n = 8$ (corn oil diet).

PGE, 3738 ± 486 and 443 ± 1482 pg/ml, respectively. Incubation of macrophages with BSA-20:5ω3 for 24 hr resulted in levels of PGE that were entirely below the level of detection for all cultures from LO-fed rats and below the level of detection for all but one culture from the CO-fed rats. When macrophages were cultured with BSA alone, those from rats receiving the LO diet synthesized approximately one-third the PGE of macrophages from rats fed the CO diet, 437 ± 180 and 1560 ± 524 pg/ml, respectively. The amount of PGE synthesized by macrophages from CO-fed rats was one-third that of identical samples which had been incubated with BSA-20:4ω6.

DISCUSSION

Fatty acid analysis of rat peritoneal macrophage phospholipids showed that when animals were fed a control diet composed of 10% CO, 20:4ω6 was the most abundant fatty acid in both the 2 major phospholipid fractions, PE and PC (31.5% and 28.0% of total fatty acids, respectively). These results are in agreement with those of Mason et al.

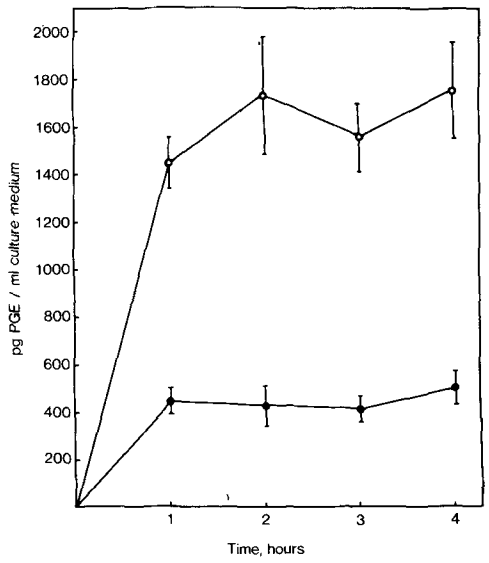

FIG. 2. Prostaglandin E recovered from cultures of peritoneal macrophages isolated from rats fed diets containing 10% by weight linseed oil (●) or corn oil (○). Approximately 1×10^6 adherent macrophages were stimulated with 100 μg zymosan in a final volume of 2 ml culture medium. After incubation for 1 hr, 28 μM indomethacin was added to cultures and incubation was continued for an additional 1, 2 or 3 hr. Results are expressed as mean ± SEM, n = 6 (linseed oil diet), n = 8 (corn oil diet).

(16) who, in characterizing the fatty acid composition of rabbit alveolar macrophages, found that 20:4ω6 was particularly high in PE (cell pellet 32.0%, vesicle fraction 22.7%).

Comparison of the fatty acid composition of macrophage phospholipids when rats were fed CO, with a relatively high content of 18:2ω6 (71%) and a low amount of 18:3ω3 (2%) as opposed to LO with a much higher content of 18:3ω3 (62%) and a relatively lower content of 18:2ω6 (18%), showed that phospholipids contained substantially lower amounts of 20:4ω6 when the diet contained a high ratio of 18:3ω3 to 18:2ω6. In PE, the amount of 20:4ω6 decreased by 40% and was replaced by 18:1ω9 as the most abundant fatty acid. In PC, 20:4ω6 decreased by 50% from 28.0 to 13.9% in macrophages from rats fed the diet high in 18:3ω3. In this case, the levels of 18:2ω6, 16:1ω7, and 18:1ω9 were all higher, respectively, than 20:4ω6.

The trend toward increased 18:2ω6, but the relatively low levels of 18:3ω3 found in the phospholipids of rats fed the high 18:3ω3 diet support the finding that 18:3ω3 is desaturated preferentially to 18:2ω6 (17,18). Given this preference of the Δ6 desaturase for ω3 fatty acids, a decrease in 20 and 22 carbon ω6 fatty acids and a corresponding increase in 20 and 22 carbon ω3 fatty acids is the expected result of increased levels of 18:3ω3 in the diet.

It is clear from these results that fatty acid composition of the macrophage is markedly influenced by the fatty acid composition of the diet and strongly reflects the presence of ω3 fatty acids when they occur in substantial amounts in the diet. It is, therefore, important that for purposes of comparison researchers analyze and report the fatty acid composition of animal diets in addition to reporting lipid composition of animal cells and tissues.

The changes in fatty acid composition of macrophages, particularly the decrease in 20:4ω6 and the increase in 20:5ω3 observed when rats were fed a diet high in 18:3ω3, are associated with a marked decrease in the synthesis of PGE. While the content of macrophage phospholipid 20:4ω6 fell by 40% in PE and 50% in PC when rats were fed the LO diet, the synthesis of PGE by macrophages from rats on this diet was decreased by ca. 67%. This finding is in agreement with the observation that 20:5ω3 is a competitive inhibitor of the synthesis of PGE_2 from 20:4ω6 (1).

The lack of evidence for increased degradation of PG by cultures of macrophages from rats fed the LO diet suggests that the decreased amount of PGE is due to decreased synthesis only. The decreased synthesis is most probably caused by both decreased availability of the substrate, 20:4ω6, as well as by competitive inhibition by 20:5ω3.

The addition of BSA-fatty acid complexes to cultured macrophages showed that the dietarily induced differences in PG synthesizing capacity could be overcome when cells were provided with either 20:4ω6 or 20:5ω3 in vitro. These results support the conclusion that the fatty acids provided in vitro were incorporated into cellular lipids and were readily available for PG synthesis. These results also provide additional evidence that 20:5ω3 is an effective inhibitor of PG synthesis in the macrophage.

No attempt was made to determine whether PGE_3 was synthesized from 20:5ω3. It has been reported that urinary bladders of frogs produce a prostaglandin or related compound from 20:5ω3 (19); however, the synthesis of PGE_3 in mammals remains controversial. The synthesis of PGE_3 in kidney medullary homogenates from rats fed fish oil has been reported (20), but it is possible that this synthesis was an artifact of aeration during homogenization. Culp et al. (1) showed that increased peroxide levels enhance the oxygenation of 20:5ω3 by purified cyclooxygenase from sheep vesicular glands and suggested the increased levels of peroxides found in phagocytic cells may allow 20:5ω3 to act as a substrate for PG synthesis. The oxygenation of 20:5ω3 by human platelets when

peroxide-forming fatty acids were present (21) lends further support to the hypothesis that 20:5ω3 may serve as a prostaglandin precursor in mammals under conditions of locally high peroxide concentration.

It seems reasonable to speculate that α-linolenic acid which so markedly alters PGE synthesis in the macrophage and may possibly provide substrate for the synthesis of PGE_3 may also influence immune cell function. Experiments to examine the possible effects of decreased PGE synthesis on macrophage function and to determine whether macrophages having a high phospholipid content of 20:5ω3 are capable of synthesizing PGE_3 are presently being conducted in our laboratory.

ACKNOWLEDGMENT

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Bovine Pulmonary Surfactant: Chemical Composition and Physical Properties[1]

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ABSTRACT

Bovine pulmonary surfactant was obtained by endotracheal lavage of lungs from newly slaughtered cows followed by differential centrifugation. Lipid extracts of bovine surfactant contained 3% neutral lipid, mainly as cholesterol and diacylglycerol and 97% phospholipid. Phosphatidylcholine (79%) and phosphatidylglycerol (11%) accounted for most of the phospholipids with smaller amounts of phosphatidylethanolamine, phosphatidylinositol, lyso-*bis*-phosphatidic acid and sphingomyelin. Fatty acid analysis revealed high levels of palmitate in phosphatidylcholine and to a lesser extent phosphatidylglycerol, but not in the other diacylphospholipids. Phosphatidylcholine was 53% disaturated and phosphatidylglycerol was 23% disaturated. Monoenoic species accounted for the major proportion of the remaining lipid. The protein content was 10% as estimated by the Lowry procedure and 5% when determined by amino acid analysis. Extraction with chloroform/methanol removed ca. 90% of the protein but had no effect on the surfactant properties as evaluated by a pulsating bubble technique.
Lipids 18:522-529, 1983.

INTRODUCTION

Normal lung function is dependent upon the presence of a specialized material, the pulmonary surfactant, which stabilizes the alveoli by reducing surface tension particularly during expiration (1-3). The presence of pulmonary surfactant is particularly important at birth (4,5). Lack of sufficient surfactant at birth appears to be an important factor contributing to the establishment of the neonatal respiratory distress syndrome (NRDS), the major cause of perinatal morbidity and mortality in North America (4,5). Treating prematurely delivered rabbits with homologous natural surfactant before the first breath promotes pulmonary expansion and prolongs survival (see 6,7 for review). More recently, Fujiwara and his associates (8) in Japan, and Smyth and coworkers (9,10) in Canada have reported that treating premature neonates suffering from established NRDS with saline dispersions containing the lipids extracted from bovine pulmonary surfactant results in improved gaseous exchange.

Despite these clinical trials, little information is available concerning bovine pulmonary surfactant. The present paper reports the chemical and physicochemical characterization of pulmonary surfactant obtained through saline lavage of lungs from freshly slaughtered cows and of lipid extract preparations similar to those utilized in the Canadian trial.

EXPERIMENTAL PROCEDURES

Isolation of Bovine Pulmonary Surfactant

Whole lungs with the heart intact from freshly slaughtered cows were placed in a large basin and a short length of rubber vacuum hose (od 3.0 cm) was inserted into the trachea and bound with household cord. The tubing was fitted to a polyethylene jerrican (Fisher Scientific Ltd., Toronto) containing 16 l of cold 0.130 M NaCl, 0.010 M $CaCl_2$, 0.008 M $MgCl_2$. This solution, hence referred to as saline-salts solution, was chosen to retard the removal of pulmonary macrophages (11). Saline-salts solution (8-10 l) were drained into the lungs by gravity until all sections became firmly extended. The fluid was allowed to flow back into an empty reservoir using gravity and gentle massaging. The remaining solution in the jerrican was drained into the lung and removed as above. Further fluid was recovered into a 2-l filter flask by mild suction from a squeeze handle hand vacuum pump (Fisher Scientific Ltd.). Lungs were obtained within minutes after slaughter. Care was taken to avoid overextension: the reason for this becomes evident with experience. Bloody or even red-tinged lavages were discarded. Approximately 12 l of white foamy fluid were recovered from each pair of cow lungs. Toluene (0.2 ml) was added to inhibit bacterial growth and the containers were kept as cool as possible during transportation back to the laboratory where they were stored at 4 C until processing. Under normal circumstances, 4-6 cows were lavaged for each preparation.

The lavages were filtered with a household strainer and centrifuged on a Sorvall RC-2B centrifuge using a Szent-Gyorgyi continuous flow attachment and an SS-34 head (Sorvall Centrifuges, Dupont Co., Wilmington, DE) at 3500 rpm (1450 g) and a flow of 200 ml/min to remove tracheal debris and cells. The resulting supernatants were concentrated ca. 10-fold with a Millipore pellicon filtration unit using a PTHK filter

[1]Some of the results contained in this paper were presented at the 73rd Meeting of the AOCS in Toronto, May, 1982.

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(Millipore Ltd., Mississauga, Ontario) with a nominal cutoff of 100,000 daltons.

The resulting suspensions were centrifuged at 8000 g for 60 min in a Sorvall GS-3-rotor. The white pellets were resuspended in saline-0.001 M $CaCl_2$ to give a final concentration of 25-50 mg phospholipid/ml. This preparation will be referred to as natural surfactant. Lipid extracts were prepared by extracting natural surfactant with chloroform/methanol (1:1). The precipitated protein was removed through centrifugation and the resulting supernatant transformed to a biphasic system by the addition of 0.9 vol of 1% KCl according to Bligh and Dyer (12). The upper phase was removed and the lower phase taken to dryness with a rotary evaporator. The washing procedure was repeated twice to reduce the protein content.

Analytical Techniques

Neutral lipids were separated from the phospholipids through chromatography on silicic acid (Bio-Rad Lab., Mississauga, Ontario) with chloroform. The neutral lipids (cholesterol, diacylglycerols, and monoacylglycerols) were converted to the *t*-butyldimethylsilyl ethers and assayed on a Hewlett-Packard gas liquid chomatograph (Model 5830A) equipped with a Hewlett-Packard capillary inlet (Model 18835B) and a fused silica capillary column coated with OV-1 as previously described (13).

Phospholipids were separated by thin layer chromatography on Silica Gel H plates (Merck Chemical Co.) using chloroform/ethanol/water/triethylamine (30:34:8:35) (14). The lipids were detected under ultraviolet light after spraying with Rhodamine 6-G. Phosphorus content was estimated on gel scrapings (15). The fatty acid patterns were determined by gas liquid chromatography of the methyl esters on a Hewlett-Packard gas chromatograph using a column of EGSS-X on Gas Chrom Q as previously described (16). Known amounts of heptadecanoate were added to act as an internal standard. Phospholipid content was calculated by multiplying the phosphorus content by 25.

Phosphatidylcholine and phosphatidylglycerol were extracted from the thin layer plates with 3 portions of chloroform/methanol/water (1:2:0.8). The pooled eluates were washed according to Bligh and Dyer (12). Positional distribution of the fatty acids on these two phosphatides were established through hydrolysis with phospholipase A_2 (hog pancreas: Boehringer Manheim GmbH, Montreal, Quebec) as described by Kates (17). The hydrolysis products were separated on thin layer as above and the fatty acid patterns determined. Molecular species of phosphatidylcholine and phosphatidylglycerol were determined after hydrolysis with phospholipase C (*Bacillus cereus;* Sigma Chemical Co., St. Louis, MO) as described previously (17). The resulting 1, 2-diacyl-*sn*-glycerols were separated on silver nitrate impregnated thin layer plates (18) with chloroform/ethanol (95:5). The individual molecular species were visualized under ultraviolet with Rhodamine 6G (0.005%). The bands were extracted with chloroform/methanol (1:1), washed according to Bligh and Dyer (12) and the free fatty acid patterns determined as above.

Protein contents were determined through the Lowry procedure (19) with sodium dodecyl sulfate (0.1% final concentration) added to dissolve the phospholipids (16) and by amino acid analysis after acid hydrolysis (20). Bovine serum albumin (Sigma) was used as standard for the Lowry procedure. Polyacrylamide gel electrophoresis was conducted in the presence of 2% sodium dodecyl sulfate as previously described (20).

The surfactant properties of bovine pulmonary surfactant and its lipid extracts were examined using a pulsating bubble surfactometer (Surfactometer International, Toronto) as described by Enhorning (21). In this technique, an air bubble is withdrawn in a 1% suspension of natural surfactant or its lipid extract in 0.15 M NaCl and 0.001 M $CaCl_2$ (Fig. 1). The bubble is pulsated at 20 rpm from a minimum radius of 0.40 mm to a maximum radius of 0.55 mm at 37 C. The pressure across the bubble is continuously monitored with a pressure transducer. The surface tensions at any point can be calculated from the Laplace equation which states that the pressure difference across a bubble equals twice the surface tension divided by the radius. The surface tensions at maximum and minimum bubble size are normally expressed (20,21).

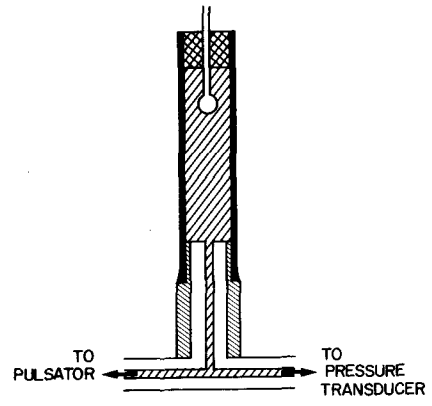

FIG. 1. Diagrammatic representation of the test chamber of the pulsating bubble model used to test pulmonary surfactant. A bubble in contact with ambient air is created by withdrawing some of the fluid in the test chamber. The bubble is then continuously pulsated between a maximum radius of 0.55 mm and a minimum radius of 0.40 mm at 37 C. The rate of pulsation is 20 rpm.

Lipid extracts of bovine pulmonary surfactant and the lipid mixtures (artificial surfactant) were dried from chloroform in polyethylene tubes with a stream of N_2. Saline containing 0.001 M $CaCl_2$ was added and the lipids dispersed by sonication with two 15-sec bursts with a Biosonik 10 (VWR Scientific, San Francisco, CA) at 175 W (20).

Materials

Standard lipids and other biochemicals were obtained from Sigma Chemical Company. Lyso-*bis*-phosphatidic acid was obtained from Serdary Research Laboratories, London, Ontario. Other reagents were obtained from Fisher Scientific Limited, Toronto.

RESULTS

Composition

Lipid extracts of bovine pulmonary surfactant contained 97% phospholipid and 3% neutral lipids (Table 1A). The neutral lipid fraction was primarily composed of cholesterol and 1,2-diacylglycerol (Table 1B). Traces of cholesteryl ester and monoacylglycerol were present at $< 1\%$ of the total neutral lipids. Triacylglycerol and free fatty acids were not detected. As anticipated, phosphatidylcholine accounted for the major proportion of the phospholipid fraction (Table 1C). Phosphatidylglycerol, the next most abundant lipid, accounted for just over 10% of the total phosphorus. Lesser amounts of phosphatidylethanolamine, sphingomyelin, phosphatidylinositol and lyso-*bis*-phosphatic acid were also detected. Lyso-*bis*-phosphatidic acid was identified only by its chromatographic properties. Phosphatidylserine was not detected.

TABLE 1

Lipid Composition of Bovine Pulmonary Surfactant

	%Total ± SEM
A. Class analysis (n = 3)	
Phospholipids	97 ± 0.34
Neutral lipids	3 ± 0.54
B. Neutral lipid composition (n = 3)	
Cholesterol	89.0 ± 1.9
Diacylglycerol	9.8 ± 0.2
Monoacylglycerol	< 1.0
Cholesterol ester	< 1.0
C. Phospholipid composition (n = 4)	
Phosphatidylcholine	79.2 ± 1.6
Phosphatidylglycerol	11.3 ± 0.5
Phosphatidylinositol	1.8 ± 0.3
Phosphatidylethanolamine	3.5 ± 0.5
Lyso-*bis*-phosphatidic acid	1.5 ± 0.4
Sphingomyelin	2.6 ± 0.5

Neutral lipids were determined by gas liquid chromatography and phospholipids by phosphorus assay as indicated in the Methods.

The fatty acid composition of the phospholipids is listed in Table 2. In keeping with the aeknowledged role of dipalmitylphosphatidylcholine in the reduction of surface tension (1-4), this phospholipid contained a high proportion of saturated fatty acids. Over half of the acyl groups released from the 2-position by phospholipase A_2 were saturated. Phosphatidylglycerol also possessed a high proportion of palmitate at the 2-position. Considerably lower levels of palmitate were observed with phosphatidylethanolamine and phosphatidylinositol. Because of their relatively low level in surfactant, the positional distribution of the fatty acids in these lipids was not examined.

The high levels of saturated fatty acids in phosphatidylcholine and phosphatidylglycerol were reflected in the presence of disaturated molecular species (Table 3). Only a small proportion of these lipids were localized in the dienoic or polyenoic species.

Examination of whole natural surfactant revealed that ca. 10% by weight could be measured as protein by the Lowry (19) procedure (Table 4). When protein was estimated by amino acid analysis of hydrochloric acid hydrolysates, the values obtained were roughly half of those determined by the Lowry procedure. Extraction of the natural surfactant with chloroform/methanol (1:1) and washing according to Bligh and Dyer (12) removed 90% of the protein. Repeated extraction did not further reduce the protein content to any great extent.

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (2%) revealed major protein bands with nominal molecular weights of 62,000 and 36,000 daltons. A considerable number of other proteins were also present (Fig. 2), including a band which streaked over the area corresponding to 9-11,000 daltons. Because the lipid extracts only contained ca. 1% protein, it was not possible to obtain clear electrophoresis patterns. Approximately 50% of the proteins in the lipid extracts could be precipitated by dispersing the chloroform/methanol (1:2) extracts in 20 vol of diethyl ether and cooling for 1 hr in a freezer. Protein constituents with minimal molecular weights of 62,000, 36,000 and ca. 10,000 daltons were clearly seen but small amounts of other proteins could also be detected in the ether precipitates of the lipid extracts (not shown).

Surfactant Properties

The surfactant properties of bovine pulmonary

TABLE 2

Fatty Acid Composition of the Major Phospholipids of Bovine Pulmonary Surfactant

Fatty acid	*Phosphatidylcholine position*			*Phosphatidylglycerol position*			Phosphatidyl-ethanolamine	Phosphatidyl-inositol	Sphingomyelin
	Total (n = 6)	1-position (n = 3)	2-position (n = 3)	Total (n = 5)	1-position (n = 2)	2-position (n = 2)	Total (n = 3)	Total (n = 3)	Total (n = 3)
14:0	3.1 ± 0.2	3.3 ± 0.9	4.0 ± 0.6	0.8 ± 0.2	0.6 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	2.3 ± 0.5
14:1	1.8 ± 0.5	1.1 ± 0.1	2.7 ± 0.4	0.6 ± 0.2	0.6 ± 0.1	0.7 ± 0.1			
14:2	0.5 ± 0.2						2.6 ± 0.3		
16:0	64.0 ± 2.3	82.6 ± 2.3	49.9 ± 1.9	51.2 ± 2.6	73.3 ± 2.5	31.2 ± 1.7	15.3 ± 1.9	26.0 ± 0.8	76.6 ± 2.3
16:1	7.5 ± 2.0	2.7 ± 0.9	7.3 ± 1.2	4.3 ± 0.7	3.4 ± 0.2	5.7 ± 1.0	4.5 ± 0.5	2.3 ± 0.2	
16:2	0.4 ± 0.1			0.4 ± 0.1			0.6 ± 0.1	1.2 ± 0.2	
18:0	2.4 ± 0.2	4.3 ± 0.4	0.7 ± 0.2	4.7 ± 0.3	11.4 ± 0.3	2.2 ± 0.4	8.9 ± 0.6	16.1 ± 1.5	9.8 ± 1.0
18:1	17.3 ± 1.8	6.1 ± 0.5	32.0 ± 1.0	33.0 ± 1.0	10.4 ± 0.7	54.6 ± 1.2	47.4 ± 2.3	33.0 ± 1.0	3.9 ± 0.5
18:2	1.6 ± 0.2		3.6 ± 0.7	2.9 ± 0.1	0.5 ± 0.2	4.8 ± 0.5	8.7 ± 0.2	3.3 ± 0.5	
18:3	0.3 ± 0.1			0.5 ± 0.2			0.9 ± 0.1	1.2 ± 0.5	
18:4	0.4 ± 0.1			0.3 ± 0.1			0.8 ± 0.1		
20:0								0.6 ± 0.2	
20:4	0.5 ± 0.1			1.0 ± 0.1			5.0 ± 1.0	7.5 ± 0.8	
20:5							0.4 ± 0.1		
22:0									4.0 ± 0.1
22:5				0.3 ± 0.1			0.5 ± 0.1	0.8 ± 0.2	
22:6							2.0 ± 0.3	4.0 ± 0.6	
24:0									3.4 ± 0.1
Saturated fatty acids (% total)	69.5	90.1	54.6	56.7	93.2	34.3	24.7	43.3	96.1

Values are presented as percent total ± SEM as determined by gas liquid chromatography.

TABLE 3

Molecular Species Composition of Phosphatidylcholine and Phosphatidylglycerol Derived from Bovine Pulmonary Surfactant

Degrees of unsaturation	Phosphatidylcholine % total ± SEM (n = 3)	Phosphatidylglycerol % total ± SEM (n = 3)
Disaturated	52.9 ± 2.5	17.2 ± 1.7
Monoenoic	45.0 ± 2.3	76.9 ± 2.3
Dienoic	1.5 ± 0.2	4.2 ± 1.3
Polyenoic	0.5 ± 0.1	1.6 ± 0.7

surfactant were determined using the pulsating bubble technique (21). Only minor differences were observed in the abilities of natural surfactant or its lipid extract to reduce the surface tension of the pulsating bubble (Table 5). Acetone precipitation of the lipid extracts resulted in a quantitative recovery of phospholipid but reduced the neutral lipid content to undetectable levels (data not shown). Nevertheless, dispersions of the acetone-precipitated lipids possess surfactant characteristics similar to those exhibited by unfractionated lipid extracts (Table 5).

The ability of lipid extracts of bovine pulmonary surfactant to reduce the surface tension of a newly formed bubble is kinetically illustrated in Figure 3. In contrast, an artificial surfactant preparation composed of purified lipids reduced the surface tension more slowly and never achieved surface tensions approaching 25 dynes/cm at maximum bubble size or 0 dynes/cm at minimum bubble size.

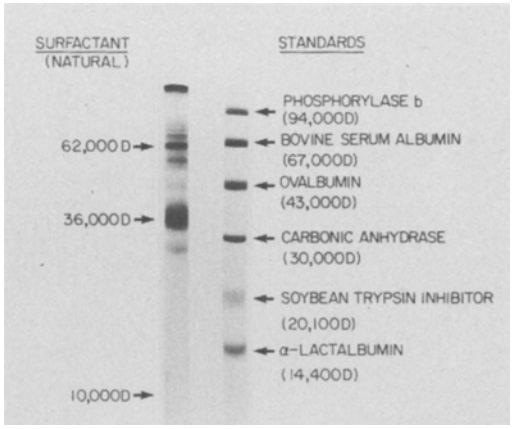

FIG. 2. Polyacrylamide gel electrophoresis of natural surfactant. The samples are stained with Coomassie Blue.

DISCUSSION

Surfactant replacement therapy appears to be the most direct approach towards prevention of respiratory failure with NRDS. The beneficial effects of treating prematurely delivered neonates with natural surfactant were first demonstrated

TABLE 4

Protein Content of Bovine Pulmonary Surfactant

	Protein (percent weight of phospholipids)			
	Lowry method	(n)	Amino acid analysis	(n)
Natural surfactant	10.3 ± 1.07	(5)	4.77 ± 0.44	(3)
Lipid extract	0.97 ± 0.07	(3)	0.46 ± 0.04	(3)

TABLE 5

Surfactant Properties of Bovine Pulmonary Surfactant[a]

Sample n = 5	Bubble size	Surface tension at various times (dynes/cm) 15 sec	30 sec	60 sec
Natural surfactant	R max	27.0 ± 1.0	25.0 ± 0.3	25.0 ± 0.4
	R min	4.0 ± 0.7	1.5 ± 0.4	0.7 ± 0.1
Lipid extract	R max	27.0 ± 0.5	25.0 ± 0.7	25.0 ± 0.7
	R min	3.0 ± 0.9	1.0 ± 0.5	0.0 ± 0.0
Acetone precipitate of lipid extract	R max	27.0 ± 0.4	26.0 ± 0.4	25.0 ± 0.4
	R min	2.5 ± 1.4	0.6 ± 0.6	0.0 ± 0.0

[a]Samples were dispersed in 0.9% NaCl, 2.5 mM $CaCl_2$ and assayed via the pulsating bubble technique.

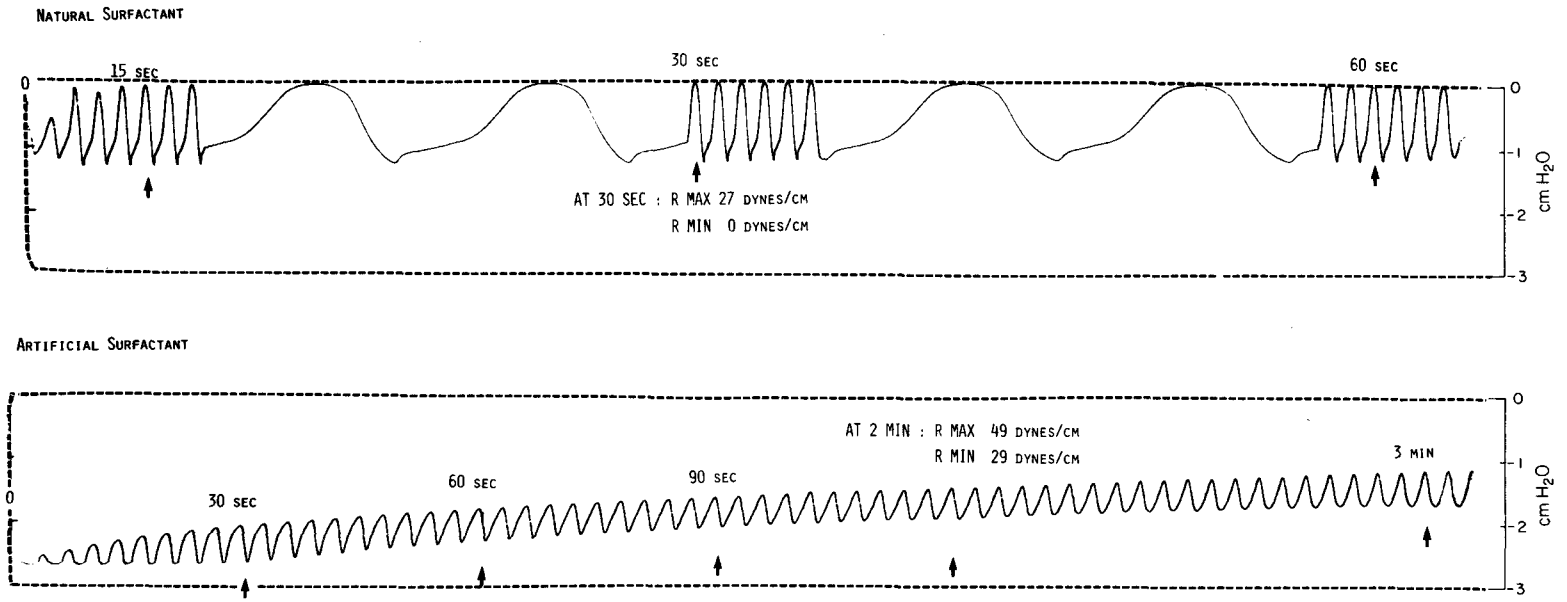

FIG. 3. Comparison of the rate of reduction of the surface tension of pulsating bubbles by natural surfactant and by an artificial surfactant preparation. The artificial mixture was composed of dipalmitoylphosphatidylcholine (70%), egg phosphatidylcholine (20%) and phosphatidylglycerol prepared from egg phosphatidylcholine (10%). The natural surfactant and the artificial mixture were dispersed in 0.154 M NaCl at a concentration of 10 mg/ml based on the phospholipid content. After the formation of the bubble, the samples were pulsated at 20 rpm at 37 C. With natural surfactant (top curve) the chart speed was increased for the 8th plus 9th and the 16th plus 17th pulsations in order to provide further detail. The arrows below the tracings indicate points of minimum bubble size for that particular pulsation. Surface tensions at the times indicated were calculated for the maximum and minimum bubble radius from the recorded pressures using the Laplace equation (see Methods).

with fetal rabbits of 27-28 days gestation (term 31 days) by Enhorning, Robertson and associates. Surfactant deposition before the first breath resulted in a marked improvement in pressure-volume characteristics and in alveolar aeration (6,7). Surfactant treatment leads to prolonged survival with prematurely delivered rabbits, rhesus monkeys and sheep (6,7,22,23). Improved gaseous exchange during artificial ventilation and a marked reduction in bronchial epitheliar necrosis were also observed (6,7).

Early clinical trials in which aerosols of dipalmitoylphosphatidylcholine were administered to premature infants proved unsuccessful, presumably because the lipid preparations did not possess the appropriate surfactant properties (7). More recently, Fujiwara and associates (8) have reported that a semisynthetic surfactant composed of an acetone precipitate of bovine lung surfactant plus dipalmitoylphosphatidylcholine and phosphatidylglycerol was effective in improving pulmonary function with infants suffering from respiratory distress. A rapid improvement in gaseous exchange was also observed by Smyth et al. (9,10), who used dispersions of the lipids extracted from bovine pulmonary surfactant prepared as described in the present investigation.

The overall lipid composition of bovine pulmonary surfactant is similar to that previously reported for other species (see 24 for review). The phospholipid composition is almost identical to previous analyses with dog (20,25), rabbit (26-28), sheep (23,25), pig (29) and rat (26,30,31) surfactant preparations. Bovine pulmonary surfactant had a lower content of neutral lipids than has been observed with surfactants from the dog (20,25) or rat (32). In contrast to these other species, triacylglycerol was not detected and the level of cholesterol ester was very low. The reason for this interesting difference is not known. Bovine pulmonary surfactant tended to have a somewhat higher content of phosphatidylglycerol (24) and a correspondingly lower level of phosphatidylinositol than surfactants from other species. A reciprocal relation exists between the levels of phosphatidylglycerol and phosphatidylinositol in human amniotic fluid during development (33), indicating that these carbohydrate-containing phospholipids share a similar function. It has been suggested that these acidic phospholipids may be involved in the transfer of phosphatidylcholine from the subphase to the air-liquid interface or in the stabilization of the subsequent monolayer, but the molecular mechanism remains vague (20,28,34). The compositions reported in the present study also show a close resemblance to the lipids present in lamellar bodies from rat and human lung (35-37).

The presence of major protein species with nominal molecular weights of ca. 70k, 35k, and 10k have previously been reported for dog, rabbit, pig, sheep, rat and human surfactant (2,20,25,29,38-40). The presence of proteins with these molecular weights in lipid extracts of pulmonary surfactant has not been reported previously. In an earlier study from this laboratory (20), it was reported that over 99% of the proteins in canine lung wash surfactant could be removed without seriously hampering the ability to reduce the surface tension of a pulsating bubble. These observations suggested, but did not prove, that the proteins associated with lung wash surfactant are not essential for the absorption of surfactant lipids to the air-liquid interface and the reduction of the surface tension to near zero. This latter phenomenon is related presumably to the squeezing out of lipids other than dipalmitylphosphatidylcholine and possibly dipalmitylphosphatidylglycerol from the monolayer (34,41,42). It is possible to prepare active lipid dispersion with surface tension-reducing properties similar to natural surfactant with mixtures of dipalmitoylphosphatidylcholine, unsaturated phosphatidylcholine and phosphatidylinositol (20). It is also possible to prepare active dispersions with phosphatidylglycerol instead of phosphatidylinositol (Metcalf, Enhorning and Possmayer, unpublished results) but in neither case can this be done consistently. The reason for the variability in the surface-active properties of such dispersions is not understood. On the other hand, studies from a number of other laboratories have shown that the presence of surfactant apoproteins can accelerate the rate at which surfactant lipids are absorbed to the air-liquid interface to produce an equilibrium surface tension of ca. 25 dynes/cm. The reason for this difference is not clear but may be related to differences in assay conditions. In the equilibrium studies, the film absorbs from a large volume of stirred hypophase with a constant surface area (25,29,43). With the pulsating bubble technique (20,21), the surface tension of the bubble created in the surfactant suspension decreases to 25-30 dynes/cm at maximum bubble size quite rapidly but the surface tension at minimum bubble radius approaches 0 dynes/cm more slowly. Adsorption of surface-active material to the bubble may be promoted by the formation of the bubble and by its pulsation (see 20, 21 for further details). The present investigations demonstrate that 90% of the proteins present in bovine pulmonary surfactant can be removed without seriously affecting the surfactant properties as estimated with the pulsating bubble technique (Table 5). Dispersions of the lipid extracts of canine surfactant are just as effective as whole lung wash in promoting lung expansion or prolonging the survival of prematurely delivered rabbit pups (44).

Lipid extracts of bovine pulmonary surfactant show biological activity with human neonates

suffering from NRDS (8-10). Nevertheless, the possibility that the proteins remaining in the lipid extracts are essential for the surfactant activity has not been eliminated.

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Sterol Synthesis from Biliary Squalene in the Jejunal Mucosa of the Rat in vivo

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ABSTRACT

Because bile contains substantial amounts of cholesterol precursors, e.g., squalene and different methyl sterols, the fate of biliary squalene was studied by incubating isolated jejunal loops of the rat in vivo with bile containing ^{3}H-squalene and ^{14}C-cholesterol. After 90 min, no radioactivity was found in plasma lipids. Closer analysis of gut epithelium revealed that both labeled compounds were preferentially taken up by the villous cells. Biliary ^{3}H-squalene was absorbed almost completely and was further cyclized to free and esterified methyl sterols and cholesterol. Whereas squalene not cyclized to sterols stayed in the mucosa, the newly synthesized sterols were transferred to lumen. The lipid patterns of gut lumen and mucosal cells were quite different, suggesting that the transfer of the newly synthesized lipid to intestinal lumen was not due to the desquamation of epithelial cells alone. The results suggest that biliary cholesterol precursors can contribute to the cholesterol production of the jejunal villous cells bypassing the rate-limiting step of the cholesterol synthesis pathway, and to the "nonexchanging" fecal neutral sterols of the rat.

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INTRODUCTION

Small intestinal mucosa is the second most active site of endogenous cholesterol synthesis in the rat as measured by methods which reflect the activity of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) (1,2). Recent experiments have indicated that the intestinal mucosa also converts dietary squalene to sterols (3). Substantial amounts of squalene and other immediate cholesterol precursors, methylated sterols in particular, are also present in bile (4,5), but their absorption and further handling in the mucosa are still obscure. The contribution of biliary cholesterol precursors to the intestinal cholesterol synthesis is of special interest, because the cholesterolgenesis from the intermediates beyond mevalonic acid bypasses the major rate-limiting step of the cholesterol synthesis pathway.

In the present in vivo study, bile labeled with ^{3}H-squalene and ^{14}C-cholesterol was injected into isolated loops of rat jejunum in order to compare the uptake of labeled biliary lipids by intestinal villous and crypt cells, to investigate the synthesis and esterification of sterols from ^{3}H-squalene, and to elucidate the fate of the newly synthesized sterols in the mucosa.

MATERIALS AND METHODS

Animals

Male rats of the Sprague-Dawley strain weighing 300-350 g were fed with standard rat chow (Hankkija Ltd., Finland) and tap water ad libitum. The animals were accustomed to a reverse lighting cycle with the dark phase from 5 a.m. till 5 p.m. The experiments were performed between 10 a.m. and 12 noon without a prior fasting period.

Labeling of Bile

Fresh rat bile was labeled with [4-^{14}C]cholesterol (58 Ci/mol, New England Nuclear, Boston, MA) and ^{3}H-squalene (969 Ci/mol, kindly supplied by Dr. R.S. Tilvis) using a method earlier described for labeling of lipoproteins (6).

Briefly, a mixture of ^{3}H-squalene (20 μCi) and ^{14}C-cholesterol (5 μCi) was dissolved in 50 μl tetrahydrofuran (THF, Fluka AG, Switzerland), warmed up to +50 C and injected rapidly into 1 ml distilled water whereafter THF was evaporated. The solution, which was homogenous and faintly opaque, was mixed with 5 ml rat bile and allowed to stay overnight in order to ensure the labeling of the micelles.

Experimental Procedure

The animals were operated under pentobarbital anesthesia (Nembutal^{R}). After opening of the abdominal wall, ligatures were placed at both ends of a 10-cm segment of proximal jejunum (7). Thereafter, 1 ml of labeled bile was injected into the jejunal loop. After checking for leaks, the intestine was replaced and the abdomen was closed. After 90 min under anesthesia, the animals were killed by decapitation and exsanguinated. The jejunal loops were drained and washed once with 0.15 M saline and the intestinal contents were collected for lipid analysis. Subsequently, isolated cells of the loop mucosa were prepared by the dual buffer technique (8). The consecutive cell layers were analyzed separately and pooled to villous, intermediate and crypt cell fractions, as described by Weiser (8).

Chemical Methods and Calculations

Nonsaponifiable lipids (NSL, including squalene and different sterols) of the lumen contents and mucosa were extracted with chloroform/methanol (2:1) (9). Squalene, free and esterified methyl sterols and cholesterol were isolated and quantitated by thin layer chromatography—gas liquid chromatography methods (10). The radioactivity (dpm) was counted in a Wallac model 1215 Rackbeta liquid scintillation counter with 10 ml of 0.5% 2,5-diphenyloxazole (PPO) in toluene. The dpm of ^{3}H-cholesterol were multiplied by 1.5 (11). Protein content of the samples was determined by the method of Lowry et al. (12).

The statistical significancies were assessed by Student's t-test and the correlation coefficients were computed by the method of least squares.

RESULTS

Analysis of blood samples after the 90-min incubation of jejunal loops with labeled bile did not reveal any detectable radioactivity in plasma lipids.

Figure 1 shows the distribution of labeled lipids and the mucosal content among different mucosal cells of isolated jejunum. After the incubation, both ^{3}H-NSL and ^{14}C-cholesterol were found predominantly in the absorptive villous cells.

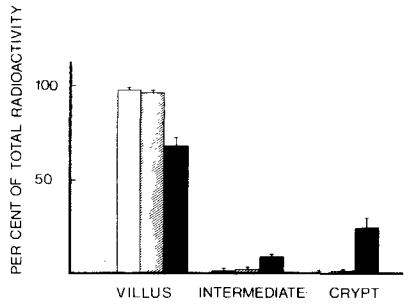

FIG. 1. Percentage distribution of mucosal ^{3}H- and ^{14}C-radioactivity and protein content between villus, intermediate and crypt cell fractions after the incubation of jejunal loops with ^{3}H-squalene and ^{14}C-cholesterol. Bars denote mean ± SE, n = 6. □ ^{3}H-nonsaponifiable lipids, ▨ ^{14}C-cholesterol, ■ protein.

The amounts of ^{14}C- and ^{3}H-radiolabels in the different mucosal cells correlated closely with each other (Fig. 2). ^{3}H-squalene was taken up by the epithelial cells almost completely as only 1% of ^{3}H-squalene was found in the gut lumen after incubation. Absorbed ^{3}H-squalene was efficiently cyclized to sterols in the mucosa. Thus, ca. 70% of ^{3}H-radioactivity was found in methyl sterols and cholesterol (intraluminal sterols included, Table 1, Fig. 3). The relative cyclization of ^{3}H-squalene was

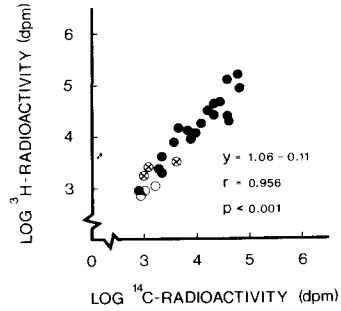

FIG. 2. Correlation of ^{3}H- and ^{14}C-radioactivity (log dpm) of nonsaponifiable lipids in jejunal cells after in vivo incubation of intestinal loops with bile containing ^{3}H-squalene and ^{14}C-cholesterol. • villous cells, ⊖ intermediate cells, o crypt cells.

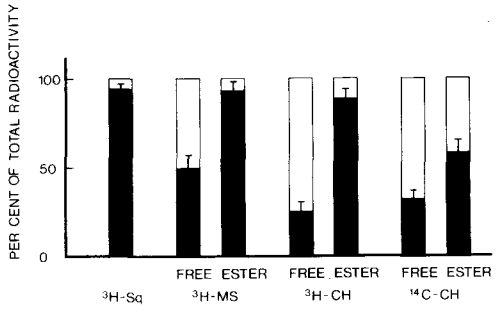

FIG. 3. Percentage distribution of radioactivity of squalene (Sq), methyl sterols (MS) and cholesterol (CH) between lumen (□) and mucosa (■) after in vivo incubation of jejunal loops with bile containing ^{3}H-squalene and ^{14}C-cholesterol. Bars denote mean ± SE, n = 6.

similar in consecutive cell layers of the villous fraction, but it was significantly higher in the crypt than villous fraction (Table 1). Both the newly synthesized ^{3}H-sterols and absorbed ^{14}C-cholesterol were partially esterified in jejunal mucosa (Table 2). The percentage esterification of ^{3}H-cholesterol and ^{14}C-cholesterol was not correlated, whereas ^{3}H-cholesterol and ^{3}H-methyl sterols were esterified similarly. The percentage esterification of luminal ^{3}H-sterols was lower than that of mucosal ^{3}H-sterols.

The distribution of ^{3}H-label between lumen and mucosa (Fig. 3) suggested that a significant portion of ^{3}H-sterols was delivered to lumen. The distribution of free but not esterified ^{14}C-cholesterol between lumen and mucosa was similar to that of ^{3}H-sterols.

In order to elucidate more fully the character of lipid transport from the mucosa, the NSL profiles (Table 3) and cholesterol specific activities (Table 4) were compared. Both were found to be clearly different in the lumen and mucosa.

TABLE 1

Cyclization of ^{3}H-Squalene to Methyl Sterols and Cholesterol in Jejunal Mucosa[a]

Fraction	Number of animals	Percentage of cyclization
Villous	6	48± 8
Intermediate	3	49±20
Crypt	3	81±6[b]
Total	6	69±6[c]

[a]Closed jejunal loops were incubated in vivo with bile containing ^{3}H-squalene and ^{14}C-cholesterol for 90 min. Luminal contents and different mucosal fractions were analyzed for nonsaponifiable lipids. Data are mean±SE.
[b]Significantly different from villous fraction, $p<0.05$.
[c]Includes also cyclized sterols transferred to lumen during the incubation.

TABLE 4

Cholesterol Specific Activities in Lumen and Jejunal Cells[a]

	^{3}H-Cholesterol		^{14}C-Cholesterol	
Site	Free	Esterified	Free	Esterified
Lumen	357±64	26± 8	1080±172	286±105
Cells	74±19[b]	70±34	145± 24[c]	134± 49

[a]Closed jejunal loops were incubated in vivo with bile containing ^{3}H-squalene and ^{14}C-cholesterol. Specific activity of cholesterol (dpm/μg) was measured in lumen and in the first layer of the villous fraction. The data are mean±SE of 6 animals.
[b]Significantly different from the luminal value, $p<0.01$.
[c]Significantly different from the luminal value, $p<0.001$.

DISCUSSION

An important point in the present study is whether the label referred to as mucosal lipid was truly internalized or merely bound to the mucosal surface. Absolute distinction is difficult because vigorous rinsing of the lumen was intentionally avoided after the incubation in order to obtain the villous fraction as intact as possible. However, active cyclization of squalene even in the most superficial layer of the villous fraction suggests that adherence of squalene on the mucosal surface did not disturb the results to any significant degree.

Recent experiments by Watanabe et al. (13) have demonstrated the dependence of the cholesterol absorption rate on the administered amount of cholesterol. In the present investigation, the luminal concentrations of both squalene and cholesterol

TABLE 2

Percentage Esterification of Labeled Sterols in Jejunal Mucosa[a]

Variable	^{3}H-Methyl sterols	^{3}H-Cholesterol	^{14}C-Cholesterol
Esterification			
Lumen	0.3±0.1	0.2±0.04	0.7±0.2[b]
Villous fraction	9.9±4.3[c]	11.3±6.1	2.0±0.4[c]
Correlation	0.97[d]	−0.56[e]	

[a]Closed jejunal loops were incubated in vivo with bile containing ^{3}H-squalene and ^{14}C-cholesterol for 90 min. Luminal contents and mucosa were analyzed for free and esterified sterols. Data are mean±SE of 6 animals.
[b]Significantly different from ^{3}H-cholesterol, $p<0.05$.
[c]Significantly different from the luminal value, $p<0.05$.
[d]Between the percentages of ^{3}H-methyl sterol and ^{3}H-cholesterol in the mucosa, $p<0.01$.
[e]Between the percentages of ^{3}H-cholesterol and ^{14}C-cholesterol in the mucosa, NS.

TABLE 3

Percentage Distribution of ^{3}H-Radioactivity in Nonsaponifiable Lipids of Lumen and Mucosa[a]

		Methyl sterols		Cholesterol	
Site	Squalene	Free	Esterified	Free	Esterified
Lumen	4±1	35±3	0.1±0.04	61±3	0.1±0.02
Mucosa[b]	52±8[c]	28±5	3±2	15±3[c]	2±1

[a]Closed jejunal loops were incubated in vivo with bile containing ^{3}H-squalene. Lumen and mucosa were analyzed for nonsaponifiable lipids. Data are mean±SE of 6 animals.
[b]Distribution of radioactivity is calculated from the total sum of radioactivity in different mucosal fractions.
[c]Significantly different from the luminal value, $p<0.001$.

were physiological and thus most appropriate for absorption studies (13). Consequently, the very efficient absorption rate of ^{3}H-squalene (Fig. 3), as compared to earlier data on dietary squalene (4), might be due to the relatively low content of intraluminal squalene. On the other hand, it is also possible that biliary and dietary squalene are not absorbed similarly, a phenomenon earlier demonstrated for dietary and micellar biliary cholesterol (14-16).

Because no fat was used in the present experiments, triglyceride-rich lipoproteins were obviously not formed and labeled lipids did not reach the circulation within the incubation time. Because the leak of the label from the mucosa and the subsequent involvement of extraintestinal factors were avoided, water-insoluble ^{3}H-squalene is preferable to mevalonate and other small molecular precursors as a metabolic source and marker for the newly synthesized sterols.

The experiments demonstrated that biliary squalene as well as dietary squalene (3) is cyclized efficiently to sterols in the gut epithelium. As luminal squalene was predominantly absorbed by villous cells, the results indicate the contribution of villous cells to the intestinal cholesterol synthesis not detected by the measurement of HMG-CoA reductase activity. On the other hand, the rate of squalene cyclization was higher in the crypt than villous cells. This may reflect the high cholesterol synthesis in the crypt cells during chow diet (17), even though different transfer of the newly synthesized ^{3}H-sterols from the 2 cell types to the gut lumen is possibly involved, too.

The mechanism of the lipid transfer is not known, but several explanations are possible. The exchange of the labels between lumen and mucosa can occur in experiments of the type I report here. Thus, the different transfer to squalene and sterols might be due to the binding of squalene by a squalene and sterol carrier protein (SCP, 18) in microsomes. However, it is unlikely that the existence of labeled esterified sterols in the lumen could be explained by the exchange mechanism. The spillage of cell contents or sloughing of mucosal cells during the incubation and the cell preparation are possible (19,20), but the patterns of radioactivity in the gut lumen and epithelium were completely different. It has been suggested that cholesterol is actively secreted from mucosa into intestinal lumen (21), but the mechanism and significance of the active secretion are obscure. Both cell sloughing and active secretion could explain the occurrence of esterified sterols in the lumen.

The present study demonstrates that biliary squalene is taken up by the jejunal mucosa and converted efficiently to free and esterified sterols. The intestinal sterols newly synthesized from biliary squalene are partly transferred to the gut lumen and may thus contribute to the "nonexchanging" fecal sterols which are not equilibrated with plasma cholesterol (22).

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Metabolism of Platelet-Activating Factor by Blood Platelets and Plasma

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ABSTRACT

High performance liquid chromatography in combination with a radioactivity detector was used to study the metabolism of platelet-activating factor (1-0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) by washed platelets, platelet-free plasma and platelet-rich plasma obtained from rabbits and humans. Degradation of platelet-activating factor to its 2-lyso derivative was observed in rabbit and human plasma. This degradation of platelet-activating factor in plasma was completely inhibited by diisopropylfluorophosphate and was partially inhibited by ethylenediamine tetraacetic acid. Washed platelets metabolized platelet-activating factor not only to the 2-lyso compound but also, by reacylation of this lyso intermediate, to an analogue of platelet-activating factor probably containing a long-chain acyl group at the *sn*-2 position. These transformations occurred, but to a lesser extent, in platelet-rich plasma.
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INTRODUCTION

PAF is a low molecular weight hydrophobic (1) phospholipid which activates platelets (2-4) and has powerful antihypertensive properties (5). PAF is known to be released into the plasma of rabbits during IgE-induced anaphylaxis and binds to circulating blood cells (6-8). The extent of PAF binding to platelets was found to be greater than to other blood cells (9). The apparent high affinity of PAF towards platelets could involve rapid association with a slow rate of dissociation, internalization within platelets and/or metabolism. Several rat tissues (10,11) as well as human leukocytes (12) have been shown to deacetylate PAF and the formation of PAF analogues by rabbit (13) and guinea pig (14) leukocytes has been reported. There is no information in the literature describing the disposition of PAF after its interaction with the platelet surface. Experiments in vivo have demonstrated that the transient hypotensive effect observed after the administration of low doses of PAF in rats is related to the degradation of PAF and its rapid removal from the circulation (15). We now show that plasma from humans or rabbits can degrade PAF enzymatically to lyso-PAF and that platelets not only carry out this transformation but reacylate lyso-PAF to a previously uncharacterized derivative formed by platelets.

MATERIALS AND METHODS

HPLC grade solvents were obtained from Fisher Scientific, King of Prussia, PA. Deionized water was filtered through a Millipore filter (0.45 μ). A Waters Associates Liquid Chromatograph equipped with a model 6000A solvent delivery system was used for HPLC. The column employed was a 250 × 4.6 mm (id) Lichrosorb Si60 (10 μ particle size) purchased from Rainin Instrument Co., Woburn, MA. The radioactivity flow detector was purchased from Radiomatics, Tampa, FL. The mobile phase consisted of two solvents, A and B. Solvent A was a mixture of propanol/ethylacetate/benzene/water (130:80:30:20, v/v/v/v) and solvent B consisted of propanol/toluene/acetic acid/water (93:110:15:15, v/v/v/v). A two-step isocratic elution scheme was used as previously described (16). TLC was done on Silica Gel GH plates obtained from Analtech Inc., Newark, NJ. 1-0-(^{3}H)-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (SA 45 Ci/mmol) was a gift from Dr. Ahern, New England Nuclear, Boston, MA. Oleic acid (1-^{14}C, SA 50 mCi/mmol), arachidonic acid (1-^{14}C, SA 55.8 mCi/mmol) and phosphatidylcholine (dipalmitoyl-1-^{14}C, SA 100 mCi/mmol) were purchased from New England Nuclear, Boston, MA. Linoleic acid (1-^{14}C, SA 57 mCi/mmol) was purchased from Amersham, Arlington Heights, IL. 1-0-alkyl-2-acetyl-glycero-3-phosphocholine and 1-0-alkyl-2-hydroxy-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar Lipids Inc., Birmingham, AL. Bee venom phospholipase A_2 and diisopropylfluorophosphate were purchased from Calbiochem-Behring, San Diego, CA. Dimethylaminopyridine and acetic anhydride were purchased from Aldrich Chemical Co., Metuchen, NJ.

Buffers

Tyrodes gelatin buffer (TG buffer) was prepared by dissolving KCl, 195 mg; $MgCl_2 \cdot 6H_2O$, 212.5 mg; NaCl, 8 g; $NaHCO_3$, 1 g; glucose, 1 g; ethyleneglycol-*bis*-(β-amino-ethylether) N,N-tetraacetic acid (EGTA), 76.07 mg; and gelatin 2.5 g; in

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Abbreviations: PAF, platelet-activating factor; "2-acyl-PAF", 1-0-alkyl-2-long chain acyl-glycerophosphocholine; PRP, platelet-rich plasma; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; PFP, platelet-free plasma.

one liter of water. The pH was adjusted to 6.5 with 1 N HCl.

Tris-Tyrodes Albumin buffer (TTA buffer) was prepared by dissolving KCl 195 mg; $MgCl_2 \cdot 6H_2O$, 212.5 mg; NaCl, 8 g; $CaCl_2 \cdot 2H_2O$, 191 mg; Tris (hydroxymethyl)-aminomethane (Tris), 1.21 g; glucose, 1 g; and BSA (bovine serum albumin), 2.5 g; in one liter of water. The pH was adjusted to 7.4 with 1 N HCL. Tris saline buffer (TS buffer) contained 445 ml of 0.15 M NaCl, 50 ml of 0.154 M Tris HCl 7.4, 0.5 g glucose, 10 ml of 0.1 M EDTA and 250 mg BSA.

Preparation of Washed Rabbit Platelets

Blood was collected by cardiac puncture of adult New Zealand White rabbits into one-tenth volume 3.8% trisodium citrate. PRP was obtained by repeated centrifugation of blood at 220×g to eliminate contaminating white cells. Washed platelets were prepared as described in ref. 17. Briefly, the PRP was centrifuged at 1400×g for 15 min at 4 C. The sediment was gently resuspended in an equal volume of TG buffer. This procedure was repeated and the platelet count was determined. The platelets were recentrifuged at 4 C. The pellet was resuspended in TG buffer and the platelet concentration was adjusted to 5×10^8/ml. The suspension was divided into 5-ml portions and each pellet was resuspended in 5 ml TTA buffer. Platelet-free plasma was prepared by centrifuging PRP at 15,000×g for 5 min at room temperature.

Preparation of Washed Human Platelets

Citrated PRP, prepared as described previously (18,19), was centrifuged twice as described above to remove white cells. This PRP was cooled to 4 C and cold 0.1 M EDTA was added to give a final concentration of 5 mM. The PRP was centrifuged at 1800×g for 6 min at 4 C and the platelet pellet resuspended in an equal volume of TS buffer and incubated at 37 C for 10 min. The suspension was cooled to 4 C and centrifuged at 600×g for 10 min. The platelets were resuspended in the same buffer, the platelet count was determined and the volume adjusted to give 5×10^8 platelets per ml. This platelet suspension was divided into 5-ml portions, each centrifuged at 600×g at 4 C for 10 min and the platelets were resuspended in 5 ml TTA buffer.

Incubation, Extraction and TLC Procedures

Unlabeled PAF (final concentration, 0.2 μM) along with 1 μCi of radiolabeled PAF was incubated at 37 C for selected time periods with 5 ml of washed platelet suspension containing 2.5×10^9 platelets, 5 ml of PRP containing 2.5×10^9 platelets, or PFP. To each incubation mixture was then added 18 ml of chloroform/methanol (1:2, v/v) followed by 6 ml of chloroform and 6 ml of 0.1 M EDTA. The samples were then mixed and centrifuged to separate the phases. The organic phase was removed, the aqueous phase was reextracted with chloroform (10 ml) and the combined organic extract was evaporated under N_2 at room temperature. Recovery of both radiolabeled PAF and lyso-PAF (see below) was 75-85%. Components of the extracts were separated by TLC (15).

Preparation of 1-O-(^{3}H)-Alkyl-2-Lyso-Glycero-3-Phosphocholine (lyso-PAF)

This was prepared following the method of Wells and Hanahan (20). Radiolabeled PAF (2 μCi) was taken up in 1 ml diethylether. To this was added 10 μl of bee venom phospholipase A_2 (10 mg/ml of 0.1 M Tris HCl, pH 7.4, 5 mM $CaCl_2$). After incubation for 3 hr at room temperature, the ether was evaporated under a stream of nitrogen and the residue was resuspended in 0.6 ml chloroform/methanol (2:1). TLC (15) of the residue using the solvent system chloroform/methanol/acetic acid/water (50:25:8:4, v/v/v/v) indicated 84% conversion to lyso-PAF (R_f, 0.20).

Reacetylation of Lyso-PAF Obtained from Rabbit Platelets

This was accomplished according to the method of Gupta et al. (21). The lyso-PAF, obtained as described above, was eluted from the TLC plate with 5 ml of methanol. Reacetylation was accomplished by refluxing with aceticanhydride (200 μl, 2.1 mmol) and dimethylaminopyridine (1 mg, 8.2 μmol) in 5 ml pyridine/methylene chloride (1:5, v/v) for 8 hr. The reaction mixture was cooled, evaporated under vacuum, and analyzed directly by HPLC.

Preparation of Labeled 1-O-Alkyl-2-Long Chain Acyl Glycero-3-Phosphocholine

Rat liver (1 g) was thoroughly washed with saline solution to remove blood and then homogenized in 45 mM KH_2PO_4 buffer, pH 7.4 (4 ml). Rat liver homogenate (1 ml, 0.25 g), ATP (25 μmol), coenzyme A (0.25 μmol), magnesium chloride (1.5 μmol) and sodium fluoride (31 μmol), buffered to pH 7.4 with 45 mM KH_2PO_4, were incubated at 37 C for 1 hr with oleic acid (1-^{14}C, 1 μCi) and ^{3}H-PAF (5 μCi). The incubation mixture (2 ml) was mixed with 7.2 ml of chloroform/methanol (1:2), 0.1 M EDTA (2.5 ml) and chloroform (2.4 ml) and extracted. The organic phase was removed and the aqueous phase was reextracted with chloroform (2 ml). The combined extract was dried under N_2. The residue was resuspended in chloroform/methanol (2:1) and analyzed directly by HPLC.

RESULTS

We first determined the time course and extent of

the degradation of radiolabeled PAF in rabbit PFP The extracts were submitted to TLC and only two products were detected. These corresponded to PAF and lyso-PAF (R_f values of 0.28 and 0.20, respectively). Figure 1 illustrates the rapid formation of lyso-PAF and decrease in PAF when ^{3}H-PAF was incubated with PFP. Lyso-PAF formation reached a plateau in 5 min. PAF was not hydrolyzed when the plasma was replaced either by TTA buffer or plasma boiled for 10 min. The degradation of PAF in rabbit plasma was almost abolished by pretreatment of plasma with 10 mM diisopropylfluorophosphate, and markedly inhibited by 10 mM EDTA (Fig. 2). The profile of radioactivity of the components of the extract obtained by HPLC following incubation of PAF with washed rabbit platelets is seen in Figure 3. In addition to unchanged PAF and lyso-PAF a third metabolite, which we are tentatively calling "2-acyl-PAF", was seen to be eluted at 59 min. This metabolite was rechromatographed together with radioactive standards of dipalmitoyl phosphatidylcholine (PC), PAF and lyso-PAF. The radioactivity profile (Fig. 4) of the separated lipids was obtained directly from the chart recorder. This metabolite is probably a 1-0-alkyl-2-long chain acyl glycero-3-phosphocholine for the following reasons. (a) The unknown metabolite produced by the rabbit platelets was eluted from the HPLC column at 59 min, very close to PC (63 min). This suggested the possibility that lyso-PAF had been reacylated with a long-chain fatty acid. Such a product has been reported to be formed by rat tissue (15). (b) We biosynthesized various PAF analogues containing oleic, linoleic or arachidonic acid in the 2-position as described in Methods. We then compared the retention times of the metabolites of these PAF analogues by HPLC. The oleic and arachidonic acid analogues of PAF had retention times of 60 min, while the linoleic analogue

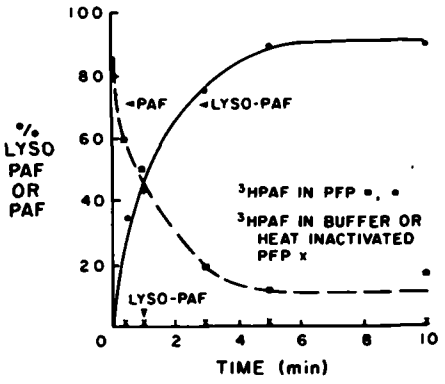

FIG. 1. Time course of degradation of ^{3}H-PAF in rabbit platelet-free plasma at 37 C.

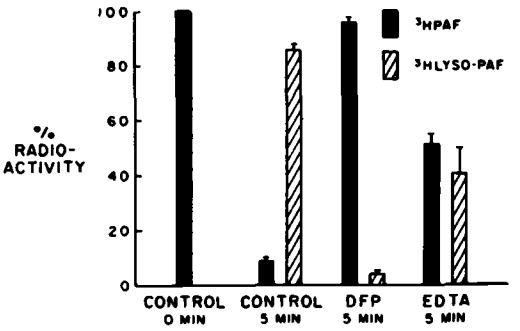

FIG. 2. Inhibition of ^{3}H-PAF degradation in rabbit platelet-free plasma by 10 mM DFP or 10 mM EDTA. Plasma was pretreated with microliter amounts of either DFP or EDTA or 0.154 M NaCl solution at 37 C for 25 min prior to incubation with ^{3}H-PAF at 37 C for 5 min. Samples were extracted and analyzed by TLC as described under Methods.

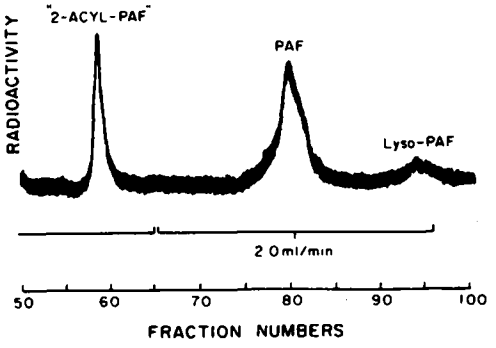

FIG. 3. Radioactivity profile of products obtained after incubation of ^{3}H-PAF with washed rabbit platelets.

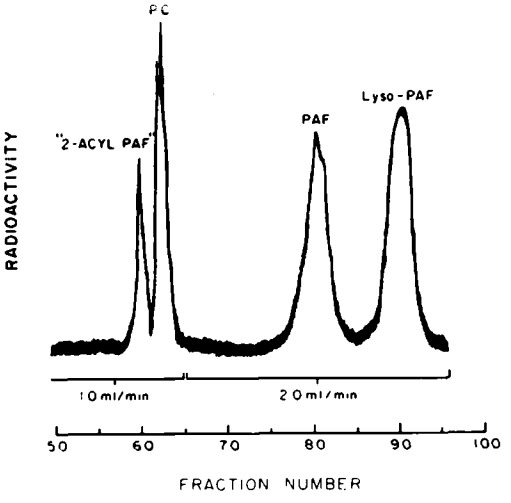

FIG. 4. Resolution of rabbit platelet metabolite from phosphatidylcholine (PC), PAF and lyso-PAF on a Lichrosorb Si60 column.

and the rabbit platelet metabolite had a retention time of 59 min. (c) Incubation of the purified metabolite with bee venom phospholipase A_2 led to the formation of a product which eluted on HPLC with the retention time of lyso-PAF (85-97 min). This product was reacetylated by refluxing in acetic anhydride (see Methods). The product of this reaction had a retention time identical to PAF (75-85 min).

Degradation of PAF and reacylation of lyso-PAF occurred in human and rabbit washed platelets as well as in PRP, but reacylation did not occur in platelet-free plasma. The extent of lyso-PAF formation by platelets or plasma as well as its reacylation by platelets to 2-acyl PAF is indicated in Table 1.

TABLE 1

Formation of "2-Acyl-PAF" and Lyso-PAF by Human or Rabbit Washed Platelets or Platelet-Rich Plasma

	Human	Rabbit
	Washed Platelets	
Lyso-PAF	3.88 ± 2.52	8.74 ± 1.6[a]
PAF	87.77 ± 2.11	66.0 ± 9.19
2-Acyl PAF	4.73 ± 1.08	19.3 ± 6.98
	Platelet-rich plasma	
Lyso-PAF	46.80 ± 9.15	62.73 ± 10.1
PAF	46.46 ± 8.98	34.27 ± 9.13
2-Acyl-PAF	2.36 ± 0.54	2.65 ± 1.19
	Platelet-free plasma	
Lyso-PAF	50.62 ± 3.0	89.75 ± 1.06
PAF	43.41 ± 5.56	9.98 ± 0.74
2-Acyl PAF	0	0

[3]H-PAF (1 μCi) plus PAF (0.2 μM) were incubated with human or rabbit washed platelets, PRP or platelet-free plasma for 5 min at 37 C. The incubation mixtures were extracted and the extracts analyzed by TLC as described in methods.

[a]Values given are mean ± SD of 5 experiments using blood from 5 different donors. Values expressed are percentage of total radioactivity extracted from each TLC plate. They do not add up to 100% because some radioactivity was always found at the solvent fronts.

DISCUSSION

It has been previously shown that platelets and other blood cells take up PAF (9) but the metabolism of PAF by platelets was not investigated. Our findings show that PAF is metabolized both by platelets and by plasma.

Metabolism by Plasma

Normal human and rabbit plasma and serum have been reported to contain an acid-labile factor which inactivates PAF (22). This factor has been reported to be associated with lipoproteins (22). It has also been reported that intravenous infusion of radiolabeled PAF in rats resulted in the accumulation of lyso-PAF in blood (15), suggesting that PAF is degraded by a plasma enzyme (10). We examined the capacity of plasma to degrade PAF in vitro. Rabbit plasma was more potent than human in the conversion of PAF to lyso-PAF. DFP inhibited the formation of lyso-PAF by ca. 90%, whereas EDTA also inhibited but to a lesser extent. Inhibition of the degradation of PAF by pretreatment of plasma with DFP, EDTA or heat indicates that the formation of lyso-PAF was an enzymatic process.

Metabolism by Platelets

The degradation of PAF to lyso-PAF by washed platelets most likely was the result of platelet phospholipase A_2 activity. In addition, we have presented evidence which suggests that lyso-PAF is reacylated to a 1-0-alkyl-2-long chain acyl glycero-3-phosphocholine analogue by rabbit and human platelets.

The formation of the PAF analogue could be explained by reacylation of the lyso-PAF via an acyltransferase. Such an enzyme exists in platelets and prefers unsaturated to saturated fatty acids (23). To obtain supporting evidence for the identity of the substituent at the 2-position of the rabbit platelet metabolite, various PAF analogues containing oleate, linoleate or arachidonate substituents at the 2-position were prepared and their retention times were determined and compared with those of the metabolites produced by rabbit platelets. The platelet metabolite with a retention time of 59 min probably contains linoleic acid at the 2-position since it coeluted with the linoleate substituted PAF analogue. Further work is in progress to identify the fatty acid(s) in the 2-position.

Our study has provided evidence that removal of the acetyl group from PAF and utilization of lyso-PAF to form other metabolites occurs in platelets. The reacylation of lyso-PAF may represent an important mechanism for conservation of 1-0-alkyl-*sn*-glycero-3-phosphocholine species for subsequent biosynthesis of PAF.

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Glycolipids and Thylakoid Proteins in Chloroplasts and Streptomycin-Bleached Lamellae of *Euglena*

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ABSTRACT

Thylakoid proteins from (a) the chloroplasts of green *Euglena gracilis* cells and from (b) the corresponding concentric lamellar bodies of streptomycin-bleached *Euglena* have been extracted in aqueous sodium dodecyl sulfate (SDS) and purified. The molecular weights of the proteins, determined by polyacrylamide gel electrophoresis in SDS, were 9 kilodaltons for (a) and 9 and 11.5 kilodaltons for the 2 polypeptide chains of (b). Each of the purified proteins consists of 55% apolar and 45% polar amino acids. The lipids of the green and bleached cells differ in polarity of components. Neutral galactolipids are abundant in green cells but not in streptomycin-bleached cells. Anionic sulfolipid content is greater in the chlorotic organisms. Polyenoic fatty acids, mainly with 16, 18 and 19 carbon atoms, occur in the green cell galactolipids. Cells grown in the presence of streptomycin are, in contrast, rich in monoenes. The opposite is observed in the sulfolipids: monoenes with 16 and 20 carbons are the main fatty acids in the green cells, while a dienoic fatty acid with 12 carbons is found in large amounts in streptomycin-bleached cultures.
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A number of chemical and physical agents can have a dramatic effect on the chloroplasts in *Euglena gracilis*, changing this unique protozoan flagellate from a green plant-like cell to a permanently bleached organism. Provasoli et al. (1) were the first to observe the bleaching effect of streptomycin in *Euglena*. The inner chloroplast membranes, which are retained in light-grown streptomycin-bleached *Euglena* cells, are found in oval or spherical bodies known as concentric lamellar bodies (CL bodies) (2). In this paper, a 9-kD thylakoid protein from green plastids and 9- and 11.5-kD thylakoid proteins from the albino plastids have been isolated and purified. The proteins were then compared for size and polarity.

Mono- and digalactosyl diglycerides, the main lipids synthesized by *Euglena* (3-5), were studied, in purified form, by Rosenberg et al. (6). Sulfolipid was also previously detected in green and bleached *Euglena* cells (4,7-9). All of these glycolipids, however, occurred in *Euglena* cells bleached by means other than by the antibiotic. A modified version for isolating and analyzing the glycolipids in the green and the streptomycin-bleached organisms is described here. A comparative study of the fatty acid compositions of these glycolipids was made in the 2 types of plastids.

METHODS

Cell Growth

Cells were grown in 1-liter cotton-stoppered Erlenmeyer flasks containing 500 ml of Wolken's complete medium (10). Cultures were maintained at room temperature (kept below 25 C by air conditioning, if necessary) and under constant illumination from a 15-watt "Cool-White" General Electric fluorescent tube.

Cells were bleached by introducing 500 mg of streptomycin sulfate into test tubes containing 10 ml of sterile complete medium followed by inoculations from the green stock culture (11). Eleven days later, the contents of the test tubes were mass transferred to Erlenmeyer flasks of streptomycin-free medium. Succeeding transfers were made to fresh streptomycin-free medium with an inoculating needle every 11 days.

Cultures were harvested after 11 days' growth by centrifugation for 5 min at 4 C and 1100×g in a Sorvall refrigerated automatic centrifuge. The cells were washed twice with cold deionized water, collected and frozen at −20 C.

Isolation of Chloroplasts and Concentric Lamellar Bodies

After thawing, *Euglena* cells were treated with trypsin for removal of their pellicles and the resulting spheroplasts were homogenized. Their chloroplasts or CL bodies were collected by centrifugation according to Rawson and Stutz (12) with the modification that the filtrate from the homogenized bleached spheroplasts was centrifuged at 120×g for 2 min. The organelles were washed with deionized water at 4 C and 12,100×g for 5 min to remove water-soluble components according to Keylock et al. (13).

Preparation of Acetone Powder Extracts

Washed organelles were mixed with cold deionized water and the suspensions slowly pipetted into vigorously stirred acetone immersed in dry ice. The ratio was 500 mg washed chloroplasts or CL

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bodies: 10 ml deionized water: 100 ml acetone. The acetone-precipitated proteins were centrifuged for 5 min at 12,100 × g, 4 C, and washed several times with cold acetone and finally with cold ether (13). Dry acetone powders were dissolved in 0.5% (w/v) sodium dodecyl sulfate (SDS), pH 7.2, in a ratio of 1 mg powder: 0.1 ml SDS solution for at least 2 hr with occasional shaking at room temperature. SDS extracts were centrifuged at 27,000 × g, 4 C, for 15 min to remove the remaining insoluble material.

Gel Filtration Chromatography

Protein was partially fractionated by gel filtration chromatography of acetone powder extracts on a column (2.5 cm × 32.6 cm) of Sephadex G-100, equilibrated and eluted with 0.5% (w/v) SDS, pH 7.2, at room temperature and flowing at ca. 12 ml/hr. Individual fractions, 1.3 ml in volume, were collected by drop counting and monitored at 280 nm in a Beckman DB-GT spectrophotometer.

Polyacrylamide Gel Electrophoresis

Approximately 25 μg of protein from the main column eluted peak were applied to each polyacrylamide gel for further purification by the procedure of Ornstein (14) and Davis (15) as modified by deVito and Santomé (16). Electrophoresis was performed in 5×10^{-2} M Tris adjusted to pH 8.3 with 7.7×10^{-2} M $NaH_2PO_4 \cdot H_2O$, diluted 10-fold and made 0.5% (w/v) in SDS. Stained gels were placed parallel to unstained gels and the corresponding major protein bands from the unstained gels dissected with a blade. The major proteins were extracted from homogenized gel slices by incubation with 0.5 ml of 0.5% (w/v) SDS/gel slice at 37 C overnight, followed by an additional rinse with 0.5 ml of SDS. Supernatant extracts were pipetted into conical centrifuge tubes and separated from residual gel particles by centrifugation at room temperature for 30 min at 200 × g. Nine parts cold acetone were mixed with one part SDS extract to precipitate the proteins (17) and centrifuged at the same speed and time as the SDS extracts. Protein precipitates were dried under nitrogen.

Molecular Weight Determinations

Using the same procedure discussed above, the purified lamellar proteins were run along with standards on separate parallel polyacrylamide gels. Electrophoretic mobilities were calculated by Weber and Osborn's formula (17) and the molecular weights of the major proteins read from the standard semilogarithmic plot of molecular weight vs electrophoretic mobility.

Amino Acid Analysis and Protein Assays

Purified proteins were transferred to acid-washed ignition tubes along with 6 N hydrochloric acid and 0.5% (v/v) phenol. The tubes were evacuated to less than 50 μHg, and the samples hydrolyzed at 110 C for 20 hr. Quantitative amino acid analyses were performed on a Durrum D-500 automatic analyzer. Ten mg of amino acids/30 ml of starting buffer was a typical load placed on the ion-exchange column.

Samples of acetone powder were hydrolyzed and analyzed for amino acids on a Durrum analyzer. Thirty mg of acetone powder protein in 5 ml of buffer was an adequate sample. Protein in the acetone powders and the polyacrylamide gel extracts was calculated from the total amount of amino acids in the hydrolysates.

Chlorophyll Estimation

Chlorophyll was determined by Arnon's method (18). The Sephadex eluant was monitored at 420 nm.

Extraction of Total Lipids

Packed cells were homogenized in a high speed Waring blendor for 2 min with a 20-fold excess (1 g/20 ml) of precooled chloroform/methanol (2:1, v/v) at ca. 4 C. The mixture was filtered and dialyzed against cold tap water in the dark. After 24 hr of dialysis, the lower chloroform phase was collected and treated overnight in the freezer with anhydrous sodium sulfate and concentrated in a flash evaporator. The chloroform/methanol was evaporated and replaced by benzene. The resulting benzene solution was diluted with 20 ml of benzene and stored under nitrogen at −20 C (6,19). Approximately 400 mg of total lipids/5 g of cells were extracted from the green and the colorless organisms.

Isolation of Glycolipids

Separation of glycolipids from total lipid material was attained through one-dimensional thin layer chromatography according to Pohl et al. (20). Each plate (12 × 20 cm) was spread with a suspension of 3.5 g of Silica Gel HF_{254} in 9 ml of deionized water, producing a layer thickness of 0.5 mm. Plates were air-dried for 30 min and then activated in an oven for 2 hr at 130 C. Warm plates were cooled in a dessicator and stored in a vacuum over fresh phosphorus pentoxide. After the plates, containing 2-4 mg of total lipids, were developed in benzene/acetone/deionized water (30:91:8, v/v/v), a 0.003% alkaline solution of Rhodamine 6 G was sprayed on an uncovered reference strip while the remainder of the chromatogram was covered with a clean glass plate. The bands, recognized by their R_f values, were scraped off with a blade and extracted 5 times with an equal volume of the following solvents: chloroform for pigment; ace-

tone for the wax-like component and the monogalactosyl diglycerides; methanol/acetone (5:1, v/v) for the cardiolipid and the digalactosyl diglycerides; and methanol for the sulfolipid and the phospholipids. Absorbent was removed from the lipid extracts by centrifugation at 500×g for 10 min at room temperature. Solvents were evaporated under nitrogen and the lipid fractions weighed.

Chemical Analyses

Hexose was quantitated by the method of Trevelyan and Harrison (21). Phosphorus was analyzed according to King (22). Sulfur was determined by Letonoff and Reinhold's procedure (23).

Gas Liquid Chromatography of Fatty Acid Methyl Esters

Fatty acid methyl esters were prepared according to Morrison and Smith (24) and separated on a Research Specialties Co_1 Series 600 gas chromatograph equipped with a hydrogen flame ionization detector and an all glass on-column injection system. Methyl esters were analyzed on 2 columns: (A) polar column, 3.5 ft long and 4 mm od, packed with 10.5% diethylene glycol succinate (DEGS) on 60-80 mesh Chromasorb W (acid washed and dimethylchlorosilane treated), column temperature 179 C, carrier gas (N_2) flow rate 47 ml/min; (B) nonpolar column, 2 ft long and 3 mm od, composed of 10.5% Apiezon L on Diatoport S, 60-80 mesh, column operated at 232 C with a carrier gas flow rate of 52 ml/min. Fatty acids were identified by comparing retention times of the peaks with those of methyl ester standards using the technique developed by James (25) and Hawke et al. (26). The percentage of fatty acid was calculated from the integrator pen trace on the chromatogram.

RESULTS

Protein Concentration of Lipid-Free Powders

By summing individual amino acid concentrations from the Durrum analysis of acetone powders, it was determined that the total protein content of the powders is 0.30 and 0.01 mg of protein per mg of green cell and bleached cell powder, respectively. Extraction of the acetone powders with SDS always resulted in white residues after centrifugation. After 5 successive extractions with SDS, the residues, analyzed for their amino acids, were found to contain 0.5% of the total protein that was acetone-precipitated from the lamellar membranes in the green powder and less than 0.01% of the acetone-precipitated protein in the bleached powder. The residues do not seem to be lipid in nature since they are not soluble in chloroform/methanol, a common solvent for *Euglena* lipids.

Fractionation and Purification of Thylakoid Proteins

Sephadex G-100 removed several minor protein components from SDS extracts of the acetone powders. Examination of column effluent by polyacrylamide gel electrophoresis (Fig. 1) revealed that there was one major protein in the green acetone powder. Streptomycin-treated cells yielded 2 major bands that were equal both in size and intensity of staining. Two major protein bands in the white cells were observed only when tris-phosphate was substituted for tris-glycine as the running buffer in electrophoresis. Tris-glycine, which produced one major band, contributed to glycine contamination in the amino acid studies. The results obtained here suggest that polypeptide dissociation in the tris-phosphate buffer may explain the observations for the bleached protein.

The gel filtration fractions that resulted in a major protein band on the polyacrylamide gels were designated as the thylakoid protein fractions. It was calculated that 25±5% of the protein in green chloroplast acetone powder is represented by the isolated protein component and 20±5% of the protein in the bleached concentric lamellae is represented by the isolated protein component.

Molecular Weights

From the electrophoretic mobilities of the isolated thylakoid *Euglena* proteins, the following molecular weights were established: 9,000 (green); 9,000 (bleached, faster moving band); 11,500 (bleached, slower moving band). Molecular weights were confirmed by gel filtration chromatography.

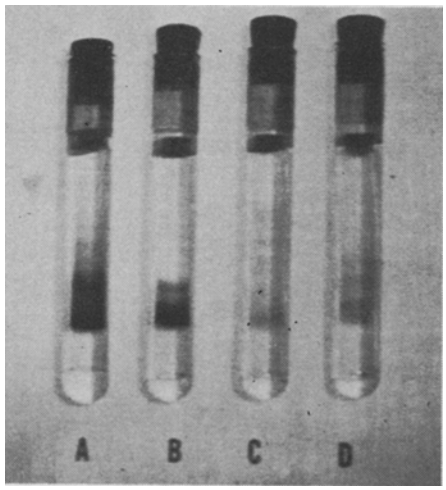

FIG. 1. Polyacrylamide disc gel electrophoretograms of the thylakoid proteins in the chloroplast lamellae from *Euglena gracilis*. (A) Acetone powder (green cells); (B) Sephadex G-100 eluant (green cells); (C) acetone powder (streptomycin-bleached cells); (D) Sephadex G-100 eluant (streptomycin-bleached cells).

TABLE 1

Amino Acid Compositions of the Thylakoid Proteins

Amino acid	Chloroplasts	Nanomole percent white CL bodies (smaller polypeptide chain)	White CL bodies (larger polypeptide chain)
Half-cystine[a]	5.2	6.0	4.3
Aspartic acid	9.6	12.4	9.3
Methionine[a]	1.8	1.5	1.6
Threonine[b]	3.9	4.4	4.5
Serine[b]	7.1	5.9	7.9
Glutamic acid	9.9	10.8	9.4
Proline	4.6	7.3	6.5
Glycine	14.6	14.4	11.9
Alanine	9.7	7.0	9.7
Valine	4.2	4.9	5.2
Isoleucine	3.5	3.6	4.0
Leucine	9.4	7.4	8.0
Tyrosine	3.4	1.6	4.2
Phenylalanine	4.5	3.4	4.2
Histidine	1.6	0.8	1.9
Lysine	2.6	3.9	3.2
Arginine	4.4	4.7	4.2
Tryptophan[c]	--	--	--
Total	100.0	100.0	100.0

[a]Proteins, oxidized by performic acid, were used to determine the content of half-cystine and methionine which were estimated as cysteic acid and methionine sulfone, respectively.

[b]Threonine and serine were corrected for hydrolytic losses of 5 and 10%, respectively.

[c]Not determined—trytophan was completely lost during hydrolysis.

Amino Acid Compositions

The amino acid compositions of the major proteins, given in nanomole percent, are listed in Table 1. The ratio 55% apolar to 45% polar amino acids in each protein was calculated from the sum of the nanomole percent of apolar or polar amino acids divided by the total nanomole percent in Table 1, excluding half-cystine, tyrosine and tryptophan. Half-cystine often participates in covalent interactions while tyrosine is ambivalent in nature. Tryptophan was completely lost during hydrolysis.

An analysis for hexoseamine in the purified protein from chloroplasts was performed on the Durrum instrument. Less than 0.02 μg of hexosamine could have been found by the ninhydrin method but none was detected.

Chlorophyll Content

At least one mole of chlorophyll remains bound to each mole of the purified major protein from the green cells, presumably in a pigment-protein complex. The pigment remains through the course of gel filtration chromatography and the electrophoretic procedure (a green band was observed at the position of the major protein on unstained gels). The chlorophyll a: chlorophyll b ratio was 2:1. No chlorophyll was recorded in the bleached samples.

Evaluation of Thin-Layer Chromatograms

The percentage of each lipid component, listed in Table 2, is based on the dry weight of each isolated constituent. The results of the spectrophotometric analysis of the total lipids are given in Table 3.

Fatty Acid Compositions of the Glycolipids

Differences between the fatty acids of the glycolipids from the green and the bleached organisms are noticed. The major fatty acids (above 5%) from the green cultures are shown in Figure 2. Figure 3 depicts the principal fatty acids from streptomycin-bleached cells. Polyunsaturated fatty acids (2-4 double bonds, 14-19 carbon atoms per chain) predominated in the mono- and digalactosyl diglycerides of green cells. Monoenes comprised ca. 20% of the fatty acids in the monogalactosyl diglycerides of streptomycin-bleached cells; however, the monoenes were still outnumbered by the polyunsaturated fatty acids in the monogalactosyl fraction of these cells. Monoenes were more abundant than polyunsaturated fatty acids in the digalactosyl diglycerides of streptomycin-bleached organisms. Sulfolipid from green cells contained chiefly monoenes, whereas achlorotic cells contained polyunsaturated fatty acids. In both cases (green and bleached), the fatty acid chains were longer (fatty acid chains of 20 carbon atoms were

TABLE 2

Components Isolated from Total Lipids by Thin Layer Chromatography

Component	Percent of total lipid weight	
	Green cells	Bleached cells
Pigment	32.8	Absent
Wax-like substance*	Absent	64.0
Monogalactosyl diglycerides	8.0	2.6
Cardiolipid + pigment	15.4	Absent
Digalactosyl diglycerides	27.6	14.8
Sulfolipid	2.4	5.3
Phospholipids	13.8	13.3
Total	100.0	100.0

*This wax-like component may be a fatty acid ester (33). Via gas chromatography, three small lipid components (less than 5% of the total lipids) which range between C_{12} and C_{14} in composition were detected.

TABLE 3

Spectrophotometric Analysis of Total Lipids

Analysis	Percent of total lipid weight	
	Green cells	Bleached cells
Hexose	11.8	1.6
Sulfur	0.6	1.2
Phosphorus	0.5	1.3

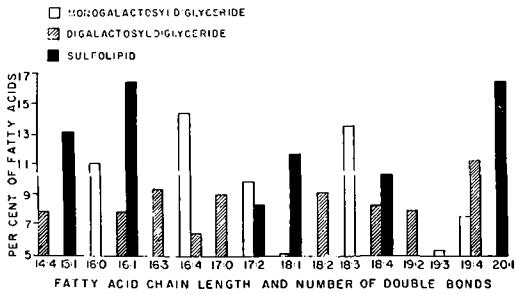

FIG. 2. The major fatty acids of the glycolipids from green *Euglena gracilis* cells.

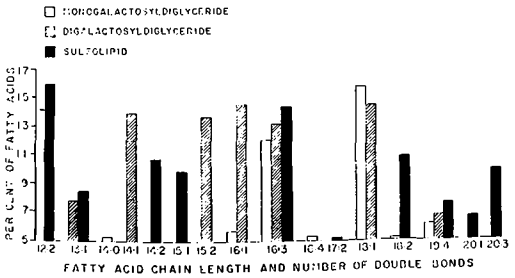

FIG. 3. The major fatty acids of the glycolipids from streptomycin-bleached *Euglena gracilis* cells.

found only in the sulfolipid but not in the galactolipids).

Rosenberg and his coworkers (6) separated the galactolipids from the total lipids of green *Euglena* cells using column chromatography. However, it was also necessary for them to free the column fraction containing the galactolipids from pigment and lipid contaminants by two-dimensional thin layer chromatography (TLC). In the present study, one-dimensional TLC was used, thereby lessening isolation time and the possibility of air-oxidation. Moreover, in this study, both polar and nonpolar columns were used for the identification of fatty acids.

DISCUSSION

It is evident from this research that streptomycin has a dramatic effect on lipid, pigment and protein biosynthesis in *Euglena*. In bacteria, streptomycin binds to and inhibits the attachment of the 30 S ribosomal subunit to messenger RNA (27). Not only does streptomycin inhibit the incorporation of an amino acid into a growing polypeptide chain, it also has the ability to induce "misreading" of the genetic code at the ribosome. Studies performed on the effects of streptomycin in cell-free systems using synthetic polynucleotides as messengers have shown that streptomycin distorts protein synthesis by causing the incorporation into a polypeptide chain of an amino acid different from that ordered by the synthetic messenger RNA (28,29).

Like bacteria, *Euglena* chloroplasts contain 70 S-type ribosomes (30). Schwartzbach and Schiff (31) have shown that *Euglena* chloroplast ribosomes selectively bind streptomycin in a manner similar to bacterial ribosomes. Extrapolated to our study, this could result in blocking of chloroplast-specific protein synthesis, such as the enzymes necessary for the biosynthesis of chloroplast proteins, lipids and pigment. In *Euglena* cells, both as green and as streptomycin-bleached, the 9 and the 11.5 thylakoid proteins are quite similar; however, the glycolipids themselves are quite different. Although the green and the bleached thylakoid proteins contain relatively the same proportions of hydrophobic and hydrophilic amino acids, it is not possible, at this time, to state whether or not streptomycin changes the sequence of amino acids. Since the isolated protein from the green cells contains bound chlorophyll, it is suggested that the protein may take part in the photosynthetic assembly along with the galactolipids and the photoreceptive pigment.

If streptomycin caused "misreading" of the genetic code, it is possible that the enzymes of chloroplast lipid biosynthesis could produce nonfunctional or incorrectly structured galactolipids. Monoenes were abundant in the digalactosyl diglycerides of streptomycin-bleached *Euglena*

cells. It is more likely that polyunsaturated fatty acids should occur in the chloroplast membranes. When fatty acids are polyunsaturated, bends or pockets are caused by the hydrocarbon chains of the galactolipids. Rosenberg (32) theorized that the methyl groups of the phytol chains of chlorophyll would fit into the pockets in the fatty acid chains of the galactolipids and thus distribute the porphyrin rings at intervals on the surfaces of the membrane where they would absorb light for photosynthesis.

ACKNOWLEDGMENTS

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The Effect of AY-9944 on Yeast Sterol and Sterol Ester Metabolism[1]

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ABSTRACT

The effects of the hypocholesterolemic drug AY-9944 (*trans*-1,4-*bis*(2-chlorobenzylaminoethyl)cyclohexane dihydrochloride) at two concentrations (10^{-4}M and 5×10^{-4}M) on the synthesis of sterols and sterol esters by *Saccharomyces cerevisiae* were investigated. Although growth was not markedly affected by the drug, there was a decrease in the free sterol to sterol ester ratio with increased drug concentration. A concomitant increase in the saturated fatty acids esterified to sterol relative to the unsaturated fatty acids was also noted in response to increased drug concentration. Ergosterol accounted for 94.7% of the free sterol in the control culture and for 87.8% of the 5×10^{-4}M drug-treated culture, respectively. However, in the sterol ester fraction, the ergosterol content decreased from a value of 45.1% in the control culture to 2.4% in the 5×10^{-4} M AY-9944 treated culture. The sterol ester fraction simultaneously showed increased levels of the Δ^8 sterol, fecosterol, in response to increased drug concentration from a 7.4% control value to 57.4% in the 5×10^{-4}M drug-treated culture. The accumulation of the Δ^8 sterol suggests that the site of action of the drug is probably at the Δ^8 to Δ^7 isomerase step in the biosynthesis of ergosterol. The fact that ergosterol is retained as the major free sterol suggests a biological advantage to the retention of this particular sterol. In addition, the near normal growth in the presence of the drug, in spite of the occurrence of an altered sterol ester profile, indicates that the composition of the sterol ester fraction is not as critical as the free sterol fraction.
Lipids 18:545-552, 1983.

INTRODUCTION

Many hypocholesterolemic drugs inhibit the biosynthesis of cholesterol at specific steps and have thus contributed to the elucidation of the biosynthetic pathway of cholesterol in animals. Thus, the chemical AY-9944 (*trans*-1,4-*bis*(2-chlorobenzylaminoethyl)cyclohexane dihydrochloride), besides its other effects, is known to be a specific inhibitor of the 7-dehydrocholesterol Δ^7 reductase and causes the accumulation of 7-dehydrocholesterol (1-3). This effect of AY-9944 was shown to take place both in vitro, using rat liver homogenates (1,2), and in vivo where 7-dehydrocholesterol accumulated and replaced the cholesterol normally present in rat brain tissue (3).

Sterol metabolism in the yeast *Saccharomyces cerevisiae* is similar to that in mammalian systems from acetate to lanosterol. Differences occur beyond this point, resulting in the formation of cholesterol in animals and in the ultimate production of ergosterol in yeast. The conversion of lanosterol to ergosterol in yeast takes place through a complex network of reactions (4-6, Fig. 1). Interestingly, *S. cerevisiae* contains no known sterol Δ^7 reductase, the established point of inhibition in cholesterol synthesis by AY-9944 in animals. Thus, *S. cerevisiae* should provide an ideal system to study any other effects of AY-9944 on sterol biosynthesis, while furnishing insights into details of the sterol biosynthetic pathway in yeast.

[1]These results form a portion of a dissertation submitted to the Graduate School, University of Maryland, College Park, MD, by Ravi Pereira, in partial fulfillment of the requirements for the Ph.D. degree in Biochemistry.

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MATERIALS AND METHODS

Reagents

Spectroscopic grade hexane (Burdick and Jackson Labs., Inc., Muskegon, MI) was used for high pressure liquid chromatography (HPLC). All other solvents were of reagent grade except for the use of technical grade hexane in bulk extractions. Technical grade hexane and diethyl ether were redistilled before use, and solvents used for HPLC were filtered and degassed prior to use. The chromatographic reference standards used have been described elsewhere (7).

Culture Conditions

Cultures of *S. cerevisiae* strain MY-304 were maintained and grown as previously described (7). Cells were grown in one liter of culture medium in Fernbach flasks under one of three conditions. The control flasks had no drug, while the drug-treated cultures had AY-9944 to a final concentration of 10^{-4}M (42.08 mg/l) or 5×10^{-4} M (210.38 mg/l), respectively. The AY-9944 was a kind gift from Dr. D. Dvornik (Ayerst Labs., Montreal, Canada) and was added as a powder and dispersed in the medium prior to autoclaving, since it had been determined to be stable to such treatment. Sterilization and inoculation were carried out as previously reported (8). Growth was followed turbidimetrically (A_{640}). The drug-treated and control

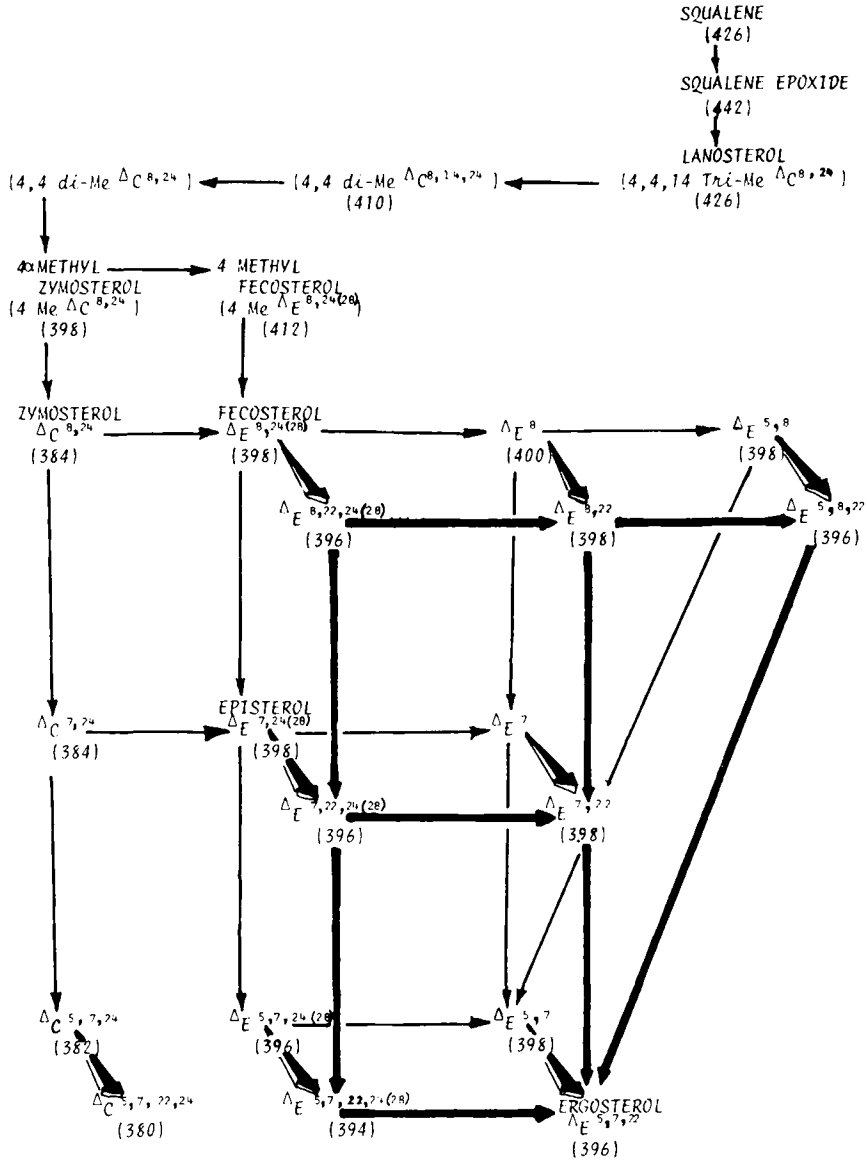

FIG. 1. Network of possible interconversions in yeast ergosterol biosynthesis as determined from identified intermediates (4-6). ΔE and ΔC correspond to the structures of ergostane and cholestane, respectively, and the numbers represent the positions of the double bonds as outlined in ref. 5. The numbers within parentheses refer to molecular weights of the free sterols. All the intermediates shown have been identified in yeast mutants.

flasks were incubated for 19 hr on a reciprocating shaker at 28 C (90 rpm). Cells were harvested and washed as described (9) and prior to harvesting an aliquot (5 ml) was taken for protein analysis using a modified Lowry reaction (7).

Lipid Extraction

The washed cell pellets were subjected to alkaline digestion and solvent extraction as outlined by Sobus and Holmlund (7) and modified by Field et al. (10). An internal standard of coprostanol (5.1 mg) was added after solvent extraction of the alkali-treated cells.

Separation of Lipid Classes

The sterols, sterol esters, fatty acids and hydrocarbons were resolved using ammonia-impregnated Biosil A columns described previously (10), except that the lipids extracted were slurried with 3 g of Biosil A and added as a dry powder to the column which was packed with 8 g of Biosil A impregnated with ammonia and slurried in hexane. Elution was effected by the sequential addition of 40 ml hexane, 75 ml hexane/diethyl ether (98:2, v/v) and 50 ml diethyl ether. Due to the ammonia treatment, the free fatty acids were retained by the column. Several minor fractions were pooled to give three major fractions A, B and C based on their reaction to iodine vapor when spotted on filter paper. The composition of the three fractions was determined by thin layer chromatography (TLC) utilizing the solvent system II of Sobus and Holmlund (7) and appropriate reference standards. Fraction A contained mostly hydrocarbons, fraction B contained sterol esters and fraction C contained the free sterols obtained from yeast cells. The three fractions were taken to dry residues on a rotary evaporator (Rotovap, Buchi Instruments, Brinkman Scientific, Westbury, NY) and redissolved in a measured amount of hexane/diethyl ether (98:2, v/v) to furnish an aliquot for mass determination. In all cases where the rotary evaporator was used, the vacuum was released with nitrogen gas to minimize possible oxidation of any component.

Saponification of Sterol Esters

Fraction B was taken to dryness and treated with a mixture of methanol/diethyl ether/benzene/20% KOH (4:3:1:0.5, v/v/v/v) containing pyrogallol (5 mg/25 ml solvent mixture). The resulting solution was refluxed gently for 0.5 hr, at which time TLC analysis indicated complete hydrolysis. The organic solvents were removed from the flasks under reduced pressure, and the residue was extracted three times with hexane (25-ml aliquots). The hexane extracts were pooled, washed twice with water, and dried over anhydrous Na_2SO_4.

Characterization of Fatty Acids Obtained From Sterol Esters

The fatty acids derived from the alkaline hydrolysis of the sterol esters were converted to free fatty acids by treatment with HCl, extracted into hexane, and methylated by treatment with 1 M HCl/methanol solution overnight at 85 C in a sealed tube. The methyl esters formed were then extracted into hexane, applied to a TLC plate, and developed with xylene. The methyl ester zone was scraped, eluted with methylene chloride and subjected to gas liquid chromatography (GLC). GLC was carried out in a 1.5 m×2 mm stainless steel column packed with 15% diethylene glycol succinate (DEGS) on Chromosorb Z, 100-200 mesh. The carrier gas was nitrogen and the flow rate was 45 ml/min. The column temperature was programmed from 150 to 185 C at a rate of 3 C/min. The injection port and flame ionization detector were at 265 C and 275 C, respectively. The instrument was a Perkin Elmer 900 gas chromatograph interfaced to a Hewlett Packard 5840A printer/integrator. Tentative identification of the fatty acids was made based on retention time comparisons for standard fatty acid methyl esters.

Resolution of Sterols Derived From the Sterol Ester Fraction (B)

The sterols released by the saponification of the sterol ester fraction were taken to dry residues in vacuo from their anhydrous hexane extracts. The sterols were converted to acetate derivatives by treatment with acetic anhydride/pyridine (1:2, v/v) and extracted into hexane as outlined previously (8). The acetate mixture was resolved using HPLC. The instrument used was a Waters Model ALC-GPC (Waters Associates, Inc., Framingham, MA) fitted with two 6.4 mm×30 cm μ-Porasil columns connected in series. The acetate derivatives were injected in hexane and eluted with a linear gradient of hexane to hexane/diethyl ether (99:1, v/v) using a solvent programmer. Fractions were collected manually and monitored using UV absorption at 220 nm by a Schoeffel Model SF 770 electroflow monitor coupled to a Schoeffel Model GM770 monochromator (Schoeffel Instrument Corp., NJ).

Resolution of Sterols Derived From Free Sterol Fraction (C)

The free sterol fraction (C) was dissolved in hexane/diethyl ether (85:15, v/v) and directly injected into the high pressure liquid chromatograph and eluted with hexane/diethyl ether (85:15, v/v). Fractions were collected as before using UV monitoring at 220 nm. The major fractions derived from the column effluent were concentrated to dry residues under nitrogen and acetylated as before,

using acetic anhydride and pyridine. The major acetylated fractions were once again subjected to HPLC using conditions outlined in the preceding section.

Characterization of Sterol Acetates Derived From Sterol Ester and Free Sterol Fractions

The HPLC fractions of the sterol acetates derived from both fractions B and C were analyzed by GLC on three glass columns (1.8 m × 3 mm id) containing the packings 3% SE-30, 1% QF-1, and 3% Hi-eff 8BP, on a gas Chrom Q support (100-200 mesh, Applied Science Labs., State College, PA). The carrier gas was nitrogen, the flow rate was 20 ml/min. Column temperatures were 220 C (QF-1), 245 C (Hi-eff 8BP), and 250 C (SE-30).

GLC of sterol acetates was done exclusively on a Hewlett Packard Model 5830A gas chromatograph equipped with a Model 7617A automatic sampler and a model 18850A GC terminal. The injection temperature was 275 C and the flame ionization detector (FID) was at 300 C utilizing hydrogen and air for combustion. The injection port was fitted with a low bleed septum (Stabiline, Pierce Chem. Co., Rockford, IL). The areas obtained from the GC terminal printout were calibrated to a known quantity of cholesterol acetate (1 $\mu g = 4.0 \times 10^{-5}$ units), which aided the quantitation of the other sterol acetates. The relative retention times (RRT) of the yeast sterols to cholesterol (as acetates) were calculated with the help of added internal standard (coprostanol acetate) using the formula:

$$RRT_{sample} = (RT_{sample} / RT_{coprostanol}) \times RRT_{coprostanol}$$

The $RRT_{coprostanol}$ was obtained from values reported for coprostanol by Patterson (11). Tentative identifications were made by comparing RRT of various sterol acetates on the three columns to reported values (10,11). The retention times for coprostanol acetate (reported in min) in this study were 5.21 ± 0.05 (QF-1); 8.51 ± 0.25 (Hi-eff 8BP) and 15.24 ± 0.58 (SE-30), respectively.

Peaks obtained on the GC system were further identified by gas chromatography-mass spectrometry (GC-MS) using a Finnigan Model 1015D GC-MS interfaced to a Model 6000 data system. The GC column contained 3% SE-30 on Gas Chrome Q (100-200 mesh), and helium was the carrier gas. Chemical ionization spectra were obtained using ammonia mixed with carrier gas. The ionizing voltage was 100 eV, source pressure and temperature were 1 torr and 100 C, respectively.

Selected samples were also analyzed by electron impact ionization GC-MS using a V.G. Micromass Model 7070F mass spectrometer coupled to a Perkin Elmer Sigma 3 gas chromatographic system. The column packing was 3% SP2340 or 3% SP2100 on Supelcoport 100-200 mesh (Supelco, Belleforte, PA) in a glass column 1.5 m × 2 mm. The carrier gas was helium and the flow rate was 20 ml/min. The ionizing voltage was 70 eV and the inlet temperature was 250 C. The data were acquired on a DS 20 data system.

RESULTS

The effects of the drug AY-9944 were investigated with respect to many possible sites of action in three separate experiments, all of which yielded similar results. No significant inhibition of growth was observed on the basis of turbidimetric (A_{640}) measurements over a 24-hr period, even at the highest drug concentration. In a representative run, a liter of culture gave a wet packed cell mass of 15.69 g (2.091 g protein/liter) for the control, 14.73 g (1.954 g protein/liter) for 10^{-4}M AY-9944, and 15.35 g (2.102 g protein/liter) for the 5×10^{-4}M AY-9944 treated cultures. Table 1 provides data on the total cellular lipid, as well as lipid recovered in fractions from Biosil A chromatography, from control and treated cultures. The fractional amount of sterol which is esterified increased in response to elevated drug concentration.

Analysis of the fatty acids derived from the hydrolysis of sterol esters did not reveal any obvious differences in pattern between the treated and control cultures (Table 2). Over 90% of the fatty acids in the control and treated samples consisted of 14:0, 16:0, 16:1, 18:0 and 18:1 carbon chains. However, the ratio of saturated to total fatty acids was significantly increased in the 5×10^{-4} M AY-9944 treated culture (Table 2).

After GC of all sterol fractions (sterol and sterol ester fractions B and C as acetates), the major fractions were subjected to HPLC again, and the major peaks recycled internally in the hope of effecting still better resolution. The results were mixed, with some fractions showing better resolution than others.

The small size of some fractions precluded mass spectral analysis after GC analysis on three columns. In these few cases, RRT on three columns had to suffice for identification. Most often, however, chemical ionization mass spectra were obtained and furnished the M+1-60 ion. Electron impact mass spectra were also carried out on several major fractions to identify the sterol in question. Sterols whose structure could not be supported by mass spectral data or information from three gas chromatographic columns were grouped together and reported as miscellaneous sterols.

The most obvious distinction between control and drug-treated cultures was the pattern of sterol ester and free sterol composition that emerged. The most abundant sterol seen in the free sterol fraction in all cultures was ergosterol. Ergosterol represented 94.7% of the total free sterol in the control

TABLE 1

The Amounts of Lipid Recovered Before and After Biosil A Fractionation of the Solvent Extracts from Yeast

	Amounts as μg/g protein					
	Before Biosil A fractionation	Biosil A fractions[a]			Total	Ratio SE total S+SE
		A	B	C		
Control	32.4	2.6	10.1	14.5	27.2	41.0%
10^{-4}M AY-9944	48.8	2.4	17.4	21.8	41.6	44.4%
5×10^{-4}M AY-9944	48.4	3.1	21.9	21.6	46.6	50.3%

[a]Fractionation was performed as described in Materials and Methods. TLC indicated that fraction A was mostly hydrocarbon, fraction B was mainly sterol ester (SE), and fraction C was mostly free sterol (S).

TABLE 2

The Effect of AY-9944 on the Fatty Acid Composition of Yeast Sterol Esters

	% of Total fatty acids		
		AY-9944 treated	
Fatty acid[a]	Control	(10^{-4}M)	(5×10^{-4}M)
14:0	2.0	2.8	2.9
16:0	8.3	7.8	12.1
16:1	39.6	46.6	38.9
16:2	0.1	0.4	0.7
18:0	5.3	3.5	7.2
18:1	37.7	30.2	30.9
18:2	1.3	0.6	3.0
Sum[b]	94.3	91.9	95.7
Saturated fatty acids	15.6	14.1	22.2
Unsaturated fatty acids	78.7	77.8	73.5
Ratio:saturated/total fatty acids (%)	16.5	15.3	23.2

[a]The fatty acids were identified as their methyl esters (FAME).
[b]The remaining fatty acid methyl esters (FAME) consisted of up to 20 different compounds.

culture, and 74.4% and 86.8%, respectively, of the 10^{-4} M and 5×10^{-4} M drug-treated cultures (Table 3). Differences were apparent in the composition of the esterified sterols between the control and drug-treated cultures (Table 4). In the control culture, the amounts of ergosterol and zymosterol in the sterol ester fractions were comparable, representing 45.1% and 36.5%, respectively, of the total sterol ester. The amount of ergosterol decreased dramatically with increased drug concentration, 25.4% (10^{-4} M) and 2.4% (5×10^{-4} M), and this was accompanied by a corresponding increase in fecosterol levels, 7.4% (control), 36.0% (10^{-4} M), and 57.4% (5×10^{-4} M). Zymosterol levels were slightly depressed by increased drug concentration.

Ergosterol, fecosterol and zymosterol acetates were identified by means of gas chromatography on three columns as well as by both chemical ionization and electron impact mass spectrometry. Ergosterol acetate had RRT very close to those reported by Patterson (11). Chemical ionization MS gave peaks of 439 (M+1) and 379 (M+1-60) corresponding to loss of acetate. The electron impact MS data gave peaks of m/e (relative intensity): 438 (M^+,5) and 378(100), 363(35), 253(65) corresponding to M^+-acetate, M^+-acetate-methyl, and M^+-side chain. Characteristic peaks of m/e at 211(20) and 143(50) further augmented the identification of ergosterol. The RRT values of fecosterol acetate also agreed well with those reported by

TABLE 3

The Effects of AY-9944 on the Free Sterol Composition of Yeast

Sterol[a]	MW[b]	% of Total		
			Concentration of AY-9944	
		Control	10^{-4}M	5×10^{-4}M
$\Delta^{8,24(28)}$-Ergostadienol (fecosterol)	398	—	7.6	3.1
$\Delta^{5,7}$-Ergostadienol	398	—	2.0	—
$\Delta^{5,7,22}$-Ergostatrienol (ergosterol)	396	94.7	74.4	87.8
"Cholesterol"	386	0.1	0.4	0.1
$\Delta^{8,24}$-Cholestadienol (zymosterol)	384	0.2	—	—
Unidentified	384	—	11.4	—
Miscellaneous sterol mixture		5.0	4.2	9.0

[a]The sterols were identified by a combination of mass spectral data and values obtained from three GC columns (SE-30, QF-1, and Hi-eff 8BP).

[b]The molecular weights were determined from chemical ionization mass spectrometry, and correspond to that of the free sterol.

Patterson (11). The MS m/e values: 440 (M^+,60), 425(40), 365(35), 313(40), 255(20), 227(70), and 213(85) were also characteristic of fecosterol, as previously reported (12). Zymosterol acetate was characterized on the basis of RRT (11) and MS data (13). The base peak m/e 69(100) is characteristic of many sterols (14). Other identifying peaks and corresponding intensities were: m/e 426 (M^+,50), 411(40), 366(18), 351(35) and 313(20).

The remaining sterol acetates, which comprised no more than 15% of the total sterol ester fraction and 10% of the total free sterol fraction, were primarily identified by their RRT on three columns and by the molecular ion obtained from chemical ionization mass spectra.

DISCUSSION

Since cell proliferation, as determined by wet cell weight and protein determination, is not markedly affected by the presence of AY-9944, it would seem that the organism can indeed tolerate, or adapt to, changes in sterol metabolism without obvious depression of growth rates.

The free sterol and sterol ester profiles obtained during this investigation show many points of interest. For instance, the tendency for yeast to retain ergosterol as the major free sterol in the presence of an inhibitor of sterol synthesis suggests that this sterol confers definite biological advantage. Retention of ergosterol in the free sterol fraction of yeast has been previously reported in the presence of the sterol synthesis inhibitors triparanol (8) and 3β-(β-dimethylaminoethoxy)-androst-5-en-17-one (DMAE-DHA) (10). The fact that sterol synthesis is indeed affected by the drug AY-9944 is conclusively shown by the dramatically altered sterol ester profiles between control and drug-treated cultures. Here ergosterol is almost completely absent at the highest drug concentration (5×10^{-4} M) used. Since growth rates are not affected by the drug, it would appear that the free sterols, especially ergosterol, and not the sterol esters, are more critical for maintaining normal growth. Alternatively, the increased presence of Δ^8 sterols in the sterol ester fraction of the drug-treated cultures may reflect differences in specificity between Δ^8 sterols and ergosterol for esterification (15). It is interesting to note that a sterol similar to cholesterol has been identified in all the samples.

In the sterol ester fraction, the greatest change in the sterol composition was the appearance of the Δ^8 sterol fecosterol in the drug-treated cultures. In the control fractions, ergosterol comprised 45% of the esterified sterol, while the Δ^8 sterol zymosterol and fecosterol accounted for 36.5% and 7%, respectively. At drug concentrations of 5×10^{-4} M, the fecosterol levels increased and accounted for 57.4% of the total sterol, while zymosterol and ergosterol comprised 21.4% and 2.4% of the total sterol, respectively (Table 4). The presence of fecosterol and other Δ^8 sterols in AY-9944 treated yeast indicates that the primary site of inhibition of the drug is at the Δ^8 to Δ^7 isomerase stage. This is in direct contrast to the effect of AY-9944 in animal systems where the primary site of inhibition is at the Δ^7 dehydrogenase step (16). Inhibition of the Δ^8 to Δ^7 isomerase by AY-9944 has also been reported

TABLE 4

The Effects of AY-9944 on the Sterol Composition of Yeast Sterol Esters

Sterol[a]	MW[b]	% of Total		
			Concentration of AY-9944	
		Control	10^{-4}M	5×10^{-4}M
4,4,14-Trimethyl $\Delta^{8,24}$-cholestadienol (lanosterol)	426	—	0.1	0.1
Δ^{8}-Ergostenol	400	1.3	3.4	1.0
$\Delta^{8,24(28)}$-Ergostadienol (fecosterol)	398	7.4	36.0	57.4
$\Delta^{5,7}$-Ergostadienol	398	3.4	6.6	1.8
$\Delta^{5,7,22}$-Ergostatrienol (ergosterol)	396	45.1	25.4	2.4
$\Delta^{8,22,24(28)}$-Ergostatrienol	396	—	—	1.5
$\Delta^{5,7,24(28)}$-Ergostatrienol	396	0.6	—	0.2
"Cholesterol"	386	1.1	0.4	0.2
$\Delta^{8,24}$-Cholestadienol (zymosterol)	384	36.5	24.3	21.4
Miscellaneous sterol mixture		4.6	3.8	14.0

[a]The sterols were identified by a combination of mass spectral data and values obtained from three GC columns (SE-30, QF-1, and Hi-eff 8BP).

[b]The molecular weights were determined from chemical ionization mass spectrometry, and correspond to that of the free sterol.

in the alga *Chlorella ellipsoidea* (17) and in bramble cells grown in culture (18). Similar inhibition has been observed in yeast in the presence of other hypocholesterolemic drugs like triparanol (8) and trifluperidol (19).

Some side-chain modification occurs in the presence of AY-9944. The drug does not seem to affect introduction of the C24 methyl group; but oxidation at C22 and C23 seems to be inhibited, as seen by a paucity of Δ^{22} intermediates. In addition, $\Delta^{24(28)}$ reduction also seems to be inhibited, as shown by the accumulation of fecosterol. These observations are in accordance with the schemes outlined by Fryberg et al. (4) and Parks (6), wherein zymosterol and fecosterol occupy pivotal positions.

The presence of coprostanol as an added internal standard allowed the calculation of the percent recovery of free sterol from the Biosil A column. Calculations based on GC values obtained on 3% SE-30 columns indicated recoveries in excess of 60%.

The fatty acid composition obtained by the saponification of the sterol ester fractions was found to agree reasonably well with values reported by Sobus et al. (19). The effects of AY-9944 on the fatty acids obtained from yeast cultures was studied by Gendelman (20), who noted a depression of the 18:1 fatty acids and an increase in the 14:0 and 16:0 fatty acids in response to increased drug concentration. In this investigation, although an increase in the amounts of 14:0 and 16:0 fatty acids was observed, no correspondingly significant reduction in the 18:1 fatty acids was evident. The drug AY-9944 did seem to increase the ratio of saturated to total esterified acids (both saturated and unsaturated).

The percentage of esterified sterols to total sterol increases with increased drug concentration. This effect parallels the more pronounced effects seen with triparanol (8) and trifluperidol (19).

The fact that ergosterol is produced at all indicates that AY-9944 does not completely block ergosterol biosynthesis. The several routes available for the further metabolic conversion of fecosterol (Fig. 1) must certainly be under the control of specific enzymes which are probably affected to different extents by the drug. The production of ergosterol takes place, albeit at a reduced rate, resulting in the accumulation of fecosterol. The production of increased total sterol may indeed be a reflection of the cell's requirement for a minimum amount of an essential free sterol—namely, ergosterol. Yeast sterols, once esterified, do not undergo additional metabolism very readily (21), and esterification may be a protective measure for the removal of biologically disadvantageous sterols; alternatively, as suggested before (15), the Δ^{8} sterols may simply function as better substrates for the sterol ester synthetase than the Δ^{7} sterols.

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Human Low Density Lipoprotein Structure: Correlations with Serum Lipoprotein Concentrations[1]

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ABSTRACT

Human low density lipoproteins (LDL) were isolated and purified from individuals having widely differing serum lipid concentrations. Very low density lipoproteins (VLDL) and high density lipoproteins (HDL) were also isolated and quantitated. HDL_2 and HDL_3 were separated by flotation velocity in the analytical ultracentrifuge and their relative weight percent determined. The mean density of LDL from 41 individuals was determined by flotation velocity at two different solvent densities. The mean density of LDL was directly proportional to the triglyceride ($r=0.65$) and VLDL ($r=0.50$) concentrations and inversely proportional to the HDL ($r=-0.55$) and HDL_2 ($r=-0.74$) concentrations (all significant at $P<0.001$). The mean molecular weight of LDL from 42 individuals was determined by flotation equilibrium centrifugation. The mean molecular weight of LDL was directly proportional to the HDL ($r=0.49$) and HDL_2 ($r=0.48$) concentrations and inversely proportional to the serum triglyceride ($r=-0.60$) and VLDL ($r=-0.48$) concentrations (all significant at $P<0.005$ except triglyceride—$P<0.001$). The molecular weight of LDL was inversely proportional to its density, and thus inversely proportional to its protein/lipid ratio which was confirmed by composition measurements. The density and molecular weight of LDL had no relationship to the concentration of LDL ($r=0.04$ and 0.03).
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LDL concentration is highly correlated with atherosclerosis. We wished to determine how these concentration differences among individuals correlate with the mean LDL density and molecular weight differences among the same individuals. The variability in LDL flotation rate (1-4) can only be caused by 3 factors: lipoprotein molecular weight, density, and/or conformational differences. We have studied the molecular weight and density of LDL and how it related to VLDL, LDL, HDL, HDL_2, and serum triglyceride concentrations of different individuals. In order to study further the interrelationships among serum lipoproteins, we have utilized quantitative methodology to isolate and characterize the major lipoproteins of human serum. These relationships may contribute to the understanding of the cause of LDL structural differences.

METHODS

Individuals used in this study were simply unselected healthy individuals whose serum lipid concentrations were determined over a 9-month period. They ranged in age from 22 to 65. Males predominated ($N=38$); 3 females were studied. The correlation of molecular weight vs triglyceride made use of additional data utilizing males and females. All serum lipoproteins were separated from other macromolecules by flotation at density 1.25 g/ml KBr followed by 6% agarose (Bio-Rad) gel filtration (5) to separate VLDL, Lp(a) plus IDL, which appear in the elution region between VLDL and LDL, and HDL. These fractions were pooled and their serum concentrations determined as cholesterol (6). The HDL concentration was, however, taken as that found by heparin-manganese precipitation (7) since it is more direct and thus more accurate. Other analyses were as follows: triglyceride (8), phospholipid (9), protein (10), and agarose electrophoresis (11). All solutions throughout contained EDTA and azide or thimerosal. Serum also contained dithio-*bis* (2-nitrobenzoic acid).

LDL Preparation

LDL from gel filtration was generally pure if the IDL fraction was low. If the amount of IDL was great, the LDL was contaminated by IDL. In those cases, the LDL peak was concentrated and ultracentrifuged at density 1.019 to remove the contaminating IDL. Upon removal of this IDL, the LDL peak observed upon flotation velocity in the analytical ultracentrifuge lost its skewed leading edge, but retained the same flotation rate (S_f).

LDL densities were determined by flotation velocity rate measurements of 2-10 mg/ml LDL at 25 C in 2 solvents of densities very near 1.2 g/ml and 1.063 g/ml KBr containing 10^{-4}M EDTA and 0.01% sodium azide at pH 7.5. The exact densities were determined pycnometrically for each sample. Calculations were carried out according to the equation

$$\frac{S_1^o}{S_2^o} = \frac{\eta_2(1-\bar{v}\rho)_1}{\eta_1(1-\bar{v}\rho)_2}$$

[1]A preliminary report of this study was given at the American Society for Biological Chemists Meeting in St. Louis, June 1981.

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Abbreviations: low density lipoproteins, LDL; high density lipoproteins, HDL; very low density lipoproteins, VLDL; intermediate density lipoproteins, IDL.

as has been described (12), where 1 and 2 refer to measurements in each solvent: $\bar{v}$ is the reciprocal of the lipoprotein density, ρ the solvent density determined by pycnometry, η the measured solvent viscosity, and S° the flotation rate coefficient extrapolated to zero lipoprotein concentration (c) by means of standard S vs c curves determined from a number of samples for each of the 2 solvents. The hydrated $\bar{v}$ obtained (0.9676 ml/g) was the same as the anhydrous (0.967) (13). Furthermore, Fisher et al. (12) found that no preferential binding of KBr takes place, and the value of $\bar{v}$ in D_2O was in agreement (0.966). This method has a very high precision.

LDL Molecular Weight

Flotation equilibrium molecular weights of 0.3-0.5 mg/ml LDL were determined in KBr at densities near 1.3 at 6-7 C and 3400 rpm after equilibration (48 hr) using cell sector volumes of 0.15 ml of solvent and 0.15 ml of LDL which had been dialyzed against this solvent as previously described (14). Exact solvent densities for each determination were measured at the temperature employed with a Westphal balance.

HDL_2 and HDL_3

HDL, upon flotation velocity in density 1.2 g/ml KBr at 48,000 rpm in a double-sector cell, separated into 2 components which were clearly defined when HDL_2 was high. HDL_2 and HDL_3 were estimated by deconvolution of the HDL double or asymmetric peaks as shown in Figure 1. The photographs were enlarged 10× and traced on graph paper. The peak tracings were redrawn to a straight line. The 2 components were estimated by completing tracing of the HDL peak or peaks symmetrically. All area under the HDL peak can be accounted for by 2 components. The relative areas were corrected for the sector shape of the ultracentrifuge cell. no correction was made for the Johnston-Ogston effect since it is ca. 2-3% (15). There was no relative area change of HDL_2 and HDL_3 upon dilution.

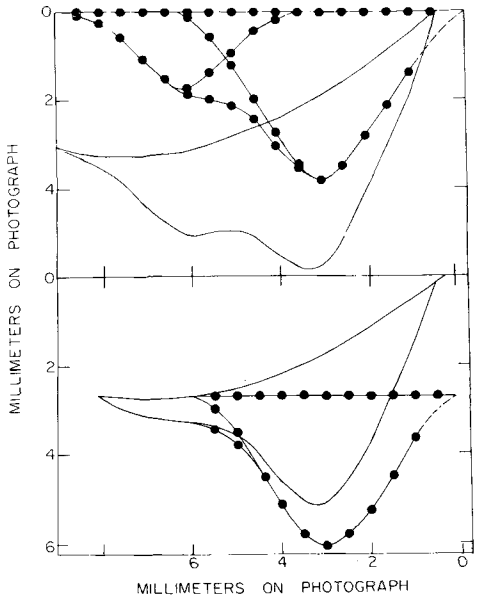

FIG. 1. Deconvolutions (closed circles) of tracings (solid lines with no points) of typical HDL photographs taken 96 min after reaching top speed in the analytical ultracentrifuge at 48,000 rpm in a 1.2 g/ml KBr solvent. The points represent the tracings redrawn with linear baselines plus the symmetrical completion of each peak. The right side was occasionally foreshortened, being at the cell bottom, and was drawn in as shown by the dashed lines.

Remixing purified HDL_2 and HDL_3 yielded the ratio expected.

RESULTS

Individuals Studied

The range, mean values, and standard deviations of lipoprotein parameters studied are given in Table 1. Where occasional values were obtained

TABLE 1

Mean and Range of Individual Lipoprotein Parameters Studied

	Serum				Concentration of			LDL	
	cholesterol (mg/dl)	triglyceride (mg/dl)	VLDL	IDL + Lp(a)	LDL (mg/dl cholesterol)	HDL (mg/dl cholesterol)	HDL_2 (mg/dl cholesterol)	Density (g/ml)	Molecular weight (millions)
Mean	215	163	23	14	136	44	6.6	1.0335	2.87
SD	±46	±106	±21	±7	±45	±16	±7.4	±0.0048	±0.29
Range	127-326	38-457	8-93	5-26	65-223	23-77	0-31	1.026-1.042	2.3-3.5

N = 40-43.

which lay 2 standard deviations outside the mean, they were omitted from analysis. The mean serum cholesterol was above 200 mg/dl, reflecting an older population (very few individuals under 30 years) than we studied previously (1) where the mean age was 22. Likewise, the mean triglyceride was high and the mean HDL_2 was low, the latter since the population studied was almost all older males.

Density of LDL

The mean density of LDL is plotted as a function of triglyceride concentration in Figure 2. The correlation coefficient was 0.65 and was significant at $P<0.001$. Samples of LDL from individuals with a low serum triglyceride concentration were heterogeneous in the analytical ultracentrifuge in solvent of density 1.2 g/ml. We reported this phenomenon previously (1). The major component in the heterogeneous LDL was reconfirmed to be the faster. Thus, the density measured of this type of LDL was that of the faster component.

The mean density of LDL is plotted as a function of HDL concentration for 41 individuals as shown in Figure 3. The correlation coefficient was −0.55 and was significant at $P<0.001$.

The mean density of LDL was also correlated with VLDL, LDL and HDL_2 concentrations and all of these correlations and levels of significance are shown in Table 2. LDL concentration exhibited no correlation (0.04) with LDL mean density. The mean density was 1.0335 g/ml with a range from 1.029 to 1.040 g/ml.

Molecular Weight of LDL

The molecular weight of LDL is plotted as a function of triglyceride concentration for 42 individuals in Figure 4. The correlation coefficient was −0.60 and was significant at $P<0.001$. Most of these values were determined by flotation equilibrium in which the mean of any heterogeneity in molecular weight was obtained.

The additional correlation of LDL molecular weight with VLDL, LDL, HDL and HDL_2 concentrations are shown in Table 2 along with levels of significance. LDL concentration was not correlated (0.03) with LDL molecular weight. The mean molecular weight was 2.87 million with a range of 2.3-3.5 million.

Since the density of LDL was correlated inversely with the molecular weight of LDL ($r=-0.62$; $P<0.001$), the larger the LDL, the lower the percentage protein it should contain. Measurements of LDL composition of samples did confirm this. The correlation of weight percent protein in LDL as an inverse function of molecular weight was significant ($r=-0.51$, $P<0.005$).

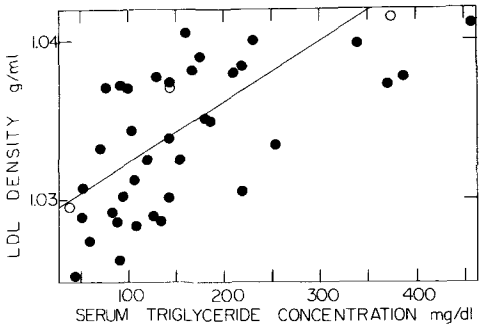

FIG. 2. The density of LDL from 41 individuals plotted as a function of their serum triglyceride concentration. The closed circles are males and open circles are females. $r=0.65$; $P<0.001$.

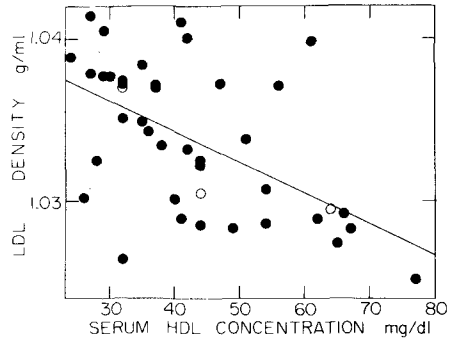

FIG. 3. The density of LDL from 41 individuals plotted as a function of their HDL concentration in mg/dl of cholesterol. The closed circles are males, open circles are females. $r=0.55$; $P<0.001$.

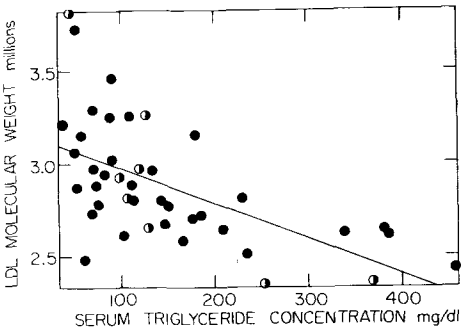

FIG. 4. The molecular weight of LDL from 42 individuals plotted as a function of their serum triglyceride concentration. The closed circles represent determinations by flotation equilibrium, and the half-open circles represent weights obtained from sedimentation plus diffusion. $r=-0.60$; $P<0.001$.

TABLE 2

The Correlation of LDL Density and Molecular Weight with Serum Lipid and Lipoprotein Concentrations

		Triglyceride	VLDL	LDL	HDL	HDL_2
LDL Density	r=	0.65	0.50	0.04	−0.55	−0.74
Significant at (N=41)	P<	0.001	0.005	N.S.	0.001	0.001
LDL Molecular weight	r=	−0.60	−0.48	0.03	0.49	0.48
Significant at (N=42)	P<	0.001	0.005	N.S.	0.005	0.005

DISCUSSION

This study clarifies and explains the changes we found in our previous observation that the LDL flotation rate, which is a function of LDL density, molecular weight and shape, was inversely correlated with the serum triglyceride concentration of individuals ($r=-0.71$; $P<0.001$; $n=55$) and not correlated with their serum cholesterol concentration ($r=-0.22$; $n=55$) (1). Both males and females were studied, and all fell on the same line of regression, even though females tended to have lower serum lipid levels than males. Consequently, in the current study, we simply obtained serum from individuals without regard to sex in obtaining a wide range of serum triglyceride concentrations.

We separately studied LDL density and molecular weight, and individually correlated these with serum triglyceride, VLDL, LDL, HDL and HDL_2 concentrations. Significant positive and inverse correlations were found between LDL structure and all of the concentrations listed above, except serum LDL concentration. Serum LDL, surprisingly, had no correlation with LDL structure ($r=0.03$, 0.04).

Shen et al. (16) separated LDL from 12 subjects by density gradients and subsequently analyzed the fractions. They also found a correlation in the LDL of an individual of a high amount of a lower density LDL fraction (#2) with HDL and negatively with VLDL concentrations. Likewise, the presence of a high amount of a higher density LDL fraction (#4) correlated with VLDL and negatively with HDL concentrations. In addition, they found the LDL density inversely proportional with its molecular weight. We have confirmed these types of correlations for the mean LDL densities and molecular weights for different individuals, and have extended these correlations to HDL_2 and to serum triglyceride concentrations. Our most significant original finding was the total lack of any relationship of serum LDL concentration with LDL molecular weight or density. It is a key to the cause of the disparity in LDL size and percentage of lipid in the serum of one individual and between individuals. The difference in densities (percentage lipid content) in LDL populations is a consequence of the metabolic pathway of LDL prior to cell internalization.

The correlation of LDL density and molecular weight with VLDL and HDL concentrations but not LDL concentration must have a metabolic explanation. Since VLDL is normally the precursor of LDL, apparently the more VLDL (triglyceride) present, the more thoroughly VLDL is catabolized to (a smaller) LDL, possibly because of increased lipoprotein lipase. The major pathways by which LDL size is determined is the conversion of VLDL to LDL by lipoprotein lipase which primarily removes triglyceride (17) and a factor in serum which removes excess cholesteryl esters (18). Lipoprotein lipase alone will remove triglyceride but leaves cholesteryl ester. There is twice as much cholesteryl ester per apo B in VLDL as in LDL (18). Incubation of LDL with VLDL plus lipoprotein-free plasma yields a cholesteryl ester poor LDL (18) indicating there is some mechanism or enzyme present in serum removing cholesteryl ester from LDL, perhaps a cholesteryl ester triglyceride exchange enzyme (19). A large amount of VLDL brings about less HDL_2 production as it takes all the available C peptides (which appear only in HDL_2) and maintains the HDL at a low concentration (only HDL_3) (20). The following schemes are possibilities: a stimulation of triglyceride/VLDL on lipoprotein lipase and/or on a cholesteryl ester exchange enzyme, or an inhibition of LDL entry into cells by some fraction we have not measured, such as apo E.

The finding that smaller LDL molecules have a larger percentage of protein agrees with the general finding that as the surface/volume ratio increases (as lipoproteins decrease in size), a higher percentage content of protein is found—since protein is a surface constituent. This is also consistent with the results of Tanford and Reynolds' groups (21,22) that there is a natural protein subunit of LDL. Our apoprotein weight varied little for LDL and was 675-725,000 daltons, which does not yield an integral number of subunits since their weights

were 255,000 daltons/subunit. While our present protein is based on the Hartree method (10), with bovine serum albumin as a standard rather than an absolute method, we do agree with other investigators. Recently, virtually full agreement has been obtained that LDL has a molecular weight of at least 2.7 million (23). Ultracentrifugal equilibrium methods were shown to be a superior method of determining molecular weights (23).

Since high serum cholesterol and high LDL levels are related to atherosclerosis and the levels are unrelated to LDL density and molecular weight, the process of atherosclerosis may be unrelated to the composition or size of LDL. Contrary to this, HDL levels are related both to atherosclerosis and to LDL composition and size.

The importance of LDL in atherosclerosis has been studied in monkeys. Cholesterol feeding increases monkey LDL molecular weight and serum LDL concentration. Rudel et al. (24,25) found serum LDL concentration was not proportional to coronary atherosclerosis, while LDL molecular weight was correlated with coronary atherosclerosis.

These studies extend our initial finding of the correlation of LDL flotation rate with serum triglyceride concentration and lack of correlation with serum cholesterol concentration (1). The cholesterol concentration is due mostly to serum LDL which was not correlated with LDL structure. The serum triglyceride concentration, however, is due mostly to VLDL, which was correlated with LDL structure.

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COMMUNICATIONS

How Do Polyunsaturated Fatty Acids Lower Plasma Cholesterol Levels?

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ABSTRACT

For 30 years it has been known that linoleic acid can lower elevated cholesterol levels. Large increases in linoleic acid have been widely recommended as a way of reducing the risk of cardiovascular disease. Such recommendations have resulted in major dietary shifts in some countries, including the USA. Yet the precise characteristics of the linoleic acid molecule which confer on it cholesterol-lowering properties are unknown. γ-Linolenic acid, the first essential fatty acid metabolite of linoleic acid, has been found to have cholesterol-lowering actions ca. 170 times greater than the parent molecule, suggesting that linoleic acid must be converted to γ-linolenic acid to exert its desirable effects on cholesterol metabolism. Aging, sex, diabetes mellitus, alcohol, catecholamines and *trans* fatty acids and saturated fats can all modulate the Δ-6-desaturase enzyme which converts linoleic acid to γ-linolenic acid. This provides a possible unifying explanation for the actions of these known risk factors for cardiovascular disease.
Lipids 18:558-562, 1983.

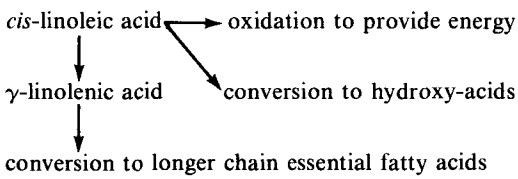
INTRODUCTION

With all its limitations, total plasma cholesterol remains the best single predictor of coronary heart disease risk in a population, especially if a time lag is introduced into the calculation (1). The predictive value of the total cholesterol may be improved slightly by consideration of the ratio between low density lipoproteins (LDL) cholesterol and high density lipoproteins (HDL) cholesterol.

When the importance of cholesterol was first appreciated, a number of studies were carried out on the effects of various diets on blood cholesterol levels. These studies culminated in the publication of two equations, one derived by Hegsted et al. (2) and the other by Keys et al. (3), which summarized the effects of changes in saturated and polyunsaturated fat intake on cholesterol. These effects were disappointingly small. A rise in polyunsaturated fatty acid (PUFA) intake equal to 1% of total calorie intake would produce a fall in plasma cholesterol of only 1.31 mg/100 ml (Keys) or 1.65 mg/100 ml (Hegsted) (mean 1.48 ml/100 ml). A fall in saturated fat intake equal to 1% of total calorie intake would produce a fall in cholesterol of only 2.74 mg/100 ml (Keys) or 2.16 mg/100 ml (Hegsted). Thus, in order to produce changes in plasma cholesterol substantial enough to be worthwhile, very large changes in dietary patterns must be achieved. The magnitude of the required changes has led to bitter arguments among experts concerning the practicality of achieving them. Nevertheless, most organizations concerned with cardiovascular health have felt confident enough to recommend substantial increases in PUFA intake and decreases in saturated fat intake. The arguments for this have been admirably summarized in the most recent diet/heart statement from the American Heart Association (4). The sustained campaign has led to large increases in PUFA intake in some countries, such as the USA, and such increases may in part be responsible for the recent fall in coronary disease mortality (5,6).

Given the importance of the issue and the number of investigators involved, astonishingly little attention has been paid to the question of how PUFA lower plasma cholesterol. There has been some discussion of this from the cholesterol end (7): effects of PUFA on cholesterol synthesis, cholesterol excretion and cholesterol redistribution have all been considered. But there has been almost no discussion of the PUFA aspect. Just what characteristics of a polyunsaturated fat molecule enable it to lower cholesterol levels?

Almost all studies have been done with a single PUFA, *cis*-linoleic acid (cLA). cLA has three major metabolic fates and there are thus 4 major possibilities by which the effect of cLA may be explained.



(a) cLA itself, in an unmetabolized form, may be the relevant factor, perhaps as a result of becoming incorporated into lipoproteins and changing their characteristics. Although rarely explicitly stated, most investigators seem to assume this is the case.

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(b) cLA may be oxidized to provide energy. It is highly improbable that this route of metabolism accounts for the cholesterol-lowering effect since as far as this route is concerned there is no obvious reason why cLA should differ from any saturated fat.

(c) cLA may be metabolized to γ-linolenic acid (GLA) and then on to other essential fatty acids and their metabolites (8). It is this route of metabolism which explains most of the essential fatty acid (EFA) effects of cLA. If the metabolism of cLA to GLA is blocked, as in cats in which the Δ-6-desaturase enzyme is genetically absent, cLA has few if any of its expected effects as an EFA (9). Sinclair has long argued that it is the EFA properties of cLA which are crucial for any action in reducing the risk of coronary heart disease (10). There is some experimental evidence for this since Kingsbury et al. found that arachidonic acid, an EFA metabolite of cLA, has a much greater cholesterol-lowering action than cLA itself (11).

We have obtained evidence that the last of these possibilities may be the correct one. GLA in the form of evening primrose oil (Efamol) had a cholesterol-lowering effect far greater than that of linoleic acid as predicted by the Hegsted-Keys equations.

PATIENTS AND METHODS

Eighty-four patients were entered into trials of the effects of oil (evening primrose oil) extracted from the seeds of a strain of *Oenothera biennis* selected for its ability to produce oil of constant composition (Efamol). Twelve patients were attending physicians for the treatment of hyperlipidemia and/or hypertension and received either 6 or 8 0.5 g capsules of Efamol per day for not less than 12 weeks. There were no controls for these patients. Twenty-seven patients were obese women attending Dr. J. Munro's clinic at the Eastern General Hospital, Edinburgh, Scotland. They were entered into a double-blind, placebo-controlled crossover trial in which they received 8 capsules of either Efamol or placebo for 12 weeks and then crossed over to the other treatment for a further 12 weeks. Their mean age was 42 years (range 16-69). Cholesterol measurements were made at the start, the crossover period and the end. The effects of Efamol and placebo on weight loss did not differ (12). Forty-five patients were attending Dr. S. Wright's eczema clinic at Bristol Royal Infirmary, England. The mean age of these patients was 26 (range 15-58). They were randomly assigned to receive 4, 8 or 12 capsules per day in a placebo-controlled, double-blind, crossover study. They received Efamol or placebo for 12 weeks and then the opposite treatment for a further 12 weeks. Fifteen patients (11 female) received 4 capsules per day, 16 patients (7 female) 8 capsules per day, and 14 patients (6 female) 12 capsules per day. Cholesterol levels were measured at the beginning, the crossover point and the end.

Total cholesterol levels were measured using SMA autoanalyzers. In all the Bristol patients and in some of the 12 patients attending physicians for hypertension, HDL and LDL cholesterol levels were measured after separation by precipitation with heparin and manganese chloride and ultracentrifugation.

The Efamol was supplied as gelatin capsules containing 0.5 g of evening primrose oil by Efamol Ltd., London. Evening primrose oil contains ca. 72% of *cis*-linoleic acid in the form of triglycerides but is unusual among polyunsaturates in also containing ca. 9% of GLA. The other major fatty acids present are oleic 9%, palmitic 8% and stearic 1%.

The patients attending the obesity clinic were given simple instructions about calorie restriction but such instructions were given equally to the Efamol and placebo groups. The other patients were given no dietary advice and Efamol was simply taken as a supplement to their usual diet. Blood samples were not taken in the fasting state but again this applied to patients taking both Efamol and placebo.

RESULTS

Initially male and female results were analyzed separately but, since there were no significant differences between them, the results from the 2 sexes were grouped together. Table 1 shows the changes in serum cholesterol from the beginning to the end of Efamol treatment in all 84 patients divided according to the initial value for total cholesterol. The falls were significant in all groups of patients with starting values above 5 mmol/l. Patients with levels below 5 mmol/l, accepted as normal by most authorities, showed no changes in response to Efamol.

Table 2 compares the effects of the 3 doses of Efamol given in the Bristol study. Starting values in the 3 groups were not significantly different from one another. There was a dose-dependent effect with the 2 higher doses producing a progressively greater response.

Patients in the Bristol and Edinburgh studies acted as their own controls. None of these patients had starting cholesterol levels above 8 mmol/l. Since Table 1 shows that Efamol had no effects when starting cholesterol values were below 5 mmol/l, patients with cholesterol levels above 5 mmol/l were selected from the 2 groups and the effects of Efamol and placebo compared (Table 3). At the start of the placebo period, 36 patients had cholesterol levels of 5 mmol/l and above. At the

TABLE 1

The Effects of γ-Linolenic Acid in the Form of Evening Primrose Oil (Efamol) on Total Plasma Cholesterol Levels

All starting values: mmol/l	n	Beginning mean ± SD	End	Fall (mmol/l)	Fall (mg/100 ml)	p
Above 8.0	7	10.90 ± 2.43	7.64 ± 1.71	3.26	125.4	0.0001
Above 7.0	15	9.15 ± 2.46	6.92 ± 1.57	2.23	85.8	0.0001
Above 6.0	31	7.79 ± 2.21	5.98 ± 1.54	1.81	69.6	0.0001
Above 5.0	50	6.94 ± 2.07	5.58 ± 1.37	1.36	52.3	0.0001
Below 5.0	34	4.32 ± 0.56	4.27 ± 0.69	0.05	1.9	ns

TABLE 2

Effects of 4, 8 and 12 Capsules/Day of a Special Variety of Evening Primrose Oil (Efamol) on Total Blood Cholesterol Levels[a]

Dose (caps/day)	n	Starting value	Finishing value	Change	p
4	15	5.67 ± 0.91	4.87 ± 1.12	−0.80 or 14.1%	0.05
8	16	5.60 ± 1.29	4.63 ± 0.77	−0.97 or 17.3%	0.02
12	14	5.88 ± 1.19	4.55 ± 0.82	−1.33 or 22.6%	0.01

[a]Samples for analysis were taken at the beginning and the end of the Efamol treatment period. Values are means ± SD. Statistical analysis was by Student's t test.

TABLE 3

Comparison Between the Cholesterol-lowering Effects of Efamol and Placebo in Patients with Starting Values above 5 mmol/l and below 8 mmol/l[a]

Treatment	n	Starting value	Finishing value	Change	p
Efamol	43	6.20 ± 0.78	5.17 ± 0.87	−0.82	0.0001
Placebo	36	5.87 ± 0.85	5.66 ± 0.86	−0.21	ns

[a]Values are in mmol/l and indicate means ± SD. Statistical analysis was by Student's t test.

start of the Efamol period, 43 patients had cholesterol levels of 5 mmol/l and above. There were fewer patients in the placebo group because half of this group had received Efamol first and so had experienced cholesterol lowering. Cholesterol levels fell in both groups, but the fall was 0.61 mmol/l greater in the Efamol group.

In 54 patients, HDL and LDL cholesterol levels were measured separately. Efamol had no effect on HDL cholesterol levels and all the cholesterol-lowering action was attributable to a fall in LDL cholesterol. As a result, there was a fall in the LDL/HDL cholesterol ratio from 4.13/1.29 (3.20) to 3.05/1.28 (2.38). There was no effect on triglyceride levels in these individuals.

DISCUSSION

Evening primrose oil (Efamol) is clearly an effective cholesterol-lowering agent in those with plasma cholesterol values above 5 mmol/l, i.e., in all but the lowest quintile of cholesterol levels and of risk in the Framingham study (13). The failure of evening primrose oil to lower cholesterol in those whose values are unequivocally normal suggests that it is acting physiologically to regulate cholesterol metabolism and is not having a pharmacological effect.

If one assumes that the cholesterol-lowering effects of evening primrose oil are due to its known constituents, then only cLA and GLA are reasonable candidates. The other major constituents, oleic, palmitic and stearic acids, are unlikely to have any effects on cholesterol, especially at the doses given. The oil contains ca. 1% of non-saponifiable matter; this has been analyzed in detail by Dr. B. Hudson of Reading University and found to have no constituents not present in safflower, corn or sunflower oils. If one further assumes that the Hegsted/Keys studies are broadly correct, as many follow-up studies have shown them to be, then only a very small part of the effect

can be attributed to cLA and most must be due to GLA. Taking the mean of the Hegsted and Keys values for the effect of polyunsaturate supplementation on cholesterol, a 1% rise of kilocalorie intake as polyunsaturated fatty acids should lower plasma cholesterol by 1.48 mg/100 ml. The majority of the patients took 4 g of evening primrose oil per day, containing 2.88 g of linoleic acid and 0.36 g of γ-linolenic acid. If one assumes a daily calorie intake of 2700 kcal, then 1% of that would be 27 kcal which would be provided by 3 g of fatty acids. The predicted fall due to the linoleic acid in Efamol is therefore $2.88/3 \times 1.48$ or 1.4 mg/100 ml. At a kcal intake of 1800/day, the predicted fall would be 2.1 mg/100 ml. The actual fall produced by Efamol over and above that produced by placebo was 0.61 mmol/l or 31.5 mg/100 ml. Thus, a fall in cholesterol of ca. 30 mg/100 ml could not be explained by the linoleic acid content of the evening primrose oil.

Given the assumptions made in the calculations, GLA is ca. 170 times more potent than cLA in the group with starting cholesterol levels above 5.0 and below 8.0 mmol/l. Changing the assumptions (e.g., by changing the total daily calorie intake) would change the precise result but not its order of magnitude. Table 1 suggests that the cholesterol-lowering effect of GLA becomes greater the higher the starting cholesterol level. In the group with starting values between 5.0 and 6.0 mmol/l, GLA was ca. 80 times more potent than the predicted effect of cLA. In the group with starting cholesterol levels above 8.0 mmol/l, GLA had a potency ca. 700 times greater than the predicted cLA effect. The following conclusions can therefore be drawn.

(a) The cholesterol-lowering effect of cLA is probably not due to linoleic acid itself but to that fraction which is converted to GLA and beyond. No conclusions can be drawn from this study as to whether GLA, dihomo-γ-linolenic acid, arachidonic acid, or any other GLA metabolite is finally responsible for the effect.

(b) If endogenous GLA formed from cLA and exogenous GLA provided as a supplement or in the diet are equipotent in their cholesterol-lowering effects, then it is possible to draw conclusions about the proportion of dietary cLA which is converted to GLA. In the individuals with starting cholesterol levels below 5.0 mmol/l in whom GLA as a supplement has no cholesterol-lowering effect, substantial amounts of endogenous GLA are presumably being formed from the 5-15 g of linoleic acid in the daily diet. In those with cholesterol levels between 5.0 and 6.0 mmol/l in whom GLA was ca. 80 times more potent than the expected effect of cLA, only ca. 1/80th of dietary cLA must be converted to GLA. As starting cholesterol levels rise, the relative potency of GLA increases, so that at cholesterol levels above 8 mmol/l only ca. 1/700th of dietary cLA seems to be converted to GLA. Thus, in spite of taking substantial amounts of cLA in the diet, it is possible to be functionally EFA-deficient as a result of defective conversion of cLA to GLA. An elevated level of plasma cholesterol may be one indicator of such defective conversion.

(c) In those with elevated cholesterol levels, the Δ6-desaturase enzyme which converts cLA to GLA appears to be functioning inefficiently. This enzyme is therefore likely to be of major importance in understanding risk of cardiovascular disease. Brenner and others have found that aging, sex, diabetes mellitus, alcohol, catecholamines, *trans* fatty acids found in processed vegetable oils and probably saturated fatty acids can all lead to reduced Δ6-desaturase activity (14-16). The actions of these agents on this enzyme may provide a biochemical basis for their known status as risk factors for cardiovascular disease. Independent evidence of weak desaturase activity in humans is provided by the failure of very large doses of α-linolenic acid (18:3n-3) to be converted to eicosapentaenoic acid (17). Since the desaturase system has a higher affinity for n-3 than for n-6 fatty acids, its failure to metabolize 18:3n-3 is good evidence for only weak enzyme activity in adult humans.

(d) The desirable effects of an increased amount of PUFA in the diet may be achievable without any drastic change in dietary habits by using GLA rather than cLA as the supplement of choice. Human milk, but not cow's milk, contains substantial amounts and on a per-kg basis breast-fed infants are receiving GLA doses several times higher than those used in the present study (18).

In view of the very large numbers of people currently taking high levels of linoleic acid as a result of publicity about the importance of the ratio of dietary polyunsaturated to saturated fats, the question of the precise mechanism of action of linoleic acid in lowering cholesterol deserves careful investigation. Our studies suggest that it is not linoleic acid itself which is active, but γ-linolenic acid or some further metabolite.

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The Effect of Developing Solvents on Lipid Class Quantification in Chromarod Thin Layer Chromatography/ Flame Ionization Detection

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ABSTRACT

Undeveloped lipids on Chromarods give peaks of variable shape and of variable area when scanned through the flame ionization detector (FID). With a brief exposure to developing solvents of suitable polarity, it is possible to produce sharp peaks of less variable area without appreciably moving peak axes. Longer developments or developments in more polar solvent systems result in broader-based peaks of lower area. These observations, based on many analyses of a triglyceride and of a ketone, suggest that the FID response of lipids can be modified according to their distribution around or along Chromarods.
Lipids 18:563-565, 1983.

INTRODUCTION

The Chromarod thin layer chromatography/ flame ionization detector (TLC/FID) system separates and measures low concentrations of lipids with reliability and rapidity. Despite the many successful quantitative applications of TLC/FID (1-6 and references therein), it has recently been stated that "we need a much better understanding of the manner in which all tested compounds behave under analysis conditions before quantitation can become feasible" (7).

In this communication, we examine one of the factors which affects the FID response of two lipid compounds. A triglyceride was chosen for in-depth study because of the widespread occurrence of triglycerides in lipid samples. Parallel experiments were conducted with a ketone which, in this case because of its lack of occurrence, has been suggested as a possible internal standard for marine lipid class analyses (1). Also, the use of both these lipids as references for the relative responses of several compounds (1,2) means a greater understanding of factors affecting their FID response is obviously desirable. We believe, however, that the observations described here extend beyond these two compounds, and that they apply to a greater or lesser extent to all lipid classes. Preliminary results from further experiments indicate that this is indeed the case.

MATERIALS AND METHODS

Solutions of tripalmitin (Supelco, Belleforte, PA) in chloroform/methanol, 2:1 (5 μg/μl) and of hexadecan-3-one (K and K Laboratories, Plainview, NY) in chloroform (3 μg/μl) were prepared. All the quantitative data presented in this communication are from the tripalmitin solution. However, data from the hexadecanone solution are entirely analogous. Standards were spotted on Chromarods-SII with disposable 1-μl pipettes (Drummond Scientific Co., Broomall, PA) with a minimum of 5 spotting actions per μl. A set of 10 rods was spotted at the origin and usually at 4 other places along each rod (Fig. 1). The rods were then developed, dried for 2 min at 120 C, and transferred to an Iatroscan TH-10 Mark III analyzer (Iatron Laboratories, Tokyo). In some experiments, the rods were not developed and were transferred directly to the Iatroscan after the spots had dried.

The air flow on the Iatroscan was 2000 ml/min, the hydrogen pressure was 0.73 kg/cm^2, and the scan speed was 3.1 mm/sec. Peak areas were obtained from a Spectra-Physics SP4200 computing integrator and, in some cases, a comparison was made with peak areas obtained manually using a Technicon integrator/calculator.

After each day's analyses, the rods were transferred to numbered test-tubes containing 60% H_2SO_4, and placed in a sonic bath for 15 min. Each rod was then washed thoroughly in distilled water, replaced in its original position in the frame, and stored in a constant humidity chamber (32% relative humidity).

RESULTS AND DISCUSSION

Undeveloped Rods With 5 Separate Spots Per Rod

The FID scan of undeveloped rods gave peaks of variable shape and of variable area (Figs. 1a and 2). The peaks were generally broad for their entire height and they were often split near the top (Fig. 1a). Peak broadening or separation in undeveloped spots has already been noted (3). This peak shape is undoubtedly the result of the spreading of the solvents in which the standards were dissolved; the outer limits of the peaks must be close to the limits to which solvents used for spotting have moved.

The inter- and intrarod precision for the undeveloped tripalmitin spots was 13% or better

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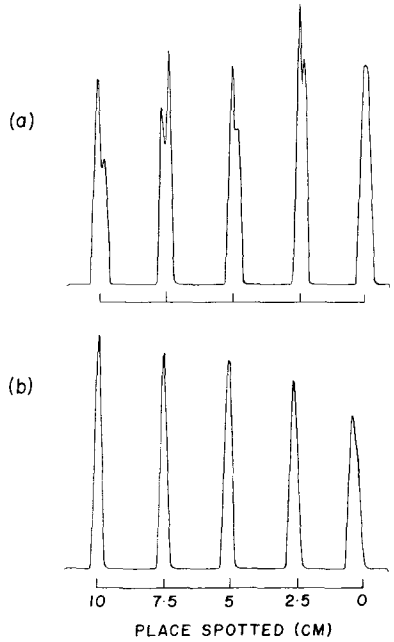

FIG. 1. Chromatograms for 5 μg of tripalmitin spotted at 5 places along a Chromarod. (a) Rod transferred directly to the latroscan and burnt without development. (b) Rod developed for 40 min in hexane/diethyl ether (99:1) from right to left in the figure and then dried and scanned. Note the slight proportional shift of peaks with time in solvent.

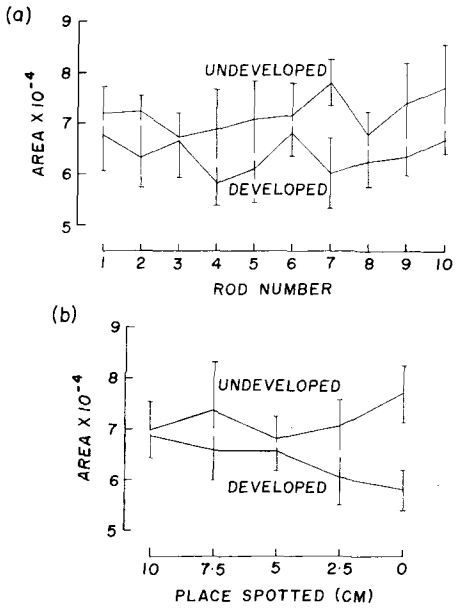

FIG. 2. Mean peak areas for 5 μg of tripalmitin spotted at 50 places on a set of 10 Chromarods. Error bars are one standard deviation from the mean. The upper curve in both graphs is for undeveloped rods and the lower curve is for rods developed in hexane/diethyl ether (99:1). (a) Mean peak areas and intrarod variability (n=5) for 10 Chromarods. (b) Mean peak areas and interrod variability (n=10) for 5 places along each Chromarod.

(standard deviation expressed as a percentage of the mean; Fig. 2). This experiment has been repeated many times with the tripalmitin solution and with the solution of hexadecanone, and no pattern to the distribution of peak areas within the 10 rods has been found. The lack of any consistent pattern in peak areas implies that the variability in peak areas obtained on undeveloped rods is mainly a function of spotting and of the distribution of material around and along the rod and is not, as claimed (7), a problem of rod "nonuniformity."

Developed Rods with 5 Separate Spots Per Rod

After a 40-min development of tripalmitin in hexane/diethyl ether, 99:1, the peaks rarely show any signs of splitting, even though none of the peaks has migrated appreciably (Fig. 1b). The intrarod precision is improved (better than 12%; Fig. 2a) and the interrod precision is improved significantly (better than 10%; Fig. 2b). The improvement in intrarod precision was less than anticipated. The reason for this is that with development, a pattern in the distribution of peak areas emerges. This pattern is consistent and applies to repeated experiments with the tripalmitin solution as well as to experiments with hexadecanone spots developed in a less polar solvent system.

A comparison of Fig. 1a with peaks from spots that have spent the least amount of time in the developing solvent (Fig. 1b: 10 cm and 7.5 cm) indicates that development causes a more even distribution of material on Chromarods. The areas are slightly smaller and the precision is improved (Fig. 2b). For these spots, development, in effect, causes solvent focusing which has already been described as a method for obtaining more reproducible peak areas (3).

Developed rods show a gradation in peak heights, in peak widths and in peak areas (Figs. 1b and 2b). The lipid material is affected in proportion to the extent of exposure to solvent, so that the shortest and broadest-based peak is located at the 0 cm mark (Fig. 1b). This corresponds to the familiar band spreading found in other chromatographic techniques. However, what was not anticipated was the concomitant reduction in FID response for tripalmitin (Fig. 2b).

Since the interrod precision for the 10 cm spots and for the 0 cm spots is similar (Fig. 2b), it would appear that the apparent differences in solvent

mobility on different rods of a set (7) are not a major contributing factor to interrod differences in FID response.

Experiments were performed to ensure that none of the patterns in peak areas were caused by a bias resulting from experimental technique. Different spotting orders were used for different rods; standard solutions were frequently renewed from stock solutions; rods were reburnt after every experiment; integrations were performed manually and compared with areas obtained on the computing integrator; and rods were scanned by the FID in different directions.

It should be noted that if a less polar solvent system (e.g., 100% hexane) is used to develop the tripalmitin spots, then the pattern produced is intermediate between that described in Figure 1a and that described in Figure 1b. Hexane is presumably not polar enough to redistribute evenly all the tripalmitin around and along Chromarods. However, if hexadecanone spots are developed in hexane, then the situation is similar to that described in Figures 1b and 2. Development of hexadecanone in hexane/diethyl ether, 99:1, or else of either compound in more polar solvent systems (e.g., hexane/diethyl ether, 80:20) causes a significant migration of material spotted at 0 cm. As would be anticipated, the peaks produced under these circumstances are broad-based and have a lower area than in Figures 1 and 2.

The fact that development causes a reduction in peak areas and in their variability means that it is unwise to draw inferences from data obtained on undeveloped rods (7) unless they are routinely used in this way. One of the less obvious examples where rods may be routinely used in this way is in the analysis of total phospholipids after neutral lipids have been eluted along the rod (1,2). The solvent systems used for neutral lipid separations leave polar lipids at the point of application and are unlikely to be polar enough to cause an even distribution of polar material around or along Chromarods. The more polar and less volatile the solvents used for spotting, the more likely it is that the polar lipid peak will be split.

The interrod precision of 10% obtained in this study is similar to published values for analyses on 5-10 rods of a set (2-6). These data cover a wide range of compounds and a wide range of sample loads: a precision of 10% or better has been obtained for loads as low as 0.5 μg (3) and for loads as high as 100 μg (4). Thus, any concern with a narrow working range on Chromarods (7) is not warranted.

In the set of rods used in these experiments, rods 1, 6 and 10 gave the highest responses after development (Fig. 2a). Since the difference between rods 4 and 6, for instance, is significant at the 98% confidence level (paired or 2 sample t-tests), an improved precision may be obtained if samples are analyzed on rods which give similar responses. The necessity of grouping rods has already been noted (8). The significant difference between rods 4 and 6 also indicates that, if one peak on a Chromarod is notably larger than the average at that position over a set of 10 Chromarods, then all the peaks are likely to be larger than the average at each position. Since 5 separate spots have been applied to each rod, then it is unlikely that the main reason for interrod differences is small differences in volume or in the standard solution concentration delivered to each spot. The difference in response between Chromarods may be due, in part, to small differences in the overall characteristics of the silica gel coatings. However, it should be possible to compensate for interrod differences by using an internal standard for lipid class analyses. Data from different Chromarod lots with rods of different ages and of different types (S and SII; [1]) and a detailed statistical analysis of rod to rod and lot to lot variation (5) show that variability can indeed be significantly reduced in this way. Even greater precision is possible if each rod is used as an individual analytical unit and only intrarod precision is considered (6).

The data shown in Figure 2 are from the 28th and 29th scans of this set of 10 Chromarods. The interrod precision for the developed rods (29th scan) is good and comparable with the precision obtained from other compounds scanned on rods of different ages (2-6). Figure 2 and data from rods which have been used many more times than this (1,3,9) suggest that any concern about a short working life for Chromarods (7) is unwarranted.

ACKNOWLEDGMENTS

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Fatty Acid Metabolism and Cell Proliferation: IV. Effect of Prostanoid Biosynthesis from Endogenous Fatty Acid Release with Cyclosporin-A

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ABSTRACT

Cyclosporin-A (Cyc-A) stimulates prostanoid (PGI_2) synthesis in confluent smooth muscle cells from guinea pig aorta through the release of endogenous fatty acid. Cyc-A, like other stimulatory agents for prostanoids, promotes smooth muscle cell proliferation and prostanoid synthesis in these proliferating cells. Indomethacin, a cyclooxygenase inhibitor, and exogenous arachidonic acid block the Cyc-A effect on cell proliferation.

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INTRODUCTION

Cyclosporin-A (Cyc-A) is a potent immunosuppressive agent used clinically to prevent tissue rejection following transplantation. Initial studies showed that Cyc-A inhibits T-cell mediated events such as graft-vs-host disease, yet Cyc-A has no effect on B-cell mediated events (1). A number of studies show that Cyc-A has an inhibitory effect on peripheral blood and bone marrow T-cell colony formation (2-5). T-cell colony formation is suppressed by PGE_1 and $PGF_{2\alpha}$ level in the 0.001-0.1 μM range (2-4) and T-cell mitogenesis is promoted by cyclooxygenase inhibitors (6). B-cell mitogenesis is unaffected by prostaglandins and cyclooxygenase inhibitors (2-4,7). These data suggest that Cyc-A may affect T-cells by promoting prostanoid synthesis.

We have previously studied prostanoid synthesis in aorta smooth muscle cells (4,8-12). Agents that stimulate endogenous fatty acid release enhance prostanoid synthesis in these cells. The proliferation of smooth muscle cells, unlike the proliferation of T-cells, is promoted by increased prostanoid synthesis. In the present study, we have examined the hypothesis that Cyc-A stimulates prostanoid biosynthesis by studying its effect on aorta smooth muscle cells.

MATERIALS AND METHODS

Materials

Arachidonic acid [20:4(n-6)] (NuChek, Elysian, MN) was shown to be peroxide-free by thin layer chromatography (8). Cyc-A was kindly supplied by Sandoz Pharmaceuticals (East Hanover, NJ). Indomethacin was purchased from Sigma Chemical Co. (St. Louis, MO). Antiserum for the radioimmunoassay (RIA) of PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) was kindly supplied by Dr. L. Levine. Medium for growing cells to confluency (growth medium) was prepared from 1× Eagle's minimum essential medium which contains Hank's salts and 25 mM HEPES buffer (GIBCO, Grand Island, NY) supplemented with 50 μg/ml gentamycin sulfate (Schering, Kenilworth, NJ), 2 mM glutamine, 1× nonessential amino acids (Microbiological Associates, Walkersville, MD), 1 mM pyruvate and 1.3 mg/ml of sodium bicarbonate. This medium was supplemented with 10% fetal bovine serum (FBS) (Sterile Systems, Logan, UT; Hyclone lot 100348).

Tissue Culture

Primary cultures of smooth muscle cells were established from the dissected medial layer of guinea pig aorta from prepubertal males (13). Smooth muscle cells were identified by their reactivity to antibodies prepared from smooth gizzard muscle (14). Cells were maintained in growth medium.

Prostanoid Synthesis

Cells were used at passage level 3 and were seeded at 1.3×10^4 cells/cm^2 in Corning T-25 flasks containing 4 ml of experimental medium (growth medium supplemented with 1× essential vitamins, 1× essential amino acids and 20% FBS). The cells were allowed to grow to confluency and then treated with ethanol solutions of Cyc-A and/or 20:4(n-6). Control cultures were treated with equal quantities of ethanol. The 6-keto-$PGF_{1\alpha}$ (PGI_2) in the media was measured after a 24-hr incubation period by a standard RIA procedure (15). The cross-reactivity of 6-keto-$PGF_{1\alpha}$ antibody was: PGE_2, 0.15%; PGD_2, 0.02%; $PGF_{2\alpha}$, 0.10%; 20:4 (n-6), 0.005%. The 6-keto-$PGF_{1\alpha}$ antiserum did not cross-react with Cyc-A.

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Cell Proliferation

Smooth muscle cells, passage 3 and 3-5 days postconfluent, were seeded at low density (80 cells/cm^2) on Costar tissue culture dishes (60 × 15 mm). Cells were allowed to attach to the plastic petri plates for one day and then treated with ethanol solutions of Cyc-A and/or indomethacin in fresh experimental media containing 10% FBS. Control cultures were treated with equal quantities of ethanol. Cells were retreated with a media change on day 5 of the incubation period. After an 8-day incubation period, cells were fixed with 3.7% phosphate buffered formalin and stained with filtered Giemsa. Prior to fixing, aliquots of media were removed for RIA analysis of 6-keto-PGF$_{1\alpha}$.

Total cell area was measured by image analysis using the Optomax visual analysis system and reported in arbitrary area units (5361 units/cm^2). A relative cell count was obtained from the total cell area on the plate. The relationship between cell area and cell number has been validated with a microscope and with a Coulter counter (10,11,16). Cells from the same primary culture and the same batch of growth medium were compared in each treatment group.

Statistics

The significance of differences between means was determined by an analysis of variance including a trend analysis and a multiple range test (Tukey-HSD procedure) for the significance level.

RESULTS AND DISCUSSION

Effects of Cyc-A on PGI$_2$ Biosynthesis

The effect of varying concentrations of Cyc-A on prostanoid biosynthesis was measured in confluent cultures of smooth muscle cells. Following 24 hr incubation with Cyc-A, media were removed from the cultures and analyzed for 6-keto-PGF$_{1\alpha}$ by RIA. The results of these studies are summarized in Table 1. Cyc-A shows a concentration-dependent stimulatory effect on the synthesis of PGI$_2$ in these confluent cultures. The stimulatory effect reaches a maximum at 50 μM Cyc-A. Less prostanoid is generated and floating cells (dead cells) are seen in cultures when the Cyc-A concentration is increased to 100 μM.

Preliminary studies showed that Cyc-A was, like many other agents (8-12), toxic in cultures of proliferating cells seeded at low densities. Thus, it was necessary to work at lower concentrations of Cyc-A in proliferating cultures than in confluent cultures. When cultures were incubated with Cyc-A during an 8-day period of cell proliferation, Cyc-A showed a concentration-dependent stimulatory effect on the synthesis of 6-keto-PGF$_{1\alpha}$ (Table 1). A trend analysis showed a highly significant correlation between Cyc-A concentration and the 6-keto-PGF$_{1\alpha}$ yield (F ratio 40.615). Enhanced prostanoid yields in this experiment could merely reflect enhanced cell proliferation. This question is addressed in a subsequent section.

Cyc-A does not have a stimulatory effect on the

TABLE 1

Effect of Cyclosporin-A on Prostacyclin Synthesis (6-keto-PGF$_{1\alpha}$) in Aorta Smooth Muscle Cells

Treatment	Prostanoid (nmol/culture)	
	Mean ± SEM	
I. Confluent cells		
Media	0.90 ± 0.11	
Media + 0.1 μM Cyc-A	0.86 ± 0.08	
Media + 1.0 μM Cyc-A	0.93 ± 0.08	
Media + 10 μM Cyc-A	1.45 ± 0.15	P < 0.02
Media + 50 μM Cyc-A	2.41 ± 0.17	P < 0.001
Media + 100 μM Cyc-A	1.57 ± 0.19	P < 0.02
120 μM 20:4 (n-6)	4.21 ± 0.23	
120 μM 20:4 (n-6) + 0.1 μM Cyc-A	4.14 ± 0.30	
120 μM 20:4 (n-6) + 1.0 μM Cyc-A	4.23 ± 0.35	
120 μM 20:4 (n-6) + 10 μM Cyc-A	4.40 ± 0.31	
120 μM 20:4 (n-6) + 50 μM Cyc-A	4.86 ± 1.01	
120 μM 20:4 (n-6) + 100 μM Cyc-A	4.22 ± 0.46	
II. Proliferating cells		
Media	0.0215 ± 0.0005	
Media + 0.005 μM Cyc-A	0.0227 ± 0.0004	
Media + 0.01 μM Cyc-A	0.0232 ± 0.0008	
Media + 0.02 μM Cyc-A	0.0253 ± 0.0006	P < 0.001
Media + 0.05 μM Cyc-A	0.0269 ± 0.0009	P < 0.001

synthesis of 6-keto-$PGF_{1\alpha}$ when both Cyc-A and exogenous arachidonic acid [20:4(n-6)] are added to the cultures (Table 1). These data show that Cyc-A stimulates prostanoid synthesis through endogenous 20:4(n-6) release rather than the conversion of free 20:4(n-6) to prostanoids (4,12).

Effects of Cyc-A on Cell Proliferation

Previous studies from our laboratory have shown that prostanoid synthesis from either low concentrations of exogenous arachidonic acid or the action of agents that stimulate endogenous fatty acid release, promotes the proliferation of aorta smooth muscle cells (4,8,12). These studies involve cultures seeded at low cell densities (80 cells/cm^2). The second aspect of our studies with Cyc-A investigated its effect on smooth muscle cell proliferation. Cyc-A showed a concentration-dependent inhibition of cell proliferation in the 0.1-10 μM concentration range (Table 2A) and a concentration-dependent stimulation of cell proliferation in the 0.005-0.05 μM concentration range (Table 2B,C,D). Two experiments supported the concept that Cyc-A stimulated cell proliferation through prostanoid synthesis. Indomethacin, at concentrations that block prostanoid synthesis (8,11,12), abolished the stimulatory effect of Cyc-A on cell proliferation (Table 2B,C). 20:4(n-6), at a concentration that would overwhelm endogenous 20:4(n-6) release and begin to generate inhibitory lipid peroxides (4,10,12), abolished the stimulatory effect of Cyc-A on cell proliferation (Table 2D).

Prostanoid data for proliferating cells in Table 1 and cell number data for proliferating cells in Table 2C were obtained from the same experiment. A trend analysis showed that cell number, like the 6-keto-$PGF_{1\alpha}$ yield, was correlated with increasing Cyc-A concentration (F ratio 15.390). If the prostanoid increment only reflects increased cell number, then the ratio of 6-keto-$PGF_{1\alpha}$ to cell number will not show a significant trend. When the 6-keto-$PGF_{1\alpha}$ content of each plate was divided by the cell number for the plate, a trend analysis showed that

TABLE 2

Effect of Cyclosporin-A on the Proliferation of Aorta Smooth Muscle Cells

Primary	Treatment	Cell number[a]	
		Mean ± SEM	
A.	Media	32,100 ± 2,340	
	Media + 0.1 μM Cyc-A	27,000 ± 1,730	
	Media + 1.0 μM Cyc-A	22,700 ± 1,330	$P < 0.01$
	Media + 10 μM Cyc-A	no cells	
B.	Media	14,800 ± 908	
	Media + 0.005 μM Cyc-A	15,700 ± 807	
	Media + 0.01 μM Cyc-A	15,600 ± 782	
	Media + 0.02 μM Cyc-A	18,600 ± 1,050	$P < 0.03$
	1 μM Indomethacin	15,000 ± 1,130	
	1 μM Indomethacin + 0.005 μM Cyc-A	13,000 ± 1,140	
	1 μM Indomethacin + 0.01 μM Cyc-A	13,000 ± 1,500	
	1 μM Indomethacin + 0.02 μM Cyc-A	13,200 ± 590	
C.	Media	18,300 ± 525	
	Media + 0.005 μM Cyc-A	20,000 ± 457	
	Media + 0.01 μM Cyc-A	19,700 ± 605	
	Media + 0.02 μM Cyc-A	19,800 ± 312	
	Media + 0.05 μM Cyc-A	21,800 ± 772	$P < 0.01$
	10 μM Indomethacin	18,000 ± 396	
	10 μM Indomethacin + 0.005 μM Cyc-A	19,000 ± 660	
	10 μM Indomethacin + 0.01 μM Cyc-A	19,300 ± 410	
	10 μM Indomethacin + 0.02 μM Cyc-A	19,200 ± 559	
	10 μM Indomethacin + 0.05 μM Cyc-A	18,300 ± 755	
D.	Media	5,470 ± 554	
	Media + 0.005 μM Cyc-A	6,830 ± 544	
	Media + 0.01 μM Cyc-A	7,830 ± 506	$P < 0.01$
	Media + 0.02 μM Cyc-A	6,220 ± 196	
	60 μM 20:4 (n-6)	3,490 ± 215	
	60 μM 20:4 (n-6) + 0.005 μM Cyc-A	3,030 ± 243	
	60 μM 20:4 (n-6) + 0.01 μM Cyc-A	2,940 ± 298	
	60 μM 20:4 (n-6) + 0.02 μM Cyc-A	3,200 ± 201	

[a]Arbitrary area units.

the ratio increased significantly with increasing Cyc-A concentration (F ratio 4.735, $P < 0.04$).

Many investigators show that Cyc-A has a selective effect on T-cell mediated, as opposed to B-cell mediated, immune responses (17-20). Our experiments with confluent and growing smooth muscle cells demonstrate that the biological activity of Cyc-A in these systems may be explained by the stimulatory effect of this agent on fatty acid release and subsequent prostanoid synthesis. We suggest that the effect of Cyc-A on prostanoid synthesis in other cell lines is worthy of further study.

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Sterols from the Gorgonian *Lephogorgia subcompressa*

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ABSTRACT

The free sterols of the gorgonian *Lephogorgia subcompressa* were isolated and characterized by means of gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) techniques. The mixture contains C_{27} derivatives as the major sterols besides minor quantities of C_{26}, C_{28}, C_{29} compounds and also a C_{30} compound, gorgosterol.
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Gorgonians have been normally characterized by their content of C_{30} sterols, the principal one being gorgosterol, which was first isolated by Bergmann et al. in 1943 (1). Later studies of the sterols of various coelenterates have demonstrated that C_{30} sterols are not always present in this type of marine organism (2-8).

We have studied by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) the constituents of the sterol fraction of the gorgonian *Lephogorgia subcompressa* and the results are presented and discussed in this report.

MATERIALS AND METHODS

Fresh specimens of *Lephogorgia subcompressa* (phylum cnidaria, class anthozoa, subclass octocorallia, orden gorgonacea) (5 kg) collected at La Boca, VI Region, Chile, were ground and extracted with acetone for 36 hr. The extract was evaporated under reduced pressure and the remaining aqueous residue was extracted with chloroform. The organic extract was washed with water, dried over magnesium sulfate and evaporated. The syrup obtained (55 g) was chromatographed on a silica gel column by elution with mixtures of petroleum ether/ethyl acetate of increasing polarity. The crude sterol mixture was recrystallized from acetone (9.97 g).

The mixture was analyzed by GC using a Hewlett-Packard 5840 instrument equipped with a 12 m × 0.2 mm fused silica capillary column coated with methyl silicone fluid (Hewlett-Packard). The steroids were chromatographed between 200 and 280C at a rate of 8C/min with helium as the carrier gas. Quantification was made by automatic integration of the peaks before and after the addition of a known amount of stigmasterol used as internal standard. Detector relative responses were determined using an artificial mixture of the most common sterols (C_{27}, C_{28} and C_{29}). The identity of the steroids was assigned by GC-MS using a Varian MAT CH7-A MS coupled to a Varian 1440 GC and interfaced to a Varian MAT Data System 166 computer. Analysis of the acetate (acetic anhydride/pyridine, 1:1, v/v) and trimethylsilyl ether derivatives (hexamethyldisilazane/trimethylchlorosilane/pyridine, 3:3:10, v/v/v) were performed with the same conditions. The relative amounts of 24-methylcholest-5-en-3β-ol and 24-methylcholesta-5,24(28)-dien-3β-ol were discerned by single ion monitoring (SIM) of the sterol's base peaks in the mass chromatogram of the mixture. Data accuracy were checked by SIM quantification of artificial mixtures of the two sterols.

RESULTS AND DISCUSSION

The sterol mixture was analyzed by GC and characterized by GC-MS of the free sterol mixture and of their acetylated derivatives as summarized in Table 1. A GC diagram of the free sterol mixture is shown in Figure 1. MS analysis of the trimethylsilyl ether derivatives allowed unequivocal assignments of Δ^5 sterols (9,10), which were done in all cases by comparison with authentic samples. As shown in Table 1, we identified various mono- and diunsaturated Δ^5 compounds in the sterol mixture, cholesterol being the most abundant of them. Among the diunsaturated sterols, $\Delta^{5,22}$ and $\Delta^{5,24\,28}$ are ubiquitous in marine invertebrates (11,12) and this is not exceptional. The presence of $\Delta^{5,7}$ sterols have never been reported in Cnidaria which, to the best of our knowledge, contain mainly Δ^5 sterols (11). In this case, cholesta-5,7-dien-3β-ol may be the precursor to cholesterol, as it is in other types of marine invertebrates, e.g., molluscs, which are supposed to use $\Delta^{5,7}$ sterols as precursors of the Δ^5 analogs (11). The previously unusual 22-*trans*-24-norcholesta-5,22-dien-3β-ol (13) is now recognized as being widespread in the marine environment and has been already reported from other gorgonians (14,15). 22-*trans*-24-Methyl-27-norcholesta-5,22-dien-3β-ol (occelasterol) and 22-*cis*-cholesta-5,22-dien-3β-ol have identical retention times and mass

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TABLE 1

Sterol Composition of the Gorgonian *Lephogorgia subcompressa*

Sterols	MS Characteristic fragments	RRT[a]	Estimated % by GLC	by SIM
22-*trans*-24-Norcholesta-5,22-dien-3β-ol	370(M⁺),355,352,300,271,255, 213,97,55	0.82	1.03	
22-*trans*-24-Methyl-27-norcholesta-5,22-dien-3β-ol	384(M⁺),366,351,300,273,271, 255,213,111,69,55	0.94	1.76	
22-*trans*-Cholesta-5,22-dien-3β-ol	384(M⁺),366,351,300,273,271, 255,213,111,69,55	0.95	5.31	
Cholest-5-en-3β-ol	386(M⁺),371,368,301,275,273, 255,231,213,145,43	1.00	51.49	
22-*trans*-24-Methylcholesta-5,22-dien-3β-ol	398(M⁺),380,365,300,271,255, 213,69,55	1.02	2.57	
Cholesta-5,7-dien-3β-ol	384(M⁺),366,351,271,253,211, 43	1.03	7.25	
24-Methylcholest-5-en-3β-ol	400(M⁺),385,382,367,315,289, 273,213,105,43	1.08	20.26	12.56
24-Methylcholesta-5,24(28)-dien-3β-ol	398(M⁺),383,380,314,299,281, 271,255,229,213,55	1.08		7.70
22-*trans*-24-Ethylcholesta-5,22-dien-3β-ol	412(M⁺),397,394,379,351,300, 273,271,255,69,55	1.12	0.89	
24-Ethylcholest-5-en-3β-ol	414(M⁺),396,381,368,329,303, 255,213,91,55	1.18	5.08	
24-Ethylcholesta-5,24(28)-dien-3β-ol	412(M⁺),397,394,314,299,281, 273,271,255,229,213,55	1.19	3.70	
22,23-Methylene-23,24-dimethylcholest-5-en-3β-ol	426(M⁺),411,408,337,314,300, 283,281,271,255,229,215,213	1.26	0.64	

[a]Relative retention times (RRT) of the free sterols to cholest-5-en-3β-ol.

spectra (16) making it impossible to distinguish between them by these methods. As the occurrence of the last one as a natural product has been questioned (16), we are here proposing that the compound with relative retention time 0.94 is indeed occelasterol and not its isomer.

Gorgonians are characterized by their content of gorgosterol (1,17) and related C_{30} sterols with a cyclopropane ring in the side chain, although Block (14) has pointed out that gorgonians did not always contain it. In our case, only gorgosterol was detectable. Although it is known that gorgonians may play host to a large number of intracellular symbiotic algae (zooxanthellae) (18), it must be considered a priori that sterols isolated from this type of coelenterate may have been produced by the algae, by the animal itself, by the association of both, or they could have been of exogenous origin. There are examples where the occurrence of gorgosterol was particularly associated with symbiotic zooxanthellae from which it was isolated (17), although Kokke et al. (19) reported that the gorgonian *Lophogorgia cuspidata* lacks both gorgosterol and zooxanthellae.

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FIG. 1. GC analysis of free sterols from *Lephogorgia subcompressa*.

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Reduction of Hepatic Stearoyl-CoA Desaturase Activity in Rats Fed Iron-Deficient Diets

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ABSTRACT

The effect of feeding iron-deficient diets to rats on the hepatic stearoyl-CoA desaturase activity was examined since iron is present in the Δ9 desaturation system. Separate groups of rats were fed low iron diets without fat (FF-Fe) or containing either 14% hydrogenated coconut oil (HCNO-Fe) or 14% corn oil (CO-Fe) for 10 weeks. Diets supplemented with iron (FF + Fe, HCNO + Fe and CO + Fe) were fed to the corresponding control groups. Stearoyl-CoA desaturase activity in the liver microsomes of rats in the CO + Fe group (2.55 ± 0.17 nmol oleate produced/min/mg protein) was about half of that in the HCNO + Fe (4.76 ± 0.15) and FF + Fe (5.38 ± 0.18) diet groups. In rats which were fed iron-deficient diets, hepatic desaturase levels were reduced significantly as compared to those of controls (1.0 ± 0.06, CO − Fe; 2.11 ± 0.13, HCNO-Fe; 3.65 ± 0.1, FF-Fe). The hemoglobin (Hb) and hematocrit (Hct) levels in blood showed moderate iron depletion only in the CO-Fe group. Hence, dietary polyunsaturated fat promotes the onset of iron deficiency. Furthermore, even before the blood Hb and Hct values express iron depletion, the effect of feeding low iron diets was observed by the reduction of hepatic Δ9 desaturase activity in rats fed HCNO-Fe and FF-Fe diets.

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INTRODUCTION

Rats maintained on a low iron corn oil diet (CO-Fe) become iron deficient, as evidenced by the reduced hemoglobin (Hb) and hematocrit (Hct) levels in blood, but those fed low iron fat-free diet (FF-Fe) for the same duration do not (1). The present study confirms this finding and shows that not only the exclusion of diet fat but also the presence of saturated fat in the diet retard the onset of iron deficiency.

Although rats fed FF-Fe diet did not become iron deficient, the ratio of the monoenoic to saturated fatty acids (16:1/16:0; 18:1/18:0) in their tissue lipids was reduced when compared to that in controls which were fed iron-supplemented FF diets (1). This observation had suggested that the Δ9 desaturase, which contains iron (2-4), was less active in tissues of animals consuming iron-deficient diets. Results from the assay of hepatic microsomal stearoyl-CoA desaturase activity reported in the present study show that the enzyme activity is indeed reduced markedly in rats which were fed low iron diets as compared to that in rats fed iron-supplemented diets.

MATERIALS AND METHODS

Thirty-six male Sprague-Dawley rats weighing ca. 75 g were purchased from Charles River, Wilmington, MD. They were divided into 6 groups of 6 each and housed in plastic cages with plastic ventilated covers. Each group was fed ad libitum one of the following low iron diets: fat-free (FF-Fe), 14% hydrogenated coconut oil (HCNO-Fe), 14% corn oil (CO-Fe) or the corresponding iron-supplemented diets (FF + Fe, HCNO + Fe, CO + Fe). Rats had free access to deionized-distilled water fed through glass sipper tubes. Cages had sawdust bedding which was changed twice a week. The animal room had a 12-hr light and 12-hr dark cycle. It was maintained at 24 C with 40-50% relative humidity. Diets were custom-made by ICN Nutritional Biochemicals, Cleveland, OH. The composition of FF-Fe diet was the same as that of the fat-free diet given in the ICN animal research diet manual[1] except that ferrous ammonium citrate was excluded from the USP salt mixture XIV. The compositions of the CO-Fe and HCNO-Fe diets were same as the low iron diet given in the ICN diet manual[1] except that the vegetable oil was replaced with corn oil and hydrogenated coconut oil, respectively. Low iron diets contained 13 μg Fe/g

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[1]Fat-free diet contained vitamin-free casein (21.1%), alphacel (16.45%), sucrose (58.45%), salt mixture U.S.P. XIV (4.0%) and ICN vitamin diet fortification mixture with choline chloride (6.008 g/kg). Low iron diet contained vitamin-free casein (27.0%), corn starch (56.0%), salt mixture H.M.W. without ferric phosphate (3.0%), fat (14.0%) and ICN vitamin diet fortification mixture. The vitamin mixture contained vitamin A concentrate 200,000 units/g, 4.5 g; vitamin D concentrate 400,000 units/g, 0.25 g; alpha tocopherol, 5.0 g; ascorbic acid, 45.0 g; inositol, 5.0 g; choline chloride, 75.0 g; menadione, 2.25 g; *p*-aminobenzoic acid, 5.0 g; niacin, 4.5 g; riboflavin, 1.0 g; pyridoxine hydrochloride, 1.0 g; thiamine hydrochloride, 1.0 g; calcium pantothenate, 3.0 g; biotin, 20 mg; folic acid, 90 mg; and vitamin B_{12}, 1.35 mg in 100 lb. The ingredients were titurated in dextrose and 1 kg of the mixture was added to 100 lb of diet.

U.S.P. XIV salt mixture contained calcium carbonate, 6.86%; calcium citrate, 30.83%; calcium biphosphate, 11.28%; magnesium carbonate, 3.52%, magnesium sulfate, 3.83%; potassium chloride, 12.47%; potassium phosphate dibasic, 21.88%; sodium chloride, 7.71% and 1.62% of a mineral mixture containing cupric sulfate 0.48%; ferric ammonium citrate, 94.33%; manganese sulfate, 1.24%; ammonium alum, 0.57%; potassium iodide 0.25% and sodium fluoride, 3.13%.

Salt mixture H.M.W. was formulated as suggested by Hubbell, Mendel and Wakeman (J. Nutr. 14, 273, 1937).

diet. The supplemented diets contained 80 μg Fe/g diet.

Determination of Hb and Hct

After 10 weeks, rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/ml/300 g rat) and exsanguinated using heparin-washed syringes and needles. Whole blood was transferred without pressure into heparinized vacutainer tubes. Hemoglobin determinations were carried out by the cyanmethemoglobin method. Packed red cell volumes were measured using a Phillips-Drucker hematocrit centrifuge and an IEC microcapillary reader.

Preparation of Liver Microsomes

Immediately after the removal of blood, livers were excised, blotted and washed with ice cold saline. Pieces of liver (ca. 1.5 g) were homogenized in 5 vol of 0.25 M sucrose at 0-2 C using a Potter-Elvehjem homogenizer. All subsequent preparative procedures were performed at 0-2 C. The homogenate was centrifuged at 1000×g for 15 min to separate cell debris and floating fat. The resulting supernatant fraction was centrifuged at 15,000×g for 20 min and the pellet was discarded. The microsomal fraction was obtained by centrifugation of the supernatant at 100,000×g for 1 hr. Microsomal pellet was washed with 0.154 M KCl and a suspension was prepared (5 mg protein/ml) in 3 ml 0.154 M KCl using a Potter-Elvehjem homogenizer by a few up and down gentle strokes of the pestle.

Assay of Stearoyl-CoA Desaturase Activity

Incubation conditions for the determination of stearoyl-CoA desaturase activity were essentially those described by Raju and Reiser (5). Reaction medium contained the potassium salt of [1-^{14}C] stearic acid (0.2 mM), CoASH (0.2 mM), NADH (2 mM), ATP (5 mM), GSH (10 mM), $MgCl_2$ (10 mM), DL-glycerol 3-phosphate (10 mM), potassium phosphate buffer (pH 7.4, 0.1 M) and microsomal protein, all in a total volume of 1 ml. With each liver, two assays were performed using ca. 0.5 mg and 1 mg microsomal protein, respectively.

Reactions were carried out at 37 C in a Dubnoff metabolic shaker for 5 min with air as gas phase. They were stopped by the addition of 5 ml chloroform/methanol (2:1, v/v) and lipids were extracted (6). Fatty acid methyl esters of lipids were prepared by transesterification catalyzed by sulfuric acid (7). Methyl oleate was separated from methyl stearate by $AgNO_3$-impregnated silica gel thin layer chromatography (TLC) (5). Plates were sprayed with a 0.2% solution of 2',7'-dichlorofluorescein in methanol and visualized under UV. Spots containing the 18:1 and 18:0 fatty acid methyl esters were scraped. The esters were extracted with diethyl ether. The ether extracts were passed through a small column containing $KHCO_3$, collected into scintillation vials and they were evaporated to dryness. After the addition of scintillation fluid, the [^{14}C] activity was determined using a Mark II scintillation spectrometer. The radioactivity in the 18:1 and 18:0 fractions accounted for more than 98% of the activity in the total fatty acid methyl esters.

Microsomal protein concentration was determined by the method of Lowry et al. (8) using bovine serum albumin as a standard. The stearoyl-CoA desaturase activity was expressed as nmol oleate produced/min/mg microsomal protein.

Pure stearic acid was obtained from Applied Science, State College, PA. [1-^{14}C]Stearic acid was purchased from New England Nuclear Corp., Boston, MA, and purified by TLC. ATP, CoASH, NADH, GSH and bovine serum albumin were obtained from Sigma, St. Louis, MO.

RESULTS AND DISCUSSION

Determination of the Hb content and Hct values of blood from various diet groups showed that moderate iron deficiency was produced only in the rats which were fed corn oil (Table 1). In the animals which were fed either the FF or HCNO diet, iron deficiency was not apparent. It would, therefore, appear that either the absence of fat or the presence of saturated fat in the diet can retard the onset of iron deficiency. The mechanism by which the type of dietary fat influences iron deficiency is not understood at the present time.

TABLE 1

Hemoglobin and Hematocrit Values of Blood from Rats Fed Iron-Deficient and Iron-Supplemented Diets[a]

Diet[b]	Hb (g%)	Hct (%)
FF + Fe	14.1 ± 1.3	43.7 ± 1.8
FF - Fe	12.7 ± 2.0	41.8 ± 2.0
HCNO + Fe	14.0 ± 1.0	44.2 ± 1.7
HCNO - Fe	12.7 ± 0.8	42.5 ± 1.4
CO + Fe	12.9 ± 0.7	42.7 ± 2.4[d]
CO - Fe	8.4 ± 1.2[c]	34.1 ± 4.6[d]

[a]Values are given as mean ± SD. These were obtained by duplicate determinations with blood from each of the 6 animals in the various diet groups.

[b]Diet abbreviations are: FF, fat-free; HCNO, 14% hydrogenated coconut oil diet; CO, 14% corn oil diet. Iron-deficient and iron-supplemented diets are designated as - Fe and + Fe, respectively.

[c]$p < 0.001$.

[d]$p < 0.01$.

In the past, numerous investigations have been conducted on the alterations of Δ9 desaturase activity due to dietary factors such as fat (9-15) and cyclopropene fatty acids (16-18) to pathologic conditions such as diabetes (19,20), obesity (21), phenyl ketonuria (22) and neoplasia (12-15,23,24), or to changes in the physiological conditions in the animal such as starvation (10) and lactation (25). The enzyme complex which catalyzes the desaturation of stearoyl-CoA consists of cytochrome b_5, NADH-cytochrome b_5 reductase, terminal desaturase enzyme which is a nonheme iron protein and lipids (2-4). Thus, although iron is a component of the desaturation system, to our knowledge, effect of dietary deprivation of iron on the tissue desaturase activity has not been reported.

When animals are fed a fat-free diet, the activity of stearoyl-CoA desaturase in their tissues is increased (9-15). However, if dietary iron is excluded from the fat-free diet fed to rats, the relative level of monoenoic to saturated fatty acids is decreased as compared to the iron-supplemented controls, suggesting that desaturase activity may be reduced in tissues (1). The specific activity of hepatic microsomal Δ9 desaturase from animals fed various diets show that the omission of dietary iron causes marked reduction of this enzyme activity (Table 2). As observed earlier (15), desaturase activity was significantly depressed in animals fed the corn oil diet as compared to those fed a fat-free or saturated fat containing diet (Table 2). When rats were fed a diet containing fat (CO or HCNO) but no iron, desaturase activity was lowered by ca. 60%. In animals fed neither fat nor iron, the desaturase activity was also lowered significantly (30%), but not to the same extent as in those fed the iron-deficient CO and HCNO diets (Table 2).

TABLE 2

Specific Activity of Stearoyl-CoA Desaturase in the Microsomal Fractions of Livers of Rats Fed Iron-Deficient and Iron-Supplemented Diets[a]

Diet[b]	nmol oleate produced/min/mg protein
FF + Fe	5.38 ± 0.18[c]
FF − Fe	3.65 ± 0.10
HCNO + Fe	4.76 ± 0.15[c]
HCNO − Fe	2.11 ± 0.13
CO + Fe	2.55 ± 0.17[c]
CO − Fe	1.00 ± 0.06

[a]Enzyme specific activity is given as mean ± SD. These were obtained by separate determinations with livers from 6 rats in each diet group. Enzyme activity was determined as described in the text.

[b]See Table 1 for diet abbreviations.

[c]Value is significantly different ($p < 0.001$) as compared to that obtained with rats fed the corresponding iron-deficient diet.

Reduction of hepatic microsomal stearoyl-CoA desaturase activity may be due to a decrease in the content either of the enzyme complex or of its iron-containing components—cytochrome b_5 and terminal enzyme. Earlier studies have shown that the lack of stearoyl-CoA desaturation in Novikoff hepatoma microsomes is due to a complete absence of cytochrome b_5 and terminal desaturase (24). Additional studies on the quantitation of the components of the Δ9 desaturase enzyme are needed to understand the mechanism by which dietary iron regulates hepatic monoenoic acid synthesis.

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The Distribution of Cholesterol and Phospholipid Composition in Submitochondrial Membranes from Bovine Adrenal Cortex: Fundamental Studies of Steroidogenic Mitochondria

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ABSTRACT

The cholesterol contents and phospholipid compositions of mitochondria, microsomes and submitochondrial membranes from bovine adrenal cortex have been analyzed quantitatively. From our results, the following cholesterol contents were obtained: mitochondria, 6.2 ± 0.9 mol %; microsomes, 18.4 ± 2.8 mol %; mitochondrial inner membrane, 2.8 ± 0.6 mol %; and mitochondrial outer membrane, 8.3 ± 1.3 mol %. In addition, the phospholipid compositions of the mitochondrial inner and outer membranes were determined for the first time. Cardiolipin was found to be enriched in the inner membrane, whereas phosphatidylinositol was richer in the outer membrane. The general features of phospholipid compositions in the submitochondrial membranes resembled that of rat liver mitochondria. *Lipids* 18:xxx-xxx, 1983.

INTRODUCTION

The intramitochondrial cholesterol movement is believed to be the rate-limiting step in adrenocortical steroidogenesis regulated by ACTH (1). The topological studies of the enzyme system in bovine adrenocortical mitochondria revealed that cytochrome P-450$_{scc}$ and P-450$_{11\beta}$ were located in the matrix side of the inner membrane (2,3). Many experiments have been done to elucidate the catalytic function of cytochrome P-450$_{scc}$, showing that the availability of cholesterol to the cytochrome depends on membrane cholesterol (1). Moreover, the mitochondrial cholesterol was never utilized completely by the cytochrome P-450$_{scc}$-dependent enzyme system. Thus, steroidogenic and nonsteroidogenic pools of cholesterol were suggested in the mitochondria (4,5). In this context, it is important to study the submitochondrial localization of cholesterol molecules for understanding the action of ACTH. In addition, recent work by Farese's group indicates that in adrenal cortex phospholipid metabolism is closely related to the ACTH influence (6,7). Under these circumstances, we decided to investigate cholesterol distribution and phospholipid composition in mitochondria and submitochondrial membranes from bovine adrenal cortex.

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Abbreviations and trivial names: ACTH, adrenocorticotropic hormone; BSA, bovine serum albumin; Cholesterol, *cholest-5-en-3β-ol*; EDTA, *ethylenediaminetraacetic acid*; EGTA, ethyleneglycol-*bis*-(β-aminoethyl ether)-N,N'-tetraacetic acid; cytochrome P-450$_{11\beta}$, adrenocortical mitochondrial cytochrome P-450 which functions in the steroid 11β-hydroxylation reaction; cytochrome P-450$_{scc}$, adrenocortical mitochondrial cytochrome P-450 which functions in the cholesterol side-chain cleavage reaction.

METHODS AND MATERIALS

Preparation of Adrenocortical Mitochondria, Microsomes and Cytosol

Adrenocortical mitochondria were prepared according to Churchill et al. (3) with slight modifications. Bovine adrenals from animals which had just been slaughtered were collected and trimmed to remove fat and immediately placed into a beaker containing 10 mM Tris-HCl buffer (pH 7.6), and 0.32 M mannitol at 0 C, and brought back to the laboratory within 1.5 hr following the death of the animals. Subsequent manipulations were carried out at 5 C. The glands were bisected longitudinally and the adrenal medulla was scraped away and discarded. The cortical tissue obtained was immersed into the buffer and rinsed several times to remove blood. The suspension was homogenized with a teflon pestle. The homogenate was centrifuged at 750 × g for 10 min in a Sorvall centrifuge (model RC 2-B) with an SS-34 rotor. The pellets were discarded and the resulting supernatant fluid was centrifuged at 1940 × g for 3 min and then at 9750 × g for 10 min. The centrifuged tubes were rapidly inverted, allowing the fluffy layer to slough off the pellet. The pellets containing mitochondria were gently resuspended in buffer. The supernatant fluid was saved for further fractionation. The mitochondrial suspension was washed 3 times with buffer by centrifugation at 7710 × g for 10 min to remove possible microsomal contamination. The final pellet was resuspended in buffer at ca. 20 mg protein/ml.

The supernatant fluid obtained after the sedimentation of mitochondria was centrifuged at 12,100 × g for 15 min. The pellet was discarded and the supernatant fluid was then cen-

trifuged at 105,000 × g for 60 min to sediment the microsomes using a Beckman centrifuge (model L2-65B) with a type 65 rotor. The solution remaining after removal of the microsomes was called cytosol.

In some experiments, the washed mitochondria were further purified by centrifugation through a discontinuous 2-layer sucrose density gradient in a Beckman centrifuge with a type SW41 rotor: 39.0% (w/v) and 44.3% (w/v) of sucrose in 50 mM Tris-HCl (pH 7.4) buffer. After centrifugation at 70,000 × g for 2 hr, the mitochondrial fraction was collected from the interface between 2 layers of the sucrose solution. This mitochondrial fraction was washed with buffer and centrifuged at 7710 × g for 15 min in a Sorvall centrifuge.

Submitochondrial Membrane Preparation

The separation of the outer membrane and mitoplast fractions was based on the method of Greenawalt (8) with some modifications. The isolated mitochondria were washed with 10 mM Tris-HCl buffer (pH 7.6) containing 8.6% (w/v) sucrose, and 0.1 mM EDTA. To this washed mitochondrial suspension (20 mg/ml), an equal volume of hypertonic solution containing 10 mM Tris-HCl (0H 7.6), 21.4% (w/v) sucrose, 0.1 mM EDTA and BSA 1 mg/ml, was slowly added and the suspension was allowed to stand at least 20 min. About 5-10 ml of the mitochondrial suspension was loaded into a precooled French press cell (Carver Inc., Model B) and pressure was applied at 4800 psi. Pressed mitochondria were slowly extruded through the cell into a precooled test tube and then diluted by the solution containing 10 mM Tris-HCl (pH 7.6), 15% (w/v) sucrose, 0.1 mM EDTA and BSA 0.5 mg/ml, at a 1:1 ratio (v/v). The pressed mitochondrial suspension was centrifuged at 27,000 × g for 15 min in a Sorvall centrifuge to sediment the pellet. After washing, the pellet was identified to be mitoplast. The supernatant fluid after sedimentation of mitoplast was centrifuged at 105,000 × g for 1 hr by a Beckman centrifuge to collect the supernatant fraction. After further centrifugation of this fraction at 150,000 × g for 90 min, the outer membrane was obtained from the pellets, and the supernatant fluid was called soluble phase of mitochondria.

Inner membrane was prepared by means of controlled sonication of the mitoplast fraction as follows. The mitoplast sample was diluted to 1.5 mg/ml and sonicated for 7 min at 1-min intervals with a Bronwill-Biosonik III with low power (50%) in an ice bath. The sonicated mitoplasts were then centrifuged at 12,100 × g for 10 min to remove unbroken mitoplast. The supernatant fluid was further centrifuged at 59,000 × g for 1 hr to sediment the inner membrane from matrix. All the submitochondrial samples were kept in the 10 mM Tris-HCl buffer (pH 7.6) containing 15% (w/v) sucrose, 0.1 mM EDTA and BSA 0.5 mg/ml.

Determinations of Cholesterol and Phospholipid

The samples for lipid determination were adjusted to 0.45% (w/v) by NaCl and then extracted with methanol/chloroform at saline/methanol/chloroform (3:4:8, v/v/v) (9). Cholesterol contents were determined at 37 C by the cholesterol oxidase (*Nocardia*) method according to Deacon and Dawson (10), with some modifications. The reaction mixture contained 100 mM sodium phosphate buffer (pH 6.9), 1.5% Triton X-100, 6 mM sodium cholate, 7.5 mM phenol, 0.8 mM 4-aminoantipyrine, 0.2 units of cholesterol oxidase, and 5 units of peroxidase. Recrystallized cholesterol was used as standard. Phospholipid contents were determined according to the method as described by Hallermayer and Neupert (11) with L-α-dipalmitoylphosphatidylcholine as standard. The concentration of cholesterol in membrane was expressed as mol %:

$$\frac{\text{moles of cholesterol}}{\text{moles of cholesterol + moles of phospholipid}} \times 100$$

The quantitative determination of phospholipid composition after 2-dimensional thin layer chromatography were performed as described previously (11). Briefly, the lipid extracts were taken in chloroform/methanol (2:1, v/v) and applied to a silica gel 60 thin layer plate. The solvent systems were chloroform/methanol/28% aqueous ammonia (65:35:5, v/v/v) in the first dimension and chloroform/acetone/methanol/acetic acid/water (56:20:10:10:4, v/v/v/v/v) in the second dimension. The spots detected by iodine vapor were scraped out for phosphorus assay.

Assays of Marker Enzyme Activities

The enzyme activities of lysosomal acid phosphatase (12), endoplasmic reticulum glucose-6-phosphatase (13), and plasma membrane 5′-nucleotidase (14) were determined as described previously. Glucose-6-phosphate dehydrogenase activity, a marker for cytosol, was determined by monitoring NADPH formation (ϵ = 6.22 cm^{-1} mM^{-1}) at 340 nm (15). Succinate dehydrogenase activity, a marker for mitochondria, was assayed according to the method of King (16) with minor modifications: 100 mM sodium phosphate buffer (pH 7.8), 0.1% BSA, 40 mM succinate, 150 μ M phenazine methosulfate and

50 mM 2,6-dichlorophenolindophenol (ϵ = 19.1 cm^{-1} mM^{-1}, at 600 nm). Monoamine oxidase activity, a marker for the mitochondrial outer membrane, was assayed at 37 C according to the method of Weissbach et al. (17) with kynuramine as substrate. Cytochrome oxidase activity, a marker for the mitochondrial inner membrane, was determined at 37 C in 100 mM sodium phosphate buffer (pH 7.0), 0.03% Lubrol WX, and 45 μ M reduced cytochrome c (ϵ = 19.6 cm^{-1} mM^{-1}, at 550 nm) (18). The assay of malate dehydrogenase activity, a marker for the mitochondrial matrix, was similar to the reported method (19): 50 mM sodium phosphate buffer (pH 7.8), 0.4 mM NAD^+, 140 μM 2,6-dichlorophenolindophenol, 0.3% Triton X-100, 77 μ M phenazine methosulfate and 7 mM malate.

Determination of Cytochrome P-450 and Protein Content

Cytochrome P-450 was estimated from the difference spectra between the sodium dithionite-reduced and the reduced and carbon monoxide treated samples. An extinction coefficient of 91 cm^{-1} mM^{-1} for the difference between 448 and 490 nm was used (20). Protein was determined by the biuret method (21) in the presence of 0.33% sodium deoxycholate. Spectrophotometric determinations were carried out with a Cary 118 spectrophotometer with an end-on photomultiplier.

MATERIALS

BSA (essentially fatty acid free), cholesterol, 2,6-dichlorophenolindophenol, EGTA, glucose-6-phosphate, kynuramine dihydrobromide, L-α-dipalmitoylphosphatidylcholine, Lubrol WX, mannitol, $NADP^+$, peroxidase (type VI horseradish), phenazine methosulfate, rotenone, triethanolamine, and Triton X-100 were purchased from Sigma (St. Louis, MO). EDTA was purchased from Aldrich (Milwaukee, WI). Cholesterol oxidase (*Nocardia*) was purchased from either Sigma or Calbiochem (La Jolla, CA). Other reagents were obtained from the best commercial sources.

RESULTS

Purity Criteria of the Mitochondrial Samples

As shown in Figure 1, the mitochondrial samples prepared by the conventional method are rich in succinate dehydrogenase activity and very low in 5′-nucleotidase and glucose-6-phosphate dehydrogenase activities, indicating that plasma membrane and cytosolic components are not significantly contaminated in mitochondrial samples. However, a relatively small amount of glucose-6-phosphatase and acid phosphatase activities were found in the mitochondrial fraction. The contaminating enzyme activities did not decrease on further purification using discontinuous sucrose density gradient centrifugation. This fact suggests that the mitochondrial sample contains some endoplasmic reticulum and lysosomal fragments bound to the mitochondrial membranes. Alternatively, the densities of the contaminants might be similar to those of mitochondria, so that they cannot be removed by gradient centrifugation. In fact, there is evidence that rough endoplasmic reticulum are physically associated with mitochondria (22,23). Moreover, Taguchi et al. (24) reported that in rat adrenal cortex both lysosomes and mitochondria showed unimodal distribution profiles of marker enzymes with isopycnic density of 1.165. We estimated a density of bovine adrenocortical mitochondria to be ca. 1.156. At present, we have not carried out density perturbation procedures to separate lysosomes from mitochondria. Because Ca^{2+} and detergents may cause redistribution of cholesterol and phospholipid in membranes, it was

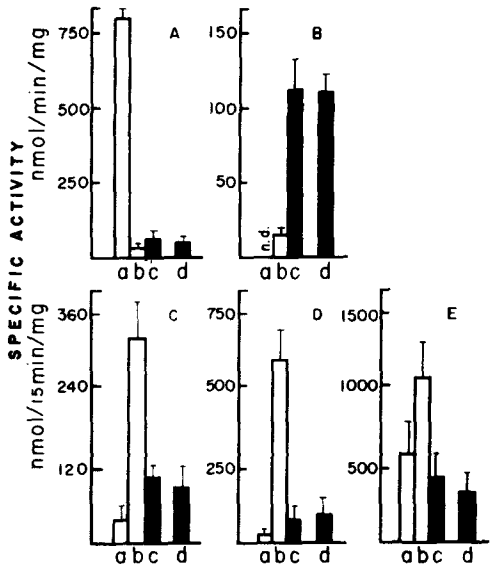

Fig. 1. The distribution of marker enzyme activities glucose-6-phosphate dehydrogenase (A), succinate dehydrogenase (B), glucose-6-phosphatase (C), 5′-nucleotidase (D), and acid phosphatase (E), in the fractions of cytosol (a), microsomes (b), mitochondria (c), and mitochondria after discontinuous sucrose density gradient centrifugation (d). The enzyme activity assays were performed as described under Methods and Materials. "n.d." refers to "not detectable." Results are expressed as means ± SD from 3-8 independent preparations.

not suitable to use either Triton WR-1339 (25) or calcium chloride (26) for separation of mitochondria from lysosomes.

Cholesterol Contents and Phospholipid Compositions in Bovine Adrenocortical Mitochondria and Microsomes

The cholesterol content of each fraction has been expressed as cholesterol mol % relative to phospholipid unless specified otherwise. The extraction efficiency of cholesterol was more than 96%. As shown in Table 1, the mitochondrial cholesterol content was 6.2 ± 0.9 mol % and further purification by gradient centrifugation did not significantly change this value. By comparison, the cholesterol content of the microsomal fraction was found to be relatively high, 18.4 ± 2.8 mol %. As shown in Figure 1, microsomal fractions are highly contaminated with plasma membranes. According to Colbeau et al. (27), the cholesterol content in rate liver is highest in plasma membrane (ca. 43 mol %), intermediate in smooth endoplasmic reticulum (ca. 19 mol %) and relatively low in rough endoplasmic reticulum (ca. 6 mol %). In our mitochondrial preparation, there was negligible contamination by plasma membrane. The fact that a residual amount of glucose-6-phosphatase activity existed in the mitochondrial fraction might be due to the mitochondria associated rough endoplasmic reticulum. Furthermore, the cholesterol/cytochrome P-450 molar ratio in the mitochondrial fraction was found to be ca. 29. This value is similar to the literature value of 30 reported by Boyd et al. (28). The amount of lysosomal cholesterol in our mitochondrial samples is unknown due to the difficulty in purifying lysosomes from either mitochondrial or plasma membrane fractions. However, the ratio of mitochondria to lysosomes in the mitochondrial preparation is higher than 3:1 in terms of their relative enzyme activities based on our calculation. Therefore, the cholesterol concentration of mitochondria reported here is reasonably close to that for pure mitochondria.

The phospholipid compositions of mitochondrial and microsomal fractions were found to be very different. In the mitochondrial fraction, the 2 main phospholipids, namely, phosphatidylcholine and phosphatidylethanolamine, appeared to be in equal amounts. The mitochondria-specific phospholipid cardiolipin was found to be 13% of total phospholipids. These results were entirely consistent with our previous finding (29). Moreover, our results on the phospholipid composition from the bovine adrenocortical mitochondria together with the data reported from guinea pig and cat adrenal mitochondria (30), suggest that the adrenal mitochondria do not contain unusually high amounts of phosphatidylethanolamine. This is inconsistent with that from previous reports (31,32). By comparison, the microsomal fraction from bovine adrenal cortex was rich in phosphatidylcholine and phosphatidylinositol but poor in cardiolipin. Furthermore, the phospholipid composition of bovine adrenocortical microsomes was close to that of bovine liver microsomes (33).

Separation of the Mitochondrial Outer and Inner Membrane from Bovine Adrenal Cortex

Although the use of digitonin is suggested to be preferable over the hypotonic swelling method for the preparation of purer inner membrane fractions (27,34), some inner mem-

TABLE 1

Cholesterol Content and Phospholipid Composition of Bovine Adrenocortical Mitochondria and Microsomes

	Mitochondria	Microsomes
Cholesterol	6.2 ± 0.9 mol % (n=10)	18.4 ± 2.8 mol % (n=4)
Phospholipid composition		
Cardiolipin	12.5 ± 1.1 %[a]	1.9 ± 1.8%
Phosphatidylcholine	39.7 ± 2.6	50.9 ± 3.4
Phosphatidylethanolamine	34.3 ± 2.5	26.9 ± 1.5
Phosphatidylinositol	6.0 ± 0.2	11.9 ± 1.4
Phosphatidylserine	3.5 ± 1.3	6.9 ± 2.1
Other	4.0 ± 2.6 (n=3)	1.5 ± 1.7 (n=3)

The determination of lipids were performed as described under Methods and Materials. Data are expressed as means ± SD, (n) is represented as the number of independent preparation.

[a]Values of phospholipid composition are expressed as percentage of total phospholipid phosphorus.

brane-bound cholesterol may be removed by the digitonin treatment. For our purposes, the French press method was used for the dissection of the outer membrane from mitochondria. In our preliminary experiments, the pressure applied to release outer membrane from mitochondria was examined from 1600 psi to 5000 psi according to suggested pressure for the separation of outer membrane from rat liver (8) and yeast (35) mitochondria. Unlike rat liver mitochondria (8), adrenocortical mitoplast prepared at 1600 psi and 10 mg protein/ml contained a large amount of the monoamine oxidase activity. Increase in the protein concentration to 30 mg/ml did not release outer membrane any further. However, the monoamine oxidase activity significantly decreased in the mitoplast at a high pressure of 4800 psi. The decrease is not due to inactivation of monoamine oxidase by pressure. In contrast, inner membrane marker enzyme activities were retained in the mitoplast fraction. Therefore, we used the French press at 4800 psi together with differential centrifugation to separate the outer membrane from mitoplast.

Figure 2 shows typical De Duve's plots of the partition of marker enzymes by submitochondrial fractionation. It is clear from this figure that the mitoplast sample is rich in cytochrome P-450, cytochorme oxidase and malate dehydrogenase but poor in monoamine oxidase. The so-called mixed membrane fractions were found to be composed of apparent outer and inner membrane markers and were not studied further for lipid analysis. 150,000 x g pellets appeared to contain the outer membrane marker as the major component, judged from the relatively high content of monoamine oxidase activity and low content of cytochrome oxidase acitivity. In agreement with Greenawalt (8), there were significant activities of monoamine oxidase and malate dehydrogenase in the soluble phase (150,000 x g supernatant fluid). A high monoamine oxidase activity in supernatant fluid might be due to incomplete sedimentation of outer membrane vesicles. In contrast, there was a small amount of cytochome P-450, and cytochrome oxidase activity could be detected in the soluble phase. We found no appreciable cytochrome P-420 (a denatured form of cytochrome P-450) during the entire process of preparation (Fig. 3), and total recoveries of cytochrome P-450 and malate dehydrogenase activity were ca. 95%. The recoveries of protein content and monoamine oxidase were ca. 90%. However, the recovery of cytochrome oxidase was relatively low at ca. 75%.

The matrix was separated from the inner membrane by sonicating the mitoplast fraction. Under our experimental conditions, ca. 55% of

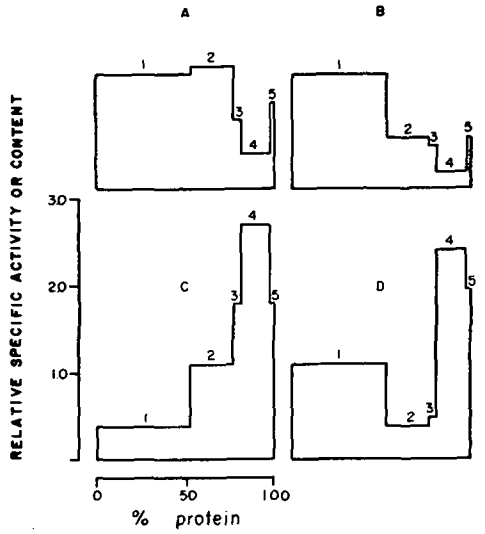

FIG. 2. De Duve's plot of the partition of marker enzymes after fractionation of French pressed mitochondria: cytochrome P-450 content (A), cytochrome oxidase activity (B), monoamine oxidase activity (C), and malate dehydrogenase activity (D); mitoplast fractions (1), mixed membrane fractions (inner + outer) (2), outer membrane fractions (3), soluble phase fractions (4), and washing pools (5). Relative specific activities or content are expressed as follows: percent of total specific activities or contents detected in the fraction/percent of total protein content recovered in the fractions. 100% was assigned as total recovery of marker enzyme activity or content after fractionation.

protein and 79% of malate dehydrogenase activity were released from inner membrane without denaturing cytochrome P-450 (Fig. 3). In a typical experiment, cytochrome P-450 content was found to be enriched from 1.8 nmol/mg in mitoplast fraction to 3.4 nmol/mg in inner membrane fraction.

Distribution of Cholesterol and Phospholipid Composition in Submitochondrial Membranes

As shown in Table 2, after removal of the outer membrane, the cholesterol and phosphatidylinositol contents decreased in the mitoplast fraction; cardiolipin, in contrast, was found to be enriched significantly in this fraction rather than in the intact mitochondria (Table 1). The cholesterol content and phospholipid composition of the inner membrane fraction were about the same as those in mitoplast, suggesting that most of these lipids in mitoplast were concentrated in the membrane. By comparison, the outer membrane contained a relatively high content of cholesterol and phosphatidylinositol but a low content of cardiolipin. The general feature of the phospholipid composition of the

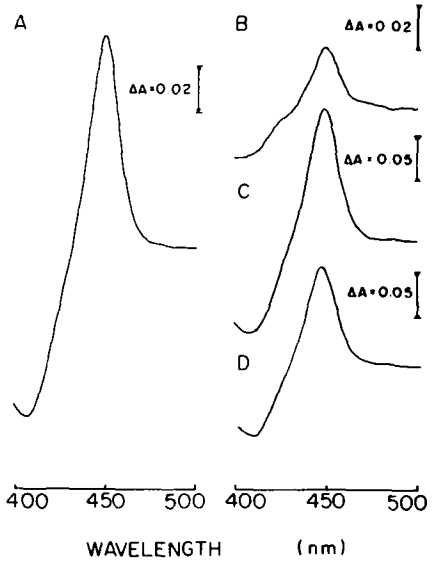

FIG. 3. Carbon monoxide difference spectra of cytochrome P-450 from bovine adrenal cortex mitochondria (0.95 mg/ml) (A), outer membrane (0.28 mg/ml) (B), mitoplast (1.02 mg/ml) (C), and inner membrane (0.37 mg/ml) (D).

outer and inner membranes from bovine adrenocortical mitochondria resembled that of rat liver submitochondrial membranes (27). Previously, Yago et al. (36) reported that the cholesterol content in the mitochondrial outer and inner membranes from hog adrenal cortex was about equal in terms of their cholesterol-phospholipid molar ratio. We prepared the inner membrane fraction from the mitoplast which was largely removed of the outer membrane, whereas they prepared the inner membrane fraction directly from the intact mitochondria. It is reasonable to assume from our present results that their outer and inner membrane samples contaminated each other to some extent.

Recently, Hall et al. (31) reported that the isolated cytochrome P-450 contained caridiolipin at ca. 4% of the total cytochrome P-450-bound phospholipids. In our present work, we observed that in the inner membrane ca. 18% of total phospholipids was cardiolipin. Cardiolipin is known to be located predominantly in the matrix side of the inner membrane (37,38), which is the same side as cytochrome P-450 (2,3). These facts suggest that the cardiolipin molecules might be removed from cytochrome P-450 during the purification process if the cardiolipin molecules are homogenously distributed in the matrix side of the inner membrane. Alternatively, cardiolipin might be poor as annular phospholipid of cytochrome P-450 if the cardiolipin molecules are heterogenously distributed in this monolayer.

DISCUSSION

It was previously implicated in the adrenal cotex that upon the action of ACTH cholesteryl ester hydrolase activity of lipid droplets is stimulated, supplying unesterified cholesterol to the mitochondrial steroidogenic reaction (39). Mahaffee et al. (40) and Crivello and Jefcoate (41) demonstrated that cholesterol, but not cholesteryl ester, accumulated in mitochondria after administration of ACTH and aminoglutethimide (an inhibitor for cholesterol side-chain cleavage reaction). The accumulated cholesterol can then be utilized by the mitochondrial steroidogenic system after the removal of aminoglutethimide. These facts suggest that the primary substrate for the steroidogenic enzyme is unesterified cholesterol in the membrane. In this context, the determination of the

TABLE 2
Cholesterol Content and Phospholipid Composition of Submitochondrial Membranes from Bovine Adrenal Cortex

	Mitoplast	Inner membrane	Outer membrane
Cholesterol	3.8 ± 1.2 (n=6)	2.8 ± 0.6 (n=6)	8.3 ± 1.3 (n=6)
Phospholipid composition			
Cardiolipin	17.4 ± 0.8%	18.1 ± 0.8%	7.9 ± 3.8%
Phosphatidylcholine	36.1 ± 2.4	37.4 ± 5.7	43.2 ± 6.2
Phosphatidylethanolamine	37.8 ± 2.5	37.3 ± 1.8	30.2 ± 4.2
Phosphatidylinositol	3.6 ± 1.5	3.1 ± 2.0	11.1 ± 1.0
Phosphatidylserine	3.0 ± 2.5	0.6 ± 0.5	5.1 ± 2.0
Other	2.1 ± 1.3 (n=3)	3.5 ± 1.8 (n=3)	2.5 ± 2.4 (n=3)

Data are expressed as described under Table 1.

endogenous cholesterol content in cellular and submitochondrial fractions has fundamental importance for understanding the complex phenomenon of ACTH-mediated transference of cholesterol from lipid droplets to mitochondria. In this study, we determined the unesterified cholesterol content in bovine adrenocortical mitochondria to be 6.2 mol %. We estimated cholesteryl ester content to be ca. 0.2 mol %.

The cholesterol concentrations of mitochondria from bovine have been previously reported as 2, 11, 6 and 23 mol % for heart, kidney, liver and adrenal medulla, respectively, by Fleischer et al. (42) and Blaschko et al. (43). These mitochondrial preparations were highly purified by differential centrifugation (42) or by sucrose density gradient centrifugation (43). We found that the cholesterol content in bovine adrenocortical mitochondria is close to bovine liver mitochondria. In addition, the mitochondrial inner membrane from bovine adrenal cortex is poorer in cholesterol than that of the outer membrane, in agreement with the feature of general mammalian mitochondria (27,44).

Based on the liposomal study (45), cholesterol below 30 mol % in phosphatidylcholine vesicles distributed equally in both inner and outer monolayers, whereas above 30 mol % cholesterol the distribution in 2 monolayers becomes uneven with predominant localization of cholesterol in the inside monolayers. From the present results, the cholesterol content of the inner membrane is ca. 3 mol %. Therefore, cholesterol molecules of the mitochondrial inner membrane bilayer should distribute equally in both the outer and inner monolayers. We have previously demonstrated that both cytochrome P-450$_{scc}$ and P-450$_{11\beta}$ are located on the matrix surface of the inner membrane (2,3). In addition, we found that the cholesterol-cytochrome P-450 molar ratio in the inner membrane was 10-16. We can then calculate that ca. 1.5 mol % or 5-8 molecules cholesterol per molecule of cytochrome P-450 are present in the matrix side of the inner membrane. Previously, Simpson et al. (4) demonstrated that only a portion of cholesterol in rat adrenal mitochondria could be depleted by isocitrate-supported steroidogenic reaction. We confirmed this finding in bovine adrenocortical mitochondria with malate-supported steroidogenic reaction (unpublished results). We suggest that the size of the available pool of cholesterol for steroidogenesis must be below 3 mol %.

The lateral diffusion of cholesterol molecule is fast, ca. 10^{-6} cm^2/sec in a diluted monolayer (46) and its transmembrane movement is slow with a half-time more than 90 min at 37 C (47), although some exceptions are known (48, 49). If the flip-flop rate of cholesterol molecule in the inner membrane of adrenal mitochondria is indeed slow, readily available cholesterol molecules must be localized in the matrix side of the inner membrane. We speculate from our results, together with previous reports (1), that the action of ACTH makes cholesterol available to cytochrome P-450$_{scc}$ from either the outer membrane and/or the outer monolayer of the inner membrane of adrenal mitochondria.

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Occurrence of Wax Esters in the Tissues of the Orange Roughy (*Hoplostethus atlanticus*)

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ABSTRACT

The skin, skeleton and a fat-filled swim bladder of the orange roughy (*Hoplostethus atlanticus*) each contained greater than 20% lipid by wet weight which was almost entirely wax esters. These had carbon numbers of 34-40 consistent with the major fatty acid being 18:1 and the major fatty alcohols being 16:0, 18:1, 20:1 and 22:1. In contrast, the liver and the roe contained appreciable quantities of glycerolipids with 18:1 and 22:6 as the major fatty acids.
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Orange roughy (*Hoplostethus atlanticus*) (1) is a deep sea fish that has recently become of commercial importance both as a food source and as a source of an oil containing very high concentrations of wax esters (2). In this report we describe the lipid composition of selected orange roughy tissues.

METHODS

Fish were caught at a depth of ca. 1000 m, frozen directly on board ship and stored at -20 C until thawed for dissection and lipid analysis. Lipids were extracted from the tissues using the method of Bligh and Dyer (3) and stored as chloroform solutions.

A preliminary qualitative analysis was performed using thin layer chromatography (TLC) with plates of Silica Gel G developed in hexane/diethyl ether/acetic acid (80:20:1) or chloroform/methanol/water (65:35:4).

Wax esters were estimated following gas liquid chromatography (GLC) of the total lipid in a glass column containing 3% OV-1, (Applied Sciences, State College, PA) with the temperature programmed from 120 C to 355 C at 10 C/min. The peaks with carbon numbers of 32-46 were summed to give the total content of wax esters. A correction for the presence of phospholipids was made for the lipid samples which contained greater than 2% phospholipid determined as described below.

Total lipid cholesterol was measured colorimetrically (4). A preliminary separation using TLC was performed for those samples which contained high concentrations of wax ester. Phospholipid was measured as lipid phosphorus (5) and converted to μg phosphatidylcholine equivalent by using bovine phosphatidylcholine (Sigma Chemical Co., St. Louis, MO) as a standard. Pooled samples of liver and roe lipids were also separated into lipid classes by chromatography on Florisil (6).

For analysis of the fatty acid and fatty alcohol compositions, lipid samples were transesterified in 6% HCl in methanol at 80 C for 20 min. Chromatography of the fatty acid methyl esters and fatty alcohols was performed using a glass column of 10% SP-2300 (Supelco, Bellefonte, PA) operated either isothermally at 195 C, or programmed from 175 C to 230 C at 3 C/min. With either condition, the fatty acid methyl esters and fatty alcohols were sufficiently well resolved to allow analysis of both on the same chromatogram. Peaks were identified using a combination of methods. For selected samples, the fatty alcohols were separated from the fatty acid methyl esters using TLC and then chromatographed separately before and after hydrogenation. Cochromatography with standard fatty acids or alcohols and semilogarithmic plots of the retention times against carbon numbers were also used.

RESULTS AND DISCUSSION

The fish examined had a mean wet weight of 1.1 kg and ranged from 0.5 to 1.9 kg. The skin, the skeleton, and an organ located anatomically in the position of the swin bladder of other teleost fish all had lipid contents of ca. 20% or greater (on a wet weight basis) with the swim bladder containing over 60% lipid (Table 1). The lipid content of the other tissues examined was lower, ranging from 2 to 5% wet weight for the muscle, liver, testes and roe.

The tissues fell into 2 groups depending on their lipid compositions. Those with the greatest lipid content had over 90% of their lipid as wax esters, whereas in other tissues the wax content was 20% or less of the total lipid (Table 1). Of the tissues examined, the muscle was the only exception to this pattern where,

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TABLE 1

Lipid Content and Composition of Orange Roughy (*H. atlanticus*) Tissues

	Total lipid	Wax ester	Cholesterol	Phospholipid
	mg/g wet weight		mg/g lipid	
Liver	55 ± 24 (6)[a]	185[b]	46[b]	230[b]
Swim bladder	620 ± 120 (6)	983	5.8	2.7
Muscle	39 ± 10 (4)	931	22	48
Skin	290 ± 57 (6)	992	12	7.0
Roe	35 ± 16 (6)	110	40	187
Testes	19 ± 10 (3)	254	37	199
Skeleton	212[d]	978	--[c]	--[c]

[a]Mean ± SD for number of observations in parentheses.
[b]Mean of triplicate estimations on pooled samples from 2-6 fish.
[c]Not determined.
[d]From ref. 2.

despite a lower total lipid content, the lipid was mainly wax esters. The composition of either the unhydrolyzed wax esters or the fatty acids and fatty alcohols derived from the wax esters was similar from tissue to tissue and from fish to fish (Tables 2 and 3), and also similar to previously published analyses of the bulk orange roughy oil (2). The predominant wax esters had carbon numbers of 34, 36, 38 and 40 consistent with the major fatty acids being 18:1 and 20:1, and the major alcohols containing 16, 18, 20 and 22 carbon atoms (Table 2).

TABLE 2

Carbon Number Analysis of Intact Waxes from Orange Roughy (*H. atlanticus*) Tissue Lipids

	Weight%			
Carbon number	Swim bladder	Muscle	Skin	Skeleton
32	5	4	5	4
34	17	15	17	17
36	21	20	20	22
38	22	22	23	24
40	18	24	19	19
42	9	8	9	10
44	3	3	2	2

The lipids of the liver, roe and testes differed from those of the other tissues in that they contained appreciable concentrations of triacylglycerols, nonesterified fatty acids, cholesterol and phospholipids. Separation of a pooled liver lipid sample by chromatography on Florisil (6) showed the triacylglycerol and nonesterified fatty acid contents to be, respectively, 6% and 53% of the total lipids. A similar separation of the pooled roe lipids gave figures of 36% and 10% (Table 4). In each case, ca. 20% of the total lipid was recovered as phospholipid reflecting the results of the lipid P determination on the total lipid (Table 1).

Nonesterified fatty acids do not occur in nature in high concentrations in normal tissues. Therefore, the high levels found in the liver particularly, and to a lesser extent in the roe, suggest that they may result from lipolysis during storage and thawing. Hydrolysis of lipids during storage of fish tissues has been reported previously (7,8). The marked similarity of the fatty acid compositions of the nonesterified fatty acids and the triacylglycerols from the liver lipids and roe (Table 4) is consistent with this.

The role of the wax esters in *Hoplostethus atlanticus* is not well understood at present, but the finding of large amounts of wax in a tissue equivalent to the swim bladder suggests that one function might be to provide buoyancy for the fish. The fish lives at depths of 1000 m or more where the low dissolved gas concentrations and the extreme pressures would make lipid an ideal flotation agent. The liver oils of elasmobranchs are also thought to assist in flotation for this group of fish (9-11). In some species, these liver oils contain appreciable concentrations of wax esters and even, of the hydrocarbon, squalene. The high wax content of the boney tissues (Table 1, also ref. 2) will also aid to provide flotation. Lee et al. (9) have discussed the role of skeletal lipids with respect to buoyancy. Interestingly, 2 deep sea species they examined, *Coryphaenoides acrolepis* and *Antimora rostrata*, both have lipid-filled swim bladders and only low concentrations of bone lipid. The castor oil fish, *Ruvettus pretiosus*, on the other hand, has high concentrations of

TABLE 3

Fatty Acid and Fatty Alcohol Composition of Total Lipids of Orange Roughy (*H. atlanticus*) Tissues

		Liver	Swim bladder	Skin	Muscle	Roe
Fatty acid methyl esters	14:0	1.6 ± 0.3[a]	1.7 ± 0.8	1.3 ± 0.6	1.4 ± 0.5	1.7 ± 0.5
	14:1	0.4 ± 0.1	0.9 ± 0.6	0.5 ± 0.2	0.5 ± 0.3	0.4 ± 0.3
	16:0	13.2 ± 0.5	2.0 ± 0.6	2.6 ± 0.5	3.6 ± 0.6	14.6 ± 1.4
	16:1	7.3 ± 2.8	13.0 ± 1.8	13.2 ± 0.6	11.0 ± 0.4	5.5 ± 1.2
	18:0	2.3 ± 0.4	0.8 ± 0.2	0.8 ± 0.3	1.3 ± 0.2	2.4 ± 3.2
	18:1	31.1 ± 3.3	54.0 ± 3.0	57.4 ± 5.3	51.7 ± 5.2	22.0 ± 0.2
	20:1	9.2 ± 1.9	17.1 ± 3.5	14.5 ± 2.6	18.4 ± 3.7	5.2 ± 1.5
	20:4	1.6 ± 0.5	--[c]	—	—	2.5 ± 0.7
	22:5	3.4 ± 1.5	—	—	—	1.0 ± 0.5
	22:1	4.6 ± 0.9	7.9 ± 2.1	6.2 ± 1.7	7.8 ± 2.1	7.2 ± 0.3
	22:6	16.9 ± 5.7	—	—	—	26.5 ± 5.2
	24:1	—	2.0 ± 1.0	3.3 ± 0.8	4.2 ± 1.8	—
	Other[b]	7.8 ± 1.6	—	—	—	9.5 ± 1.3
Fatty alcohols	14:0	—	1.7 ± 0.5	1.9 ± 0.4	11.9 ± 0.3	—
	16:0	—	24.2 ± 3.9	23.3 ± 4.1	20.6 ± 5.8	—
	16:1	—	3.6 ± 0.9	3.0 ± 0.9	3.3 ± 0.5	—
	18:0	—	7.3 ± 1.1	8.4 ± 1.5	8.8 ± 1.6	—
	18:1	—	17.4 ± 4.3	16.4 ± 4.4	16.6 ± 4.7	—
	20:1	—	26.7 ± 6.3	26.5 ± 7.6	26.2 ± 10.3	—
	22:1	—	16.0 ± 3.3	16.8 ± 3.8	19.3 ± 3.7	—
	24:1	—	3.0 ± 1.0	3.8 ± 1.3	3.3 ± 1.0	—

[a]All values are weight percentage of total fatty acids or alcohols and a mean ± SD of individual analyses on tissues from 4 fish for roe and 6 fish for other tissues.
[b]Include 15:0, 15:1, 17:0, 17:1, 18:2, 20:2, 20:3, 22:2, 22:3, 22:5.
[c]Not detected.

TABLE 4

Fatty Acid Composition of Lipid Classes Obtained Following Florisil Chromatography of Orange Roughy (*H. atlanticus*) Roe and Liver Lipids

	Roe			Liver		
Acid	Triacyl-glycerols	Free fatty acids	Phospho-lipids	Triacyl-glycerols	Free fatty acids	Phospho-lipids
14:0	1.9	2.6	1.0	2.6	1.7	2.2
16:0	14.8	19.8	9.2	13.6	12.6	14.8
16:1	9.8	11.4	2.5	11.4	9.8	6.5
18:0	1.9	2.4	4.8	2.1	1.4	4.4
18:1	31.9	32.5	14.1	36.7	36.9	18.3
18:2	1.1	1.7	0.4	0.9	1.5	1.6
20:1	8.9	5.5	5.5	13.0	9.6	6.6
20:4	5.4	7.1	5.4	1.2	3.0	4.2
22:1	2.2	0.9	6.4	8.0	5.9	2.6
22:6	16.3	10.0	40.7	3.0	11.7	26.7
Others[a]	6.1	6.0	9.9	6.2	5.0	12.0
% of total recovered from Florisil chromatography	31	10	36	6	53	29

[a]See Table 2.

skin and bone lipid (mainly wax esters) and no swim bladder (13).

It is possible that the wax esters in *H. atlanticus* might also function as an energy store. In preliminary experiments, the swim bladder tissue from frozen fish was homogenized and microsomes were prepared by differential centrifugation. These microsomes contained both acyl-CoA synthetase activity and acyl-CoA: fatty alcohol acyltransferase activity, indicating that this tissue is metabolically active (M.R. Grigor, unpublished observations). To date, it has not been possible to obtain serum samples from the orange roughy to see whether the serum lipoproteins also transport wax esters.

At this stage, it is not known whether the alcohol component of the *H. atlanticus* wax esters is derived from the diet or synthesized de novo. Wax esters figure prominently in the marine food chain being synthesized by the calanoid zooplankton where they appear to act as an energy store (14-17). Most animals consuming these calanoid copepods convert the alcohols to fatty acids and store triacylglycerols (18), whereas others (e.g., the myctophids) retain the ability to store wax esters in their tissues (19). However, Kayama and Nevenzel (20) have shown that the myctophids have the ability to synthesize fatty alcohols de novo from acetate, indicating that the alcohols need not be of dietary origin. The alkyl-chain length distribution we report for the *H. atlanticus* waxes differs from that of the myctophids (19) and is much more similar to those of the copepods (14,15). Although this would not necessarily imply that the *H. atlanticus* alcohols are derived from copepods which might form part of the diet, this observation would not be inconsistent with such an origin.

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Variation of Cyclopropenoid Fatty Acids in Cottonseed Lipids[1]

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ABSTRACT

The proportions of the cyclopropenoid fatty acids (CPA) esters, malvalate and sterculate, varied little in lipids from individual cottonseeds. Coefficients of variation were 10% and 20% for seeds from a lock and 13 varieities, respectively. Within the seed, variations in CPA concentrations were very large. Cyclopropenoid fatty acid concentration in the lipids decreased from 28% in the root tip to 2% in the top of the axis, and to 0.02% in the portion of the cotyledons nearest to the hull. The axial portion was only ca. 5% of the kernel, yet it contained 75% of the CPA. Distribution of dihydrosterculic acid, the precursor of CPA, was similar to that of CPA. High concentrations of CPA were found in immature seeds, root tip and radicle of germinated seeds, and root tips of cotton plants.
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Although they probably pose little potential danger to humans because proper processing tends to destroy them (1), it is well known that cyclopropenoid fatty acids (CPA), present in cottonseed as malvalate (18:CE) and sterculate (19:CE) esters, cause adverse physiological effects when ingested (2-4). These effects and their underlying causes have recently been reviewed (5). Carter et al. (6) identified CPA as one of the factors affecting the value of cottonseeds and recommended lowering or eliminating them by selective breeding. They stressed the need for good quantitation methods, a data base for current cultivars, and an understanding of any morphological or physiological factors affecting composition as prerequisites for attempts to lower or eliminate CPA content.

The large samples required for classical analyses of CPA in oils limited studies of their variabilities in cottonseed to pooled samples (7,8). Development of new reliable gas chromatographic (GC) methods for CPA quantitation (9) make possible studies on variations among individual seeds and tissues within a seed. This paper reports the results of such studies.

MATERIALS AND METHODS

Cottonseed

Variations among seeds from a lock, the locks of a boll, and bolls from a plant were studied with seed from mature bolls of a glanded variety. Immature seeds were from bolls on the same plant that had not opened before the first freeze. Variations among varieties were evaluated with sound mature seed left over from other studies (10). Seeds were acid delinted, if necessary, and dehulled by hand. Kernels were dissected by slicing off most of the cotyledon and then extracting the axis with a dissecting needle or by soaking them in warm water for a few minutes and separating the swollen cotyledons from the axis with a dissecting needle. The latter technique gave cleaner separations without detectable changes in lipid composition, but involved redrying of the separated parts. Root tips were excised as carefully as possible, but contained varying amounts of adjacent tissue. Seeds were germinated by rolling them in moistened paper towels which were placed in plastic bags and kept in the dark at room temperature for several days.

Extraction

Seeds or their different tissues were ground with a glass rod in a test tube or conical centrifuge tube under 5 vol of ether. After centrifugation, the extract was transferred to another container and the residual meal similarly re-extracted twice. For 12 kernels, oil content was 33 ± 3%. Very small samples were extracted once with 50 μl of ether. For wet tissue, an equal weight of a drying agent, such as 4A molecular sieves, was added during extraction. The combined ether extracts were evaporated to about one-third their original volume, giving about a 5% solution of lipids in ether.

Esterification

The lipids were converted to methyl esters by the method of Metcalf (11). Typically, 5 μl

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of tetramethylammonium hydroxide (TMAH; 20% in methanol, Aldrich Chem. Co., Milwaukee, WI) was added to 0.1 ml of ether extract containing 5 mg of oil. The mixture was agitated on a vibrating mixer for 2 min and allowed to stand at room temperature for 5 min. After addition of 0.05 ml of decane to facilitate separation, the mixture was centrifuged. Occasionally the ether-decane layer was cloudy or gave extraneous GC peaks. In these cases, the solution of esters was mixed with neutral alumina (5-10 mg) for 1-2 min. If esters were stored, the solution was transferred to a screw-capped vial, 50 μg of propyl gallate added, and the samples placed under nitrogen at 2 C.

Gas Chromatography

The methyl esters were analyzed by GC with 10- or 50-m fused silica columns coated with OV-101. Concentrations were calculated by peak-area normalization. In general, analyses were terminated as soon as arachidate (20:0) emerged and the column temperature raised to 250 C to elute small amounts of higher esters.

RESULTS AND DISCUSSION

The methods used for extraction of the lipids and their conversion to methyl esters were not critical if acidic conditions were avoided. Preliminary comparisons of the fatty acid composition of lipids extracted with hexane, ether, or chloroform/methanol (95:5) showed no major differences. Ether was chosen for the extraction step because the crude extracts could be used directly for methanolysis with TMAH-methanol. Fatty acids present as esters, either polar or nonpolar, were converted to methyl esters. Free fatty acids were convered to TMAH salts and removed with the lower polar layer. In a few cases, free fatty acid composition was determined by pyrolysis of these salts, in the injection port, to form methyl esters. Broadened GC peaks were obtained, but the chromatograms were adequate to show that there were no gross differences in the composition of the free and esterified fatty acids.

Analyses of single seeds from 13 varieties gave variations for most components that were significantly greater (P=0.01) than the 1-3% coefficients of variation (CV) for replicate analyses of lipids from the same seed; but the variations were still fairly small and 18:CE, 19:CE, and their precursor dihydrosterculate (19:CA), were not significantly more variable than other minor components (Table 1). Ranges for 18:CE, 19:CE, and 19:CA were 0.291-0.536%, 0.157-0.322%, and 0.143-0.402%, respectively. Mean total CPA content was 0.66% (CV=14%) with a difference of 0.33% between the high and low values. This is in good agreement with published values of 0.75% (CV=14%) with 0.40% range for 25 U.S. varieities (7), and 0.83% (CV=11%) with 0.33% range for 12 Indian varieities (8) obtained by hydrogen bromide titration of several g of oil.

TABLE 1

Varietal[a] Variation in Concentration of Fatty Acids in Lipids

Acid[b]	ECL[c]	Mean (%)	CV[d] (%)
14:0 Myristic	14.00	0.725	15
15:0	15.00	0.031	23
16:1 Palmitoleic	15.77	0.602	13
16:0 Palmitic	16.00	22.72	8
17:2	16.66	0.103	28
17:1	16.75	0.121	12
17.:0	17.00	0.085	10
18:CE Malvalic	17.42	0.410	15
18:2 Linoleic	17.73	51.96	5
18:1 Oleic	17.78	18.6	10
18:0 Stearic	18.00	2.16	10
19:CE Sterculic	18.40	0.246	19
19:CA Dihydrosterculic	18.82	0.244	28
19:0	19.00	0.033	76
18:2 Epoxide	19.31	0.770	24
18:1 Epoxide	19.52	0.246	15
20:0 Arachidic	20.00	0.322	17

[a]One seed of each of 13 varieities.

[b]Carbons: double bonds (name). CE = cyclopropene, CA = cyclopropane, epoxide = monoepoxide of unsaturated fatty given.

[c]Equivalent chain length.

[d]Coefficient of variation.

TABLE 2

Seed-to-Seed Variation in Concentration of Fatty Acids in Lipids

Acid	5 Runs 1 seed		4 Seeds 1 lock		5 Seeds 1 plant		1 Seed 13 varieties	
	Mean (%)	CV[a] (%)	Mean (%)	CV (%)	Mean (%)	CV (%)	Mean (%)	CV (%)
16:1	0.544	3	0.486	6	0.550	11	0.602	13
17:0	0.091	2	0.089	4	0.090	7	0.085	9
18:CE[b]	0.450	1	0.501	8	0.464	6	0.410	15
18:0	2.02	1	2.12	3	2.07	3	2.16	10
19:CE[b]	0.319	1	0.428	12	0.332	10	0.246	19
19:CA[b]	0.405	1	0.331	7	0.382	8	0.244	28
20:0	0.282	1	0.234	7	0.270	14	0.322	17

[a]Coefficient of variation.

[b]CE = cyclopropene, CA = cyclopropane.

The data presented in Table 2 are for single seeds of each variety so the variations include all sources of variation, not just varietal differences. In fact, much of the variation was not related to variety. Concentrations of CPA and most other components were significantly ($P = 0.01$) more variable even for lipids from seeds from the same lock than for replicates for lipids from the same seed. However, compared to lipids from seeds from the same lock, variability did not increase significantly ($P = 0.05$) for lipids from seeds from each lock of a boll, from different bolls on a plant, or from commercially ginned seed of a single variety. Among 16 seeds from the same plant, CV of 18:CE (7%) and 19:CA (8%), but not of 19:CE (14%), were significantly lower than among seeds from different varieities. There probably are differences in the CPA content of lipids from different varieties of cottonseed, but they are small.

Since about half of the total variation of CPA concentration is among seed within a variety, maximum progress in a breeding program aimed at reducing CPA content should be achieved by selection of individual seeds that are low in CPA. This technique, which involves analyzing a portion of the seed and planting the rest of it, played a major role in the development of erucic aicd-free rapeseed oil (12). With cottonseed, no problem was encountered in slicing a few mg of the cotyledons from the top or side of a seed, extracting the lipids and analyzing them. However, the lipids obtained in this way were not representative of the whole seed; their CPA content was much too low.

Within-Seed Variations

Analyses of the upper and lower halves of a seed confirmed the nonuniform distribution of CPA-containing lipids within a seed. Lipids from the lower half of the seed were several times richer in CPA than those from the upper half. Separate analyses of the 3 readily distinguishable parts of a seed revealed striking differences in lipid composition (Table 3). Composition was not even constant within the major parts of the seed (Table 4). There was a steady increase in the concentrations of 18:CE, 19:CE and 19:CA between the root tip and the edges of the cotyledons. The 3 cyclic acids are formed at the expense of oleic and linoleic acid. This pattern of localization of CPA was observed in seeds of glandless as well as glanded varieties. After this work was completed, Berry (13) reported a similar pattern for CPA in Durian seed, which contains ca. 2% oil that is 50% CPA. Differences in composition of lipids from germs and from cotyledons or endosperm have been observed in other seeds, including corn (14) and peanuts (15), but these differences are small.

The weights and lipid contents of the various parts of the seed need to be determined to eval-

TABLE 3

Within-Seed Variation in Concentration of Fatty Acids in Lipids

Acid	Cotyledon (%)	Axis[a] (%)	Root tip (%)
18:CE[b]	0.050	5.23	21.10
18:2	56.37	39.96	20,27
18:1	14.60	15.80	9.27
18:0	2.24	2.93	3.44
19:CE[b]	0.068	3.33	7.69
19:CA[b]	0.090	3.66	6.60
20:0	0.257	0.360	0.600

[a]With root tip removed.

[b]CE = cyclopropene, CA = cyclopropane.

TABLE 4

Variation in Concentration of Fatty Acids in Lipids Within Cotyledon and Axis

	Cotyledon		Axis		
	Top (%)	Bottom (%)	Top (%)	Middle (%)	Bottom[a] (%)
18:CE	0.015	0.073	1.56	7.16	12.78
18:2	56.0	58.9	42.4	33.6	31.1
18:1	16.6	13.8	24.8	17.4	9.2
19:CE	0.005	0.042	2.37	5.17	5.26
19:CA	0.066	0.091	1.53	5.47	5.10

[a]Includes root tip.

TABLE 5

Distribution of Weight, Oil and CPA in the Kernel

Part	Weight (%)	Oil[a] (%)	CPA[b] (%)
Cotyledon	95.1	96.4	24
Axis[c]	3.8	2.7	42
Root tip	1.1	0.9	34

[a]Oil contents were cotyledon 33%, axis 23%, and root tip 28%.

[b]Cyclopropenoid acid contents of the oils were cotyledon 0.184%, axis 11.4%, and root tip 26.6%.

[c]With root tip removed.

uate their contribution to the overall CPA content of cottonseed oil. Root tips, remainders of the axes, and cotyledons from 10 kernels were pooled to obtain these data (Table 5). Although they are only ca. 1% of the kernel, root tips contribute one-third of the total CPA. The cotyledons that make up 95% of the kernel contribute only one-fourth of the CPA.

A few cursory checks indicated that the CPA contents of the nonpolar lipids extracted by hexane and the polar lipids subsequently extracted by methylene chloride/methanol were similar; e.g., 0.184 and 0.166%, respectively, for cotyledons, and 26.5 and 18.3% for root tips. Detailed investigation was not made of the CPA content of various polar lipid classes.

The localization of CPA in the axis helps explain increases in CPA content of lipids obtained with successive hexane extractions of cottonseed (16). These increases were attributed to concentration of CPA in "specific areas of the seed which are not readily accessible to solvent," without any identification of these areas. It is now clear that they are gross structural areas, namely the axes, not polar-lipid areas, such as membranes, in cells. The axes are somewhat denser and harder than the cotelydons and tend to remain intact during flaking of the kernels, which would make their lipids more difficult to extract.

The same association of lipid composition with parts of the embryo persisted when the seeds were germinated (Table 6). Even in cotton plants, the lipids in the root tips are rich in CPA, while only traces are present in the leaves. The germinating seeds contained a higher proportion of polar lipids and free fatty acids than the dormant seed. Neutral lipids, phospholipids and free fatty acids all contained similar proportions of CPA.

There was no evidence of selective lipolysis or metabolism of CPA; so the accumulation of CPA should be a valid measure of their formation in each type of tissue. Although there may be questions regarding the precise biosynthetic pathway from 19:CA to 18:CE, the formation of 19:CA from 18:1 by transfer of a methylene group from methionine is well established (5). Oleate is abundant in all parts of the seed, so the formation of CPA should be a function of the concentrations of methionine and the enzyme that transfers the methylene group. The general similarity of fatty acid composition, especially CPA concentrations, of corresponding parts of the seed and the plant favors association of different proportions of the various lipid-modifying enzymes with different cell types as the limiting factor in CPA produc-

TABLE 6

Effect of Germination on CPA[a] Content of Lipids

	Dormant		Germinating		Seedling	
	Cot[b]	Root	Cot	Root	Leaf	Root
18:CE (%)	0.062	20.8	0.066	18.2	tr[c]	12.1
19:CE (%)	0.087	5.7	0.084	5.0	tr	1.6
19:CA (%)	0.149	6.2	0.150	3.5	tr	1.3

[a]Cyclopropenoid fatty acid.

[b]Cotyledon.

[c]tr = trace (less than 0.01%).

tion. On the other hand, in vitro formation of 19:CA when slices of cottonseed are incubated with labeled methionine (17) seems to favor methionine concentration as the limiting factor.

In view of the wide variation of CPA content within seeds, it seemed probable that much of the variation observed among seeds was related to the proportions of the various seed parts. In particular, lipids from small, immature seeds that have a high proportion of axis (18) should have higher CPA contents than those from mature seeds. Analyses of lipids from embryos weighing 6-8 mg (from 25-30 mg seeds) supported this assumption. These lipids contained 18-22% CPA – even more than was anticipated. Apparently the embryo as a whole makes a high proportion of CPA during the earliest stages of its development and as development proceeds there is a selective decrease in the porportion of CPA produced by various tissues. Kajimoto et al. (19) reported a CPA content of 27% in the small amount of triglycerides present in the ovules 3 days after fertilization with decreases to 11 and 4% for 10- and 20-day-old seed, respectively. During the period of rapid embryonic development that begins about three weeks after flowering (20), CPA content of triglycerides from the seed decreased to 2.5% at 30 days, and 0.5% at 40 days. It changed very little during the next 10 days and then increased to 0.7-0.8% at about the time the bools opened (55-60 days after flowering). On this basis, one would expect any correlation between weight and CPA content of lipids for nearly mature seed to be positive.

Although seed that obviously were immature were omitted in our studies, 8 seeds from one variety that were analyzed had higher than normal variation of weight per seed (71-136 mg), per kernel (29-91 mg), and of CPA content of the lipids (0.27-0.97%). For this variety, CPA content was highly correlated with both seed and kernel weight (r = -0.9). Correlation coefficients were lower (r = -0.71) for 12 seeds from another variety that had narrower ranges of seed weight (95-127 mg), kernel weight (59-82 mg), and CPA (0.83-1.12%), and even lower (r = -0.37) for the 11 seeds from the other varieties; but they were negative in all cases, as would be expected from the differences in rates of development of axes and cotyledons, not positive as one might expect from the data of Kajimoto (19). The lower correlations for seeds from different varieties are certainly due, in part, to genetic differences, but environmental effects, especially changes in growing conditions that occur while the embryo is developing, may be contributing factors.

SUMMARY

Variation of CPA content of lipids among varieties are only slightly larger than the variations among seeds from the same plant. Much of the variation among seeds probably reflects differences in the proportions of different tissues. Concentrations of CPA in lipids from various parts of a cottonseed were 0.1, 8 and 28% from cotyledons, axis (hypocotyl) and root tip, respectively. Three-fourths of the total CPA is concentrated in the axial tissue, which makes up only 5% of the seed. This localization of CPA is maintained from the development of the embryo through dormancy and germination. Genetic engineering or induced mutation might produce cottonseeds containing little or no CPA by eliminating the enzyme(s) that transfer a methylene group from methionine to oleate. However, one needs to be sure that CPA are not beneficial to cotton plants, as suggested by Halloin (21), before steps are taken to eliminate them.

In the interim, CPA content of cottonseed oil can be minimized by processing only mature seeds, and with chemical methods that destroy CPA during processing (22,23). Major reduction could be achieved if a practical method could be developed to separate the axes from the cotyledons prior to oil extraction.

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Studies on the Half-Life and the Distribution of Stearoyl-CoA Desaturase in the Housefly

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ABSTRACT

The half-life of the stearoyl-CoA desaturase was determined by using houseflies injected with puromycin. The LD_{50} for puromycin was determined to be 1.55 μ g/mg body wt for both sexes. By following the decay in specific activity of the desaturase after puromycin injection, the half-life of this enzyme in 4-day-old females was calculated to be 9.35 hr, while in 1-day-old females it was 3.38 hr. The inhibition curve for the 3-day-old males never reached 100%, and a biphasic curve was observed. The early phase resulted in a half-life of the desaturase of 2.41 hr, while the latter phase had a half-life of 8.45 hr. In general, it appeared that the half-life of the desaturase increased with age in either sex. Studies on the distribution of desaturase activity within the housefly showed that most of the activity was present in the integument; however, in the female, the onset of ovarian development seemed to shift the distribution of desaturase from the integument, towards the internal tissues. In the male, there appeared to be equal amounts of desaturase in integument and the fat body plus internal tissues at day 2, but by day 5 most of the activity was associated with the integument. The high specific activity and high percentage of total desaturase activity associated with integument coupled with the shift of distribution of desaturase in females during the onset of ovarian development may be indicative of a close correlation of the desaturase with alkene synthesis and possibly sex pheromone production.

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INTRODUCTION

The mammalian stearoyl-CoA desaturase which converts stearoyl-CoA to oleoyl-CoA has been extensively studied (1,2). In contrast, there have been only a few studies aimed at characterizing the desaturase in insects (3-5). Recently, we have studied the acyl-CoA desaturase activity in both sexes of housefly (6). In general, the male desaturase activity was higher than female. In males, the activity increased ca. 26-fold from emergence until the insects were between 3 and 4 days old. After day 4, the specific activity decreased gradually until about day 10 when the activity reached the level observed at emergence from the pupa. In females, the activity increased ca. 12-fold from emergence until day 2; then it sharply decreased on day 3 maintaining a 6- to 8-fold increase for the next 4-5 days. Again, the activity in females declined to emergence levels by day 10. Despite these differences, the desaturase activities were observed to parallel closely alkene synthesis in both sexes (6). The decrease in desaturase activity in females between days 2 and 3 correlated with initiation of vitellogenesis and ovarian maturation (6). Adams et al. (7,8) have shown that ovarian development reaches early vitellogenic stages at about day 2. Because houseflies are poikilothermic, the correlation of egg stage with days after emergence will vary somewhat depending on the temperature at which the flies are kept. However, our observation that the decrease in desaturase activity between days 2 and 3 correlated with alkene synthesis in females may be suggesting that desaturase activity is correlated with sex pheromone synthesis. However, it is difficult to understand how the desaturase, which decreases between days 2 and 3, could be correlated with (Z) 9-tricosene synthesis, which increases during this time period (8). On the other hand, recent studies by Dillwith et al. (9) showed that (Z)-9-tricosene, a major component of the sex pheromone complex, is formed via direct elongation and decarboxylation of oleic acid, the product of the stearoyl-CoA desaturase (6).

Because the desaturase activity is closely related to the synthesis of cuticular alkenes, which include the sex pheromone, the turnover rate and the localization of the desaturase become important parameters to define if, in fact, there is a correlation between this enzyme and the sex pheromone. The results presented in this report demonstrate a close association of desaturase activities with integument tissue from which the cuticular alkenes, including the sex pheromone, are synthesized (9).

MATERIALS AND METHODS

Materials

[1-^{14}C] Stearate was obtained from New

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England Nuclear, Boston, MA. L-[1-^{14}C] Leucine (55 mCi/mmol) was obtained from ICN, Irvine, CA. Silica gel impregnated glass fiber sheets (ITLC Type SG) were purchased from Gelman Instrument Co., Ann Arbor, MI. Bovine serum albumin, cytochrome C and puromycin were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were analytical grade or the purest available.

Insects

Housefly pupae (Fales 1958 Strain T-II) were supplied courtesy of Dr. Ted Shapas, Biology Section, S. C. Johnson and Sons, Inc., Racine, WI. Insects were maintained and handled as previously described (6).

Desaturase Assay

Insect microsomal isolation was accomplished and desaturase activities were measured as previously reported (6). The desaturase activity was determined by measuring the incorporation of radioactivity from stearoyl-CoA into oleic acid. This was accomplished via thin layer chromatography of the methylated fatty acids followed by liquid scintillation counting to determine the radioactivity.

Puromycin LD_{50}

To determine the LD_{50} of puromycin, different amounts of puromycin were injected into the houseflies and the percent survival after a 1-hr incubation at room temperature was determined. Twenty to 30 houseflies were used for each concentration of puromycin, and the concentration of puromycin ranged from 0.2 μg to 3.6 μg/mg body wt. The percentage surviving after a 1-hr incubation was plotted against puromycin concentration to determine the LD_{50}.

Protein Synthesis Inhibition Study

Female and male houseflies were injected with 1 μl containing 25 μg and 15 μg of puromycin, respectively. Saline injection (1 μl) served as a control. The inhibition of protein synthesis was monitored by injecting 1 μl containing 25 nCi of L-[1-^{14}C]leucine into each of 10 houseflies at different time intervals after the puromycin or saline injection. The houseflies were killed after 1 hr of incubation by homogenization in 8 ml of 5% perchloric acid. A polytron (Brinkman Instruments) was used by operating at full power for 30 sec. Following heating at 90 C for 15 min, the homogenate was centrifuged for 10 min at 5000 rpm in a Beckman J-21 refrigerated centrifuge. The pellet was resuspended in 4 ml cold 5% perchloric acid and the precipitate collected by centrifugation as noted above. This extraction was repeated 3 times. The final pellet was dissolved in 6 ml hot 1 N NaOH. An aliquot of this solution was taken and counted in a scintillation counter. The L-[1-^{14}C]leucine incorporation was compared between puromycin injected and control houseflies. About 20% of the injected radioactivity was incorporated into protein in control flies. In males, incorporation of leucine was inhibited 82-85% for up to 6 hr after puromycin injection, and in females the inhibition of incorporation was 92-95% for up to 6 hr.

Half-Life of the Desaturase

Amounts of puromycin which produced only 1-2% mortality and which produced complete inhibition of protein synthesis were injected into 50 female and 50 male houseflies for each time point. The houseflies were killed after different time intervals, the microsomes were isolated and the desaturase half-life was determined from the slope of the specific activity versus time curve. Untreated controls were not assayed, but in our previous study (6), we showed that the activity of the desaturase changed very little for the older flies and increased for the younger flies; therefore, this control should not be necessary.

Desaturase Distribution

For the distribution study, ca. 300 insects were used. Abdomens were excised and the fat body and other internal tissue removed by gently squeezing the abdomen. The head and throax were also excised. The head, thorax, fat body plus internal tissue and integument tissues were placed separately in isolation buffer (6) and homogenized with the polytron. Immediately after microsomal preparation, the desaturase activities were assayed as described before (6). Microsomal protein concentrations were determined by the procedure of Lowry et al. (10).

RESULTS

LD_{50} of Puromycin

In order to determine the half-life of the housefly desaturase, the amount of puromycin to be used as a protein synthesis inhibitor needed to be determined. Because nothing is known about the puromycin toxicity to this housefly strain, the LD_{50} of puromycin was determined first. The male and female houseflies were injected with the same amounts of puromycin, and the percentage remaining alive

after 1 hr was determined. This value was then plotted against the amounts of puromycin injected. Males were more sensitive to the puromycin than females. However, the females are, in general, ca. 60% heavier than males. Females weigh ca. 22 mg at emergence and increase to ca. 27 mg by day 5. Males weigh ca. 20 mg at emergence and decrease to ca. 16.5 mg at day 5. Thus, when the data were converted to a per body weight basis, the LD_{50} turned out to be the same for both sexes (Fig. 1). It was 1.55 μg/mg body weight.

Half-Life Study

Previously, we had shown that in females the desaturase activity increased until the 2nd day after emergence; whereupon, it declined markedly by day 3 (6). After day 3, there was a slight increase in desaturase activity until day 6 6; after which, the desaturase activity decreased to the same level as it was at emergence. It reached this point by day 11. Thus, we chose 2 age groups to use for determining the desaturase half-life. We used 1-day-old females because the desaturase activity was increasing at this age, and we used 4-day-old females because the desaturase activity was rather static in this age group.

Figure 2 shows the desaturase rates of females at different times after puromycin injection. The half-life of the desaturase from 1-day-old females was calculated much shorter than 4-day-old females. The half-life was 3.38 hr at day one and 9.35 hr at day 4. The initial specific activity was 0.80 and 0.36 nmol/min/mg, respectively. Thus, the half-life of the desaturase in female houseflies increased and the specific activity decreased with age.

For male houseflies, we had observed that desaturase activity increased until about the 3rd day after emergence (6). By the fifth day, the desaturase was still at a high but rather static level. The desaturase then decreased in activity reaching emergence level by day 11. Thus, we chose different time points for males. We chose day 3 because the activity was at a maximum at this age, and we chose day 5 because the desaturase activity had begun to decline slightly at this age.

As shown in Figure 3, the older males had a longer desaturase half-life. The half-life of the male desaturase at day 3 was 2.41 hr, and its specific activity was 1.37 nmol/min/mg (Table 1). At day 5, the half-life was 6.96 hr and the

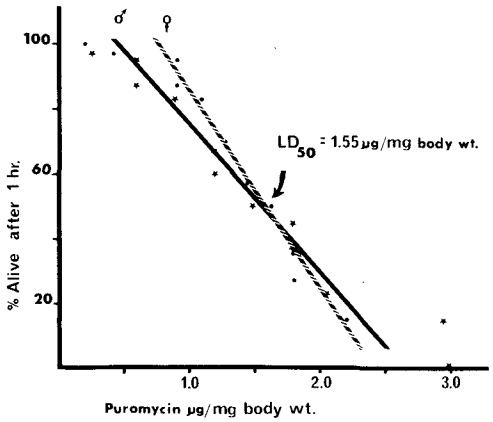

FIG. 1. LD_{50} for puromycin. Twenty to 30 houseflies were used for each concentration of puromycin. The concentration of puromycin ranged from 0.2 μg to 3.0 μg/mg body wt. The percentage surviving after 1 hr incubation was plotted against puromycin concentration to determine the LD_{50}. The stars and solid line represent the data obtained for males, and the solid dots and dashed line represent the data obtained for females. The correlation coefficient for each regression was 0.98 for the males and 0.97 for the females. When the 2 curves were compared via a least squares fit analysis, the F test for quality of variance was 4.53, and the slopes of the 2 regression curves were different with a $P < 0.01$.

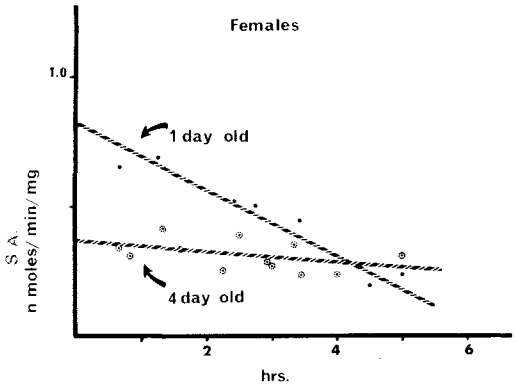

FIG. 2. Stearoyl-CoA desaturase turnover study in the female houseflies. Twenty-five μg of puromycin were injected into each of 50 female houseflies, and the houseflies were killed after different time intervals. The microsomes were insolated, and the half-life of the desaturase was calculated from slope of the specific activity vs. time curve. The solid dots represent data obtained from 1-day-old females, and the circled stars represent that obtained from 4-day-old insects. The statistical analyses of the curves in Figs. 2 and 3 were done via a least squares fit statistical program. The correlation coefficient for the 1-day female curve was 0.97 and that for the 4-day females curve was 0.43. The F test for quality of variance was 33.522; thus, the 2 curves are different with a $P < 0.001$.

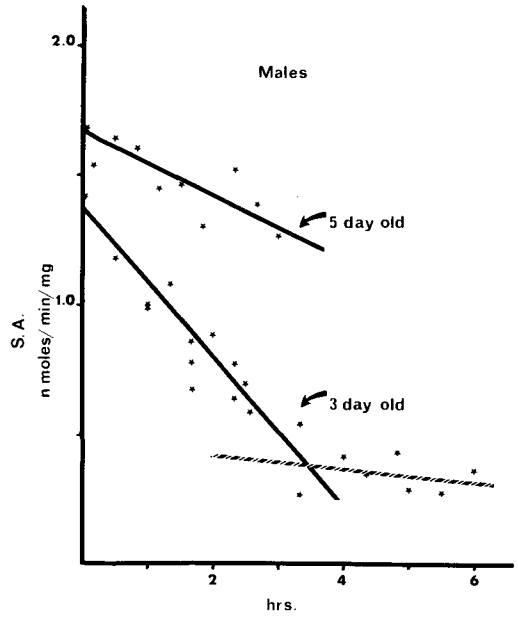

FIG. 3. Stearoyl-CoA desaturase turnover study in the male houseflies. Fifteen μg of puromycin were injected into each of 50 male houseflies. The half-life of the desaturase was obtained as described in Fig. 2. The upper solid curve represents data collected from 5-day-old males, and the dashed curve represents a statistical break in the curve for the 3-day-old males. The correlation coefficient for the 5-day males curve was 0.83 and that for the upper part of the 3-day male curve was 0.88. The lower part of the 3-day male curve had a correlation coefficient of 0.65. Using the F test for quality of variance, the F value for the 5-day curve compared to the upper part of the 3-day curve was 11.04; thus, these 2 curves are different with a $P < 0.01$. When the 2 curves for the 3-day insects were compared, the F test was 12.51 which gave a $P < 0.01$.

specific activity was 1.66 nmol/min/mg. Thus, the half-life of male desaturase increased with age and the specific activity was slightly elevated.

A biphasic inhibition curve was observed in 3-day-old males (Fig. 3). The second phase was parallel to the inhibition curve of the 4-day-old females. The half-life for this second phase of desaturase activity was 8.45 hr, and the specific activity was 0.48 nmol/min/mg.

Distribution of Desaturase Activity

In order to determine if the specific activity of the desaturase was similar in different parts of the insect body, we divided the insect into 4 parts: head, thorax, fat body plus internal organs and integument. The desaturase activity of each part was assayed, and from the specific activity, we determined the total activity in the different tissues.

TABLE 1

Half-Life of Desaturase Activity in Male and Female Houseflies of Different Ages

	Age (Day)	Half-life (hr)	SA (nmol/min/mg)
Female	1	3.38	0.80
	4	9.35	0.359
Male	3	2.41	1.368
	(3)	(8.45)	(0.484)
	5	6.96	1.663

For each time point, 50 flies of each sex were injected with puromycin (15 μg/male fly and 25 μg/female fly). After different time intervals, microsomes were prepared and the desaturase activity was determined. The half-life was determined from a plot of desaturase activity vs time after puromycin ingestion (see Figs. 2 and 3). The figures in parentheses represent the data for the lower part of the curve for 3-day males.

In females at the earliest time (1.5 days, see Table 2), the head contained the lowest total desaturase activity of the different body parts. The fat body plus internal organs and the thorax contained similar amounts of activity and were 61-109% greater than the head. The integument contained the largest amount of desaturase activity. There was 9.8-fold more activity than head, 6.1-fold more than thorax, and 4.7-fold more than fat body plus internal organs. With the onset of vitellogenesis and ovarian maturation (day 2), some very interesting changes were noted in the distribution of desaturase activity. The total activity decreased 51%, 16%and 61%, respectively, in the head, thorax and integument; whereas, it increased 243% in the fat body plus internal tissues. By day 5, the activities of the desaturase returned to the initial 1.5 day levels in the head, thorax and fat body plus internal organs. In the integument, the total activity increased towards the 1.5 day level, yet it remained at ca. 50% of this earlier value.

In males at day 2, the head and thorax contained similar amounts of desaturase activity, and these amounts were considerably lower than those observed in the integument and/or fat body plus internal organs. These last 2 tissues contained similar amounts of activity. Compared to females, a much different effect of age was observed in males. The total desaturase activity decreased 40% in the head, but it

increased 99%, 2394% and 27% in the thorax, the integument, and the fat body + internal tissues, respectively. The very large increase in desaturase activity in the integument in males may be related to maintenance of cuticular wax.

DISCUSSION

The fatty acid composition of the adult housefly, *Musca domestica,* has been studied (11-13). The results of these studies show that the housefly, in contrast to the mammalian system which generally contains a low percentage of palmitoleate and a moderate percentage of oleate, contained a relatively high percentage of palmitoleate and oleate. It is generally agreed that most insects are unable to synthesize polyunsaturated fatty acids, and no polyunsaturated fatty acids have been detected in housefly larva fed on a fat-free diet (12). Thus, in order to maintain the fluidity and proper functioning of their biomembranes, the high content of monoenes may be vital for this insect. When houseflies were fed a cholesterol-deficient diet, the fatty acid composition of the phospholipids showed an increase in saturated acids, indicating an attempt to regulate the proper membrane fluidity (13). Thus, certain dietary lipids play a role in controlling membrane fatty acid composition.

Fast (14) suggested that the high palmitoleic acid content of the housefly is linked to the unusually high content of phosphatidylethanolamine found, with a few exceptions, in all Diptera. Other insects which contain a much smaller proportion of phosphatidylethanolamine do not contain the large amounts of 16:1. Further, each class of phospholipid seems to have a specific fatty acid composition (13). In housefly larvae, the fatty acid composition of the total phospholipid fraction was reported to be different depending on which tissue was assayed (13).

The fatty acid composition of most animals and organisms can easily be influenced by dietary fatty acids. Thus, the fatty acid composition of an insect represents a combination of what it eats and what it makes itself. The facts that the dry powdered milk, used as the only dietary lipid source for the housefly, contains low 16:1 (3%) and high 18:1 (36%) and that the housefly contained a high content of 16:1 (18%) and 18:1 (20%) further support this idea. The assay of the desaturase activity showed a high activity, indicating that the housefly should have a high content of monoenoic fatty acids. A previous study (6) showed that the Δ^9 desaturase activity varied depending upon the age and sex of the housefly; however, these

TABLE 2

Distribution of Desaturase Activity within the Whole Housefly

Age	Head		Thorax		Integument		Fat body + internal organs		Body wt
(days)	SA[a]	TA[b]	SA	TA	SA	TA	SA	TA	(mg)
Female									
1.5	0.140±0.018	0.74±0.10	0.139±0.008	1.55±0.09	0.58±0.11	7.25±1.44	0.092±0.007	0.19±0.09	26.0
2.0	0.060±0.014	0.36±0.08	0.092±0.006	0.15±0.08	0.44±0.03	2.86±0.18	0.290±0.04	4.08±0.50	27.0
5.0	0.178±0.004	0.76±0.01	0.128±0.004	1.56±0.04	0.39±0.06	3.45±0.50	0.046±0.006	1.86±0.23	26.8
Male									
2	0.060±0.006	0.55±0.09	0.048±0.002	0.76±0.03	0.35±0.05	3.93±0.57	0.28 ±0.03	3.59±0.36	17.4
5	0.034±0.005	0.33±0.05	0.065±0.007	1.51±0.16	1.24±0.15	13.34±1.43	0.53 ±0.08	4.55±0.61	16.5

[a]SA = nmol/min/mg protein (specific activity).
[b]TA = nmol/min (total activity).
For each of these experiments, 300 houseflies of each sex were divided into head, thorax, integument and fat body plus internal organs. The individual tissues were homogenized and microsomes were prepared. The desaturase activity was determined for each tissue. Each value represents the mean ± SD for 2-3 assays.

alterations in desaturase activity did not correlate with changes in the percentage fatty acid composition (Ryan, unpublished). Thus, a direct link between desaturase activity and tissue fatty acid composition is difficult to establish. This also may hold true for any correlation with the desaturase and the synthesis of the (Z) 9-tricosene.

The present study showed a slightly shorter half-life for male housefly desaturase ($t_{1/2}$ = 2.4-3.4 hr) compared with those of rat hepatic tissue ($t_{1/2}$ = 3-4 hr) (15), chick liver explant tissue ($t_{1/2}$ = 4 hr) (16) and *Euglena gracilis* ($t_{1/2}$ < 10 hr) (17). However, in the male, there appeared to be a second population of desaturase which had a longer half-life similar to the female desaturase in the older age group. This long half-life desaturase appeared to have a low specific activity, while the short half-life desaturase, in the earlier age group of both sexes, had a higher specific activity. It should be noted that we have not purified nor made antibodies to the desaturase; thus, these turnover studies are simply decay analyses after protein synthesis has been stopped with puromycin. Therefore, the half-life times reported must be interpreted cautiously.

The concept of 2 populations of desaturase is supported by changes in desaturase activity depending on the location of the desaturase. Our present data on the distribution of desaturase activities within the housefly showed that, in general, most of the high activity desaturase was associated with integument. In the males, the integument contained ca. 44-67% of the total desaturase activity depending upon the age of the flies. Further, the highest specific activity of the desaturase was also associated with the integument. A shift from almost even distribution between integument and fat body plus internal organs at an early age (day 2) towards an increase in the integument at an older age (day 5) was seen. These data coupled with desaturases with 2 different half-lives indicate that there are 2 populations of desaturase. In the 1.5-day-old females, the integument contained 67% of the total desaturase activity. However, the onset of ovarian development, which occurs about day 2 in females, correlated with a shift in the percentage distribution of the desaturase towards a higher percent located in internal tissues. This shift towards internal tissues also represents a shift towards a lower specific activity desaturase. This decreased activity coupled with the shift of desaturase distribution also indicates there may be 2 populations of desaturase: one associated with integument which has a higher specific activity; and one associated with internal tissue which has a lower specific activity. The one associated with integument appears to have a shorter half-life and the other one associated with internal tissue has a longer half-life. This is suggested by the half-life study in females in which the desaturase at day 1 has a higher specific activity but a shorter half-life and the desaturase at day 4 has lower specific activity but a longer half-life. The 2 different half-life values determined for the desaturase in males also support this idea.

Previous study indicated a strong correlation between desaturase activity and alkene synthesis (6). The present study suggests that the desaturase in integument is closely associated with alkene synthesis and possibly subject to change as alkene production in housefly changes. The synthesis of cuticular hydrocarbons, including the alkenes, has been demonstrated to be closely associated with the integument (18). The biosynthesis of the hydrocarbon components of the housefly appears to follow the general pathway as reported for the cuticular hydrocarbons of other insects in which an appropriate precursor is elongated to a very long chain fatty acid and then decarboxylated (19, 20).

Recent studies by Dillwith et al. (9) have shown that oleic acid, a major product of desaturase (6), is a precursor of alkenes in housefly. This finding coupled with our studies again confirmed the strong correlation between cuticular desaturase activity and alkene synthesis.

The female sex pheromone complex contains (Z)-9-tricosene which is a major alkene produced by cuticular tissue in mature females. (Z)-9-tricosene is formed via elongation and decarboxylation of oleic acid (9), and the female starts to produce this sex pheromone around day 2. Also, during this time, the insects switch from a high percentage of alkenes to a high percentage of alkanes in their hydrocarbons. The males, in contrast, still maintain a high percentage of alkenes. Because oleic acid is the direct product of the Δ^9 desaturase and a precursor of alkenes, including sex pheromone, the switching of hydrocarbon pattern coupled with the shifting of distribution of desaturase activities from the integument to the internal tissues during this period may indicate some correlation between desaturase activity and sex pheromone production.

The detailed molecular mechanism of the shifting and changing of desaturase activities is not known. Hormonal effects on desaturase enzymes have been demonstrated in the mammalian system (21-23). That various hormonal inductions of overall desaturation result from a

specific increase in the content of the terminal desaturase has been demonstrated in chick liver explants by Joshi and Aranda (16). No information is available to suggest that insect hormones have an affect on the desaturase. However, ecdysone has been shown, primarily in Diptera, to stimulate hydrocarbon synthesis (24) and protein synthesis (25), and to control reproduction in adult insects (26). It also has been demonstrated that ecdysone injected into the male housefly can induce the synthesis of (Z)-9-tricosene (Blomquist, Dillwith and Adams, unpublished). Further, unpublished data (Wang and Reitz) suggest that ecdysone may affect desaturase activity. Thus, it is very likely that ecdysone or another insect hormone may affect this shifting and changing of desaturase activity. This area is certainly worthwhile for further investigation.

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Phospholipids of *Rhizobium meliloti* and *Agrobacterium tumefaciens:* Lack of Effect of Ti Plasmid

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ABSTRACT

We have studied the phospholipid composition of *Rhizobium meliloti* strains which do or do not contain the large, tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens.* The major phospholipids of stationary phase cells were phosphatidylethanolamine (PE) (22%), phosphatidyl-N-methylethanolamine (22%), phosphatidylcholine (PC) (27%), phosphatidylglycerol (11.4%), and cardiolipin (11%); as average percent of lipid phosphorus. Phosphatidyl-N,N-dimethylethanolamine (3.7%) was also present. The proportions of PE were higher, and PC lower, in logarithmic phase cells. No significant differences were seen in the proportions of phospholipids in strains with or without the Ti plasmid. Qualitative examination of the phospholipids of *A. tumefaciens* with or without the Ti plasmid similarly revealed no differences.
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Rhizobium species are capable of inducing nodules on the roots of members of the plant family Leguminosae This results in a symbiotic relationship in which dinitrogen is fixed into ammonia leading to the incorporation of nitrogen into plant tissue. This major ecological process is of great economic importance in agriculture (1).

The family Rhizobiaceae (2) consists of the genera *Rhizobium* and *Agrobacterium.* The latter infect diverse species of plants and produce gall hypertrophies. Thus, both groups of organisms are capable of entering into long-lasting associations with plant host cells. The presence of PC has been reported in all members of the genus *Agrobacterium* (3-5) and it has also been found in several species of *Rhizobium* including *R. japonicum* (6), *R. leguminosarum* (7) and strains infective on *Lotus pedunculatus* (8). Among the economically important species of *Rhizobium* not studied with respect to phospholipid composition are *R. trifolii, R. phaseoli* and *R. meliloti.* The first 2 are closely related to *R. leguminosarum* (1). *R. meliloti* nodulates plants of only a few genera: *Medicago* (alfalfa), *Trigonella* (fenugreek) and *Melilotus* (sweet clover).

The tumorigenicity of *Agrobacterium* is related to the presence of a large tumor-inducing (Ti) plasmid (9-11), which contains information for the ability to synthesize and catabolize opines such as opaline and nopaline and for sensitivity to antibiotics. Large plasmids are also found in *Rhizobium* species, and these have been implicated in the establishment of the bacteria-plant symbiosis (1). The tumorigenic capacity of *Agrobacterium tumefaciens* can be transferred by introduction of the Ti plasmid into strains of *R. trifoli* and *R. leguminosarum,* but transfer of the plasmid to *R. meliloti* did not confer tumorigenicity (P.J.J. Hooykaas, personal communication).

We have undertaken a study of the phospholipid composition of *R. meliloti,* including strains carrying the Ti plasmid of *A. tumefaciens,* and of strains of the latter which do and do not contain the Ti plasmid.

MATERIALS AND METHODS

Cells and Methods of Culture

The strains of *R. meliloti* studied were LPR 2120 and LPR 2121, LPR 2136 and LPR 2139, LPR 2132 and LPR 2137. The second member of each pair contained a Ti plasmid. Also studied was *R. meliloti* ATCC 9930. The two strains of *A. tumefaciens* studied were LBA 201 and LBA 202. The latter was generated by curing the Ti plasmid from virulent strain LBA 201. These strains are from the Phabagen Collection, Utrecht, The Netherlands, and were obtained through the courtesy of Dr. P.J.J. Hooykaas, University of Leiden.

Cells were grown at 30 C on a shaker in a synthetic medium containing, in g/l: K_2HPO_4, 2.05; KH_2PO_4, 1.45; $(NH_4)_2SO_4$, 3.00; $MgSO_4 \cdot 7H_2O$, 0.50; NaCl, 0.15; $CaCl_2$, 0.005; glucose, 2; $FeSO_4 \cdot 7H_2O$, 0.0025 (12). Inocula were supplemented with mannitol, 10 g/l; and

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PME, phosphatidyl N-methylethanolamine; PDME, phosphatidyl N,N-dimethylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

yeast extract, 1 g/l and the salt concentrations in g/l were: K_2HPO_4, 0.50; $MgSO_4 \cdot 7H_2O$, 0.20; NaCl, 0.20; $FeCl_3 \cdot 6H_2O$, 0.01; $CaCO_3$, 3.0. Cells were harvested by centrifugation at 4000-5000 × g at temperatures below 10 C. The pellets were washed in cold 0.05 M potassium phosphate buffer, pH 7.4, and were either extracted immediately or stored at -20 C until extracted.

Lipid Extraction

Larger amounts of cells (≥ 2 g wet wt) were extracted by a modification of the method of Folch et al. (13,14), while smaller amounts of cells were extracted according to Bligh and Dyer (15). Poly-β-hydroxybutyrate accumulates in *Rhizobium* strains (1) and it was removed from the lipid extracts (16) in order to facilitate chromatography.

Chromatography and Quantitative Determinations

Thin layer chromatography (TLC) was performed on Adsorbosil or Adsorbosil-Plus 1 Prekotes (Applied Science, Deerfield, IL) thin layer plates. For quantitative analysis by phosphate determination (17), the plates were prewashed with chloroform/methanol/7 N ammonia (42:44:3.5, v/v/v). Duplicate samples were separated by TLC with chloroform/methanol/7 N ammonia (60:35:5, v/v/v) in the first dimension, followed by *n*-butanol/acetic acid/water (60:20:20, v/v/v) in the second dimension. Plates were air-dried and placed under high vacuum between runs.

Phospholipid ratios were also determined by two-dimensional TLC of ^{32}P-labeled lipids, in the same solvent systems on either Prekotes, as described above, or on plastic-backed silica gel 60 plates (E. Merck). Lipids were located by autoradiography and the individual lipid spots were either scraped into scintillation vials or, for plastic-backed plates, cut out with scissors and placed in vials. Counting was in OCS scintillation fluid (Amersham, Arlington Heights, IL).

For qualititative analysis, plates were run in duplicate or triplicate for staining with iodine vapor, ninhydrin, molybdate or Dragendorff's reagent (18). Appropriate standards were run in parallel and stained for comparison with the unknowns.

Total phospholipids were deacylated by alkaline methanolysis (18) and the recovered glycerophosphate esters were analyzed by 2-dimensional TLC as described (19). The 10 × 10 cm plastic-backed cellulose sheets were autoradiographed and cut up for scintillation counting of ^{32}P as described above.

Tumorigenicity of the *Agrobacterium* strains was checked by injection of concentrated suspensions of cells in nutrient broth (Difco) into tomato plant stems, 2 sites on each plant. The plants were inspected for tumor formation at the sites of injection and strain LBA 201 was found to be positive while strain LBA 202 did not produce tumors.

RESULTS

A. tumefaciens

We examined qualitatively the phospholipids of *A. tumefaciens* strain LBA 201, which contained the Ti plasmid, and strain LBA 202, which did not, by both one-dimensional TLC in chloroform/methanol/7 N ammonia (60:35:5, v/v/v) or by 2-dimensional TLC, as described under Materials and Methods. PC, which had previously been identified in this species (3,4), was found in both strains (data not shown), showing that the Ti plasmid does not provide the information for synthesis of the enzymes needed for the conversion of PE to PC (20).

R. meliloti

The presence or absence of the Ti plasmid in *R. meliloti* strains similarly had no significant effects on its phospholipid composition (Table 1). Strain 2121 contains the Ti plasmid but strain LPR 2120 does not. Determination of the percent phosphorus in each phospholipid by either colorimetric assay of acid-digested lipids or by liquid scintillation assay of ^{32}P-labeled lipids gave similar results, except that the ^{32}P-labeled cells appeared to have a lower proportion of PC. Figure 1 shows a tracing of a 2-dimensional chromatogram of *R. meliloti* lipids. The relative positions of the phospholipids on the chromatograms were the same as those observed with phospholipids from *A. tumefaciens*. It should be noted that the position of PC in the second dimension is dependent on the amount of lipid chromatographed. When radioactive lipids alone were chromatographed, the PC had an R_f in the second dimension of ca. 0.5, while addition of carrier lipid (∿50 μg) resulted in the R_f shown (0.32). Under some conditions, a double spot was seen. Thus, caution should be exercised in using this system for samples containing PC.

Assay of ^{32}P after 2-dimensional TLC of the water-soluble glycerophosphate esters obtained from the phospholipids of strain LPR 2120 by alkaline methanolysis (Fig. 2, Table 2) gave results in excellent qualitative and quantitative agreement with those obtained with intact phospholipids.

TABLE 1

Phospholipid Compositions of *R. meliloti* Strains

	(% of Lipid phosphorus)							
	Strain LPR 2120				Strain LPR 2121			
	Logarithmic		Stationary		Logarithmic		Stationary	
	P analysis	^{32}P	P analysis	^{32}P	P analysis	^{32}P	P analysis	^{32}P
PE[a]	28.5	27.8	21.6	19.9	32.3	38.6	23.0	22.4
PME	26.6	27.1	23.4	21.0	23.9	24.4	21.2	23.0
PDME	2.9	3.4	2.9	4.1	2.6	3.4	3.8	4.0
PC	20.4	15.0	28.3	26.3	20.3	12.0	27.9	26.6
PG				11.2[b]				11.5[b]
CL				12.0[b]				9.7[b]
PG + CL	21.2	26.3	23.4	28.5	20.7	26.3	24.2	23.2

[a]Abbreviations: PE, phosphatidylethanolamine; PME, phosphatidyl-N-methylethanolamine; PDME, phosphatidyl-N, N-dimethylethanolamine; PC, phosphatidylcholine; PG phosphatidylglycerol; Cl, cardiolipin.

[b]Separated by 2-dimensional TLC on Adsorbosil Plus-1 Prekotes (Applied Science).

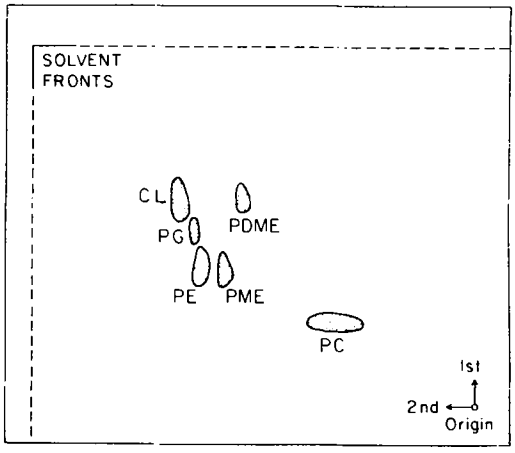

FIG. 1. Two-dimensional TLC of *R. meliloti* phospholipids. The solvent systems are described under Materials and Methods. Lipids were stained with iodine vapor.

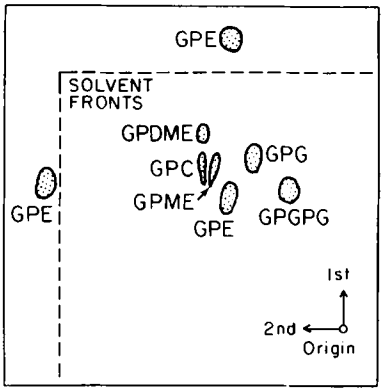

FIG. 2. Two-dimensional TLC of the deacylation products of *R. meliloti* phospholipids. Chromatography was on plastic-backed cellulose sheets as described (19). Glycerophosphorylethanolamine standards were chromatographed on the edge of the sheet, as shown. Abbreviations are given in Table 2.

The other strains of *R. meliloti*, ATCC 9930, LPR 2136, LPR 2137 and LPR 2139, analyzed for phospholipid composition by incorporation of ^{32}P followed by 2-dimensional TLC of intact lipids gave the following average compositions (as percentage of ^{32}P): PE plus PME, 50.8 ± 7.5%; PDME, 2.7 ± 0.85%; PC, 20.9 ± 5.6%, PG, 16.8 ± 1.5%; and cardiolipin, 8.0 ± 0.8%. These values are similar to those obtained with the 2 strains analyzed in detail (Tables 1 and 2). As with strains 2120 and 2121, no significant differences were seen in the proportions of phospholipids in strains without or with the Ti plasmid derived from *A. tumefaciens*.

TABLE 2

Glycerophosphate Esters Derived from *R. meliloti* LPR 2120 by Alkaline Methanolysis

Ester	% of ^{32}P
GPE[a]	21.3
GPME	17.8
GPDME	3.7
GPC	27.8
GPGPG	15.3
GPG	14.2

[a]Abbreviations: GPE, glycerophosphorylethanolamine; GPG, glycerophosphorylglycerol; GPC, glycerophosphorylcholine; GPME, glycerophosphoryl N-methylethanolamine; GPDME, glycerophosphoryl N,N-dimethylethanolamine; GPGPG diglycerophosphorylglycerol.

DISCUSSION

The phospholipid composition of *R. meliloti* closely resembles that previously described for *A. tumefaciens* (4,5,20,21), *Agrobacterium radiobacter* and *Agrobacterium rhizogenes* (5). All agrobacteria were found to contain $\geqslant$ 7% PC, which in *A. tumefaciens* increased with progression of cultures from log to stationary phase (21). In *R. meliloti,* PC also increases in stationary phase cells (Table 1). The ratio of PME to PE is higher in *R. meliloti* than in *A. tumefaciens* at all stages of growth, and is one of the highest observed among gram-negative bacteria that contain PC (22). Short et al. (19) found 42% PME, 20% PE and 1.5% PC in *Thiobacillus (Ferrobacillus) ferrooxidans.*

These data suggest that the first N-methylation of PE in *R. meliloti* and especially in *T. ferrooxidans* is rapid relative to the second and third N-methylations. Genetic evidence in *Neurospora* suggests that a single enzyme catalyzes the second and third N-methylation steps (23). The fact that no prokaryotic species has been observed to synthesize PDME, in the absence of PC (22), suggests that this may also be true for prokaryotes.

Among the *Rhizobium* species examined, none has a phospholipid composition identical to *R. meliloti.* The closest, *R. leguminosarum,* has PE, PDME, PC, PG and cardiolipin, but no PME. Presumably, PME is an intermediate in the synthesis of PC, but does not accumulate in this species. *R. japonicum* strains were found to contain PS (8.5-18.5%) in addition to PE, PC and cardiolipin. Small amounts of other phospholipids, which may have been methylated intermediates of the PC synthesis pathway, were not identified (6). Also, it is not certain that the solvent systems used for 2-dimensional TLC would have resolved PE and PME (5). Two strains of *Rhizobium* that infect *Lotus pedunculatus* were found to contain PI only in the bacteroid, but not the free-living form. Another strain also contained PS, but only in the free-living form (8).

Vincent (1) points out that agrobacteria share several properties with fast-growing rhizobia, which includes *R. meliloti* and the *R. leguminosarum-R. trifolii-R. phaseoli* group. They are most like *R. meliloti* in many biochemical, nutritional and growth characteristics. Our findings on the phospholipid composition of *R. meliloti* also argue for a close relationship with the agrobacteria; however, other criteria justify separation of agrobacteria and *R. meliloti* at the generic level (1).

The findings that the absence of the Ti plasmid of *A. tumefaciens* does not affect the ability of this organism to synthesize PC and its presence does not affect the quantitative phospholipid composition of *R. meliloti,* argue for the chromosomal location of the genes coding for the enzymes that transfer methyl groups from S-adenosylmethionine to PE. These genes have not been mapped in prokaryotes, since no mutants have been available.

The presence of PC in prokaryotes has been correlated with the presence of efficient electron transport systems (24), and with extensive intracytoplasmic membrane systems (25), 2 characteristics that are often present in the same organisms. *Agrobacterium* does not possess complex intracytoplasmic membrane systems (26) and although aerobic, it does not have the specialized, efficient electron transport systems found in photosynthetic or chemoautotrophic species. Dart and Mercer (27,28) demonstrated a well developed intracytoplasmic membrane system in the bacteroids of *R. trifolii,* which were not found in the rodform of this species. No intracytoplasmic membranes were observed in a parallel study of *R. meliloti* bacteroids in the barrel medic plant. Other recent ultrastructural studies of bacteroids in root nodules, including one of *R. meliloti,* did not provide evidence of intracytoplasmic membranes (29-32); however, the methods of fixation may not have been optimal for demonstrating these structures (27,28). In a preliminary report of a study of 2 unidentified species of *Rhizobium,* Raveed and Reed presented evidence for a system of 100-300 nm diameter vesicles, invaginating from the plasma membrane, and a set of 50-75 nm vesicles on the plasma membrane adjacent to the larger vesicles. These vesicles were stained with ferritin-labeled antibodies directed to nitrogenase (33).

As an alternative to the involvment of PC in the formation of intracytoplasmic membranes, one of us has suggested that the evolution of the PC synthesis pathway in the Rhizobiaceae may in some way be related to the requirements of the bacterial-host interactions (22). The question of the role of PC in the infection of plants or in the bacterial-host symbiotic relationship, awaits isolation of suitable mutants in this pathway.

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Biosynthesis of the Novel Fatty Acid, 17-Methyl-*cis*-9,10-methyleneoctadecanoic Acid, by the Parasitic Protozoan, *Herpetomonas megaseliae*

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ABSTRACT

Herpetomonas megaseliae, a flagellate protozoan parasite of the gut of a dipteran, *Megaselia scalaris*, is shown by chromatographic, spectrometric and radiotracer methods to synthesize de novo an *iso*-branched chain cyclopropane fatty acid, 17-methyl-*cis*-9, 10-methyleneoctadecanoic acid. Lipids 18:xxx-xxx, 1983.

Trypanosomatid flagellates are mainly parasites of the alimentary tract of insects and some other invertebrates. A few are etiological agents of economically and medically important diseases in vertebrates and higher plants. Many species contain a cyclopropane fatty acid, *cis*-9, 10-methyleneoctadecanoic acid, acylating phosphatidylethanolamine (1). This fatty acid, common among bacteria (2) but very rarely observed in eukaryotes (3,4), is formed by the methylenation of phosphatidylethanolamine-bound oleic acid (5). Its presence in bacteria appears to be associated with environmental stresses (6,7), and in trypanosomatids may be related to physical and chemical characteristics of microhabitats in the insect hosts.

Some species of the genus *Herpetomonas* also form extraordinary amounts of branched fatty acids (8), including unique *iso*-branched, even-numbered, unsaturated forms with 1-5 double bonds. The *iso* analog of oleic acid, 16-methylheptadecenoic acid, is the major component. Methylenation of the double bond of *iso*-oleic acid would yield 17-methyl-*cis*-9, 10-methyleneoctadecanoic acid, a fatty acid not reported heretofore as a natural product. For this reason, it was sought in the lipids of *Herpetomonas megaseliae*, an intestinal parasite of a dipteran, *Megaselia scalaris* (9).

MATERIALS AND METHODS

H. megaseliae, ATCC 30209, was grown at 25 C in a fatty acid-free medium; RE III of Steiger and Steiger (10). harvested by centrifugation in late logarithmic growth phase and directly saponified under N_2 with KOH (5% in 70% methanol, 3 hr). Unsaponifiables were removed with petroleum ether, the solution was acidified, and the fatty acids were extracted with $CH_3CH_2OCH_2CH_3$, dried over anhydrous Na_2SO_4, and converted to their methyl esters by reaction with CH_2N_2. Purified fatty acid methyl esters (FAME) were collected from the methylation reaction products by preparative, Silica Gel H, thin layer chromatography (TLC) (solvent, C_6H_5; eluent, $CH_3CH_2OCH_2CH_3$/CH_3OH, 9:1, v/v), and fractionated by preparative, Silica Gel H, argentation TLC ($AgNO_3$ 5%, w/w; solvent, C_6H_5; eluents, $CHCl_3$ and $CHCl_3/CH_3(CH_2)_4CH_3$, 1:1, v/v).

The saturated FAME fraction was examined by support-coated open-tabular (SCOT) column gas liquid chromatography (GLC), with stainless steel columns (0.5 mm id × 15 m) containing DEGS and OV 101 (Perkin-Elmer Corp.) as stationary phases, in a Hewlett-Packard Co. 5830A with flame ionization detection (FID), at an oven temperature of 180 C and He carrier gas column flow rate of 5 ml/min. Components were detected with the GLC properties of straight chain C_{19} (*n*-19), *iso*-branched C_{19} (*iso*-19), *anteiso*-branched C_{19} (*anteiso*-19), *cis*-9,10-methyleneoctadecanoate (cyclo-19), and what appeared to be 17-methyl-*cis*-9,10-methyleneoctadecanoate (*iso*-cyclo-19) (Table 1). The *anteiso*-19 and *iso*-cyclo-19 together comprised 85% of the C_{19} FAME.

The saturated FAME fraction was then submitted to preparative, high polarity, packed column GLC (glass, 4 mm id × 3 m, 10% DEGS on 80-100 mesh Chromosorb W-AW [Supelco, Inc.]; Hewlett-Packard Co. 5734A, TCD, oven temperature programmed from 160 C to 200 C at 2 C/min, He flow rate 100 ml/min), to separate the presumed *anteiso*-19 and *iso*-cyclo-19, which had similar RRT on DEGS, from *n*-19, *iso*-19 and cyclo-19, and to collect them. The collected fraction presumed to contain *anteiso*-19 and *iso*-cyclo-19 was, in turn, submitted to preparative, low polarity, packed-column GLC (glass, 4 mm id × 3 m, 15% OV 101 on 60-80 mesh Gas Chrom Q [Supelco,

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Inc.]; Hewlett-Packard Co. 5734A, TCD, oven 230 C isothermal, He flow rate 100 ml/min). Two major recorder peaks were observed and their corresponding fractions were collected. These fractions were identified as *anteiso*-19 and *iso*-cyclo-19 by SCOT GLC.

Thermal decomposition products of the GLC column stationary phases were removed from the presumed *iso*-cyclo-19 by silicic acid column chromatography (11), and it was then examined by proton nuclear magnetic resonance (^{1}H NMR), infrared (IR), and electron impact mass spectrometry (MS). ^{1}H NMR spectra of the methyl ester in $CDCl_3$ were recorded on a Varian XL-100-5 (100.1 MHz), in the Fourier transform mode, at 28 C with tetramethylsilane as reference. IR spectra were obtained with a Perkin-Elmer Model 457A, on thin films of the methyl ester (10 μg/μl benzene) spread between AgCl discs, with polystyrene as reference. Mass spectra of the pyrrolidide derivative of the fatty acid (12) were recorded with a Finnigan 3100 mass spectrometer, at an ionizing energy of 70 eV and a temperature of 180 C, with samples introduced by direct probe insertion.

To obtain information on the biosynthesis of the presumptive *iso*-cyclo-19, *H. megaseliae* was grown in RE III medium as before, but with radioisotopes of those amino acids known to be precursors of the functional groups of interest in an *iso*-branched, odd-numbered, cyclopropane fatty acid; i.e., L-[3,4-^{3}H]valine, source of the terminal isopropyl group (13), and L-[methyl-^{14}C]methionine, source of the methylene group donated to form the cyclopropane ring (5). The presumptive *iso*-cyclo-19 was collected from the harvested cells as described above, and its content of ^{3}H and ^{14}C assayed by standard techniques of liquid scintillation spectrometry for compounds doubly labeled with ^{3}H and ^{14}C.

RESULTS AND DISCUSSION

The ^{1}H NMR spectrum of the postulated *iso*-cyclo-19 methyl ester contained the following proton resonance intensity peaks (δ); multiplet at -0.3, proton *cis* to the alkyl substituents in a cyclopropane ring near the center of the carbon chain (1H); multiplet at +0.6, other 3 protons of such a cyclopropane ring (3H); well separated doublet at 0.9, terminal isopropyl group (6H); multiplet at 1.3, methylene groups in the carbon chain (2H each, total 22), triplet at 2.33, protons of the 2-carbon in an ester (2H); singlet at 3.7, methyl group of ester (3H). The resonance peaks and the proton count were those anticipated for a C_{19}, saturated fatty acid methyl ester with a terminal isopropyl group, and with a *cis*-cyclopropane ring near the center of the carbon chain (14).

The infrared spectrum of the methyl ester showed absorption bands at 1020 cm^{-1} and 3076 cm^{-1} characteristic of a cyclopropane ring structure (11), and a doublet in the 1360-1380 cm^{-1} range diagnostic of a terminal isopropyl group (15).

The mass spectrum of the pyrrolidide derivative gave the mass ion (m/e 349) appropriate to the presumptive identification made on the basis of GLC information. The base peak was the McLafferty rearrangement ion, m/e 113, while the peak at m/e 154 was decreased in intensity as a consequence of the rearrangement. Peaks of ion clusters were 14 amu apart except between m/e 196 and m/e 208, where the interval was 12 amu. This feature and the dominant peaks at m/e 182, m/e 222, m/e 236 and m/e 250 are diagnostic of a cyclopropane ring structure at the 9,10-position in the carbon chain (12). Other peaks in the spectrum typical of the positional isomer were also present, i.e., m/e 180, m/e 194, m/e 210, m/e 279. Finally, at m/e 320, there was a peak of low intensity,

TABLE 1

Support-Coated Open-Tubular GLC of the Methyl Esters of the Saturated C_{19} Fatty Acids of *H. megaseliae*

Methyl ester	Abbreviation	% Saturated fatty acid methyl esters[a]	RRT[b] SCOT GLC DEGS	OV 101
Nonadecanoic	*n*-19	0.5	1.38	1.30
17-Methyloctadecanoic	*iso*-19	1.8	1.23	1.20
16-Methyloctadecanoic	*anteiso*-19	9.5	1.28	1.23
cis-9, 10-Methyleneoctadecanoic	cyclo-19	1.0	1.46	1.24
17-Methyl-*cis*-9, 10-methyleneoctadecanoic	*iso*-cyclo-19	9.4	1.31	1.13

[a]Estimated from GLC recorder peak areas.
[b]Retention time relative to methyl stearate; DEGS 8 min, OV 101 4 min.

bracketed by higher intensity peaks at m/e 306 and at m/e 334. This pattern is characteristic of fatty acid pyrrolidides with a terminal isopropyl group, i.e., *iso* acids (12).

All the spectrometric analyses confirmed the structure of the unknown, suggested by the GLC analyses as 17-methyl-*cis*-9,10-methyleneoctadecanoic acid.

When *H. megaseliae* was grown in the fatty acid-free RE III medium (10), the odd-*iso* cyclopropane fatty acid must have been formed from simple precursors. Drawing upon knowledge of the biosyntheses of *iso*-branched and cyclopropane fatty acids in trypanosomatids (16) and bacteria (5,13), we speculated that the following pathway might be operating: (a) exogenous valine (from RE III medium) is deaminated; (b) the product, α-ketoisovaleric acid, is oxidatively decarboxylated in the presence of CoASH to yield the universal branched primer for the even-number *iso*-series of fatty acids, isobutyryl-CoA; (c) repeated condensations of malonyl-CoA, first with *iso*-butyryl-CoA and then with subsequent condensation products, in the presence of NADPH and catalyzed by a branched chain fatty acid synthetase complex, form isopalmitoyl-CoA; (d) chain elongation, by a microsomal system, again by malonyl-CoA condensation in the presence of NADPH, forms isostearoyl-CoA; (e) Δ^9-desaturase-catalyzed oxidative desaturation is followed by transacylation of the isooleoyl product to phosphatidylethanolamine; and (f) cyclopropane synthase-mediated S-adenosylmethionine methylenation of the double bond yields phosphatidylethanolamine-linked isodihydrosterculic acid (17-methyl-*cis*-9,10-methyleneoctadecanoic acid).

When *H. megaseliae* was grown in RE III medium in the presence of L-[3,4-^{3}H] valine (New England Nuclear; sp act 50.9 Ci/mM; 50 μCi/l) and L-[methyl-^{14}C] methionine (New England Nuclear; 54 mCi/mM; 10 μCi/l), the distributions of radioactivity observed in the major fatty acid methyl ester fractions collected from the saturated fatty acids by preparative GLC were as shown in Table 2. The even number *iso*-fatty acids (*iso*-14, *iso*-18, *iso*-20) were heavily labeled with ^{3}H, as would be expected if valine acted as precursor of isobutyryl-CoA (13). The *anteiso*-fatty acids (*anteiso*-15, *anteiso*-17, *anteiso*-19) were poorly labeled with ^{3}H. Isoleucine is the amino acid source of α-methylbutyryl-CoA, the universal branched primer for the odd number *anteiso*-series of fatty acids. The odd numbered and *iso*-branched cyclopropane fatty acid (*iso*-cyclo-19) was heavily labeled with both ^{3}H and ^{14}C, as would befit a fatty acid formed by the methylenation of the even numbered, *iso*-branched, monoenoic, 16-methylheptadecenoic acid (*iso*-oleic acid).

The radiotracer experiment, then, supports the proposed biosynthetic pathway. It demonstrates that the presence of the terminal isopropyl group of 16-methylheptadecanoic acid (*iso*-stearic acid) does not impair the Δ^9-desaturase catalyzed formation of *iso*-oleate from *iso*-stearate, the transacylase mediated linkage of *iso*-oleate in phosphatidylethanolamine, and the cyclopropane synthase catalyzed donation of the methyl group of S-adenosylmethionine to *iso*-oleate to form 17-methyl-*cis*-9,10-methyleneoctadecanoate.

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TABLE 2

Distributions of Radioactivity Among the Major Saturated FAME from *H. megaseliae* Grown with L-[3,4-^{3}H] Valine and L-[Methyl-^{14}C] Methionine

Methyl ester	Abbreviation	% Saturated fatty acid methyl esters[a]	Relative specific dpm[b] ^{3}H	^{14}C
12-Methyltridecanoic	*iso*-14	6.2	72,930	–
12-Methyltetradecanoic	*anteiso*-15	16.6	2,890	–
14-Methylhexadecanoic	*anteiso*-17	19.4	8,840	–
16-Methylheptadecanoic	*iso*-18	20.8	156,910	–
16-Methyloctadecanoic	*anteiso*-19	9.5	4,896	830
17-Methyl-*cis*-9, 10-methyleneoctadecanoic	*iso*-cyclo-19	9.4	53,040	38,090
18-Methylnonadecanoic	*iso*-20	6.8	35,700	2,400

[a]Estimated from GLC recorder peak areas.
[b]dpm associated with the fatty acid methyl ester collected by GLC, divided by the % of the ester in the sample injected.

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Enzymatic Hydrolysis in vitro of Thermally Oxidized Sunflower Oil

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ABSTRACT

The hydrolysis of thermally oxidized sunflower oil by pancreatic lipase was studied in relation to chemical changes in the acylglycerols. Four classes of compounds (monomers, dimers, trimers and polymers) formed from the acylglycerols were separated from the heated oils by column chromatography on silica gel, and further verified by thin layer chromatography. Each fraction, after analyses for generaly properties, was subjected to a time course study of hydrolysis by pancreatic lipase over a 30-min period. After 70 hr of heating, the amount of hydrolysis for the acylglycerol dimers was only about half that of the monomers, and that for the trimers was, in turn, about one-third that of the monomers. The polymers were the least hydrolyzed and showed no further reaction after 5 min. The reduction in enzymatic hydrolysis of isolated fractions from the thermally oxidized oils indicates structural differences, related to formation of polar compounds and polymerization products. Adverse effects on animals from feeding these materials can be attributed partly to inhibition of hydrolysis resulting in less available energy.
Lipids 18:000-000, 1983.

The biological properties of thermally oxidized oils are closely related to their chemical properties. Heating of oils changes their composition and 2 typical compounds are formed (1-6); one is volatile breakdown derivatives, and the other is NVOP. The amount of decomposition products increases gradually, and after a certain heating time, the oil shows defects in odor and taste. Decomposition products may accumulate to such an extent that the oil should be regarded as deteriorated. However, extensive exposure to heat and oxygen is needed for the oil to reach a state in which it could be detrimental to health (7,8).

NVOP are ingested with fried foods, and their analyses have shown the presence of potentially toxic compounds (9,10). However, any harmful effects would be dependent on hydrolysis and intestinal absorption, as well as uptake into the lymph which has been determined only in a limited number of cases (11, 12). Indeed, thermally oxidized fat is not so rapidly hydrolyzed as the unheated material (13). We have reported that among individual fractions, the monomers were hydrolyzed at least as rapidly as the corresponding unheated oils, whereas the dimers were hydrolyzed slowly (14).

In the present report, thermally oxidized sunflower oil heated for various periods of time was fractionated into 4 classes based on polarity of the acylglycerol products. Time course lipase hydrolyses were carried out for the unheated oil, heated oils and heated oil fractions to study relations to chemical properties. Effects of enzyme inhibition were observed by calculation of hydrolysis rates.

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Abbreviations: TLC, thin layer chromatography; NVOP, nonvolatile oxidation products; IPE, isopropyl ether; HEX, *n*-hexane; DEE, diethyl ether.

MATERIALS AND METHODS

Fractionation of Thermally Oxidized Oils

A commercially refined sunflower oil was heated at 180 C for 50, 70 or 100 hr in the presence of air (400-600 ml/min), using the apparatus described in a previous report (14). The thermally oxidized oils were fractionated on a column of silica gel (Bio-Sil A, 100/200 mesh, BIO-RAD Laboratories, Mississauga, Ontario) with modifications of the methods of Ota et al. (15), and Ohfuji and Kaneda (16). The columns were prepared as described previously (14), and a measured quantity (1.0 g) of thermally oxidized oil was added with 3 ml of 5% IPE in HEX. By successive elution with 150 ml of 20% IPE in HEX (fraction I), 125 ml of 60% IPE in HEX (fraction II), 125 ml of IPE (fraction III) and then 150 ml of DEE (fraction IV), the thermally oxidized oils were separated into 4 fractions. TLC was used to verify the separations, and when needed, further purification was done with a silica gel column. The acylglycerol monomers were eluted in fraction I, the dimers in fraction II, the trimers in fraction III, and the polymers containing highly polar NVOP in fraction IV, respectively (17).

Determination of Chemical Properties for Fractionated Thermally Oxidized Oils

Carbonyl values were determined as described previously (14), and iodine values

according to the AOCS Official Method Cd 1-25 (18). The ester bond was estimated by the procedure of Snyder and Stephens (19).

Hydrolysis of Fractionated Thermally Oxidized Oils by Lipase

The general technique described by Mattson and Volpenhein (20), as modified (14), was used. Briefly, at least 100 mg of the oils was suspended in 6.0 ml of 0.2 M Tris buffer (pH 7.6) containing 0.25 M $CaCl_2$. To this suspension, 100 mg of pancreatic lipase (Sigma Chemical Co., St. Louis, MO) was added. All the other reaction conditions except for incubation time were the same as described earlier (14,20). After 5, 10, 15, 20 or 30 min of incubation with continuous rapid agitation, 3 ml of alcohol was poured into the reaction mixture, and it was extracted 3 times with 10 ml of DEE. The hydrolysates were recovered from the upper layer, and then titrated with 0.02 N KOH in alcohol using 0.1% phenolphthalein in alcohol as an indicator. Control experiments without added enzyme were carried out under the same conditions, and the values obtained were deducted as blanks to estimate the acids released at various times due to the hydrolysis procedure. These data are compared with the relative values from the hydrolysis of the thermally oxidized oils after 50, 70 and 100 hr of heating, and those values for unheated sunflower oil.

RESULTS

Analytical data for fresh and thermally oxidized sunflower oils are in Table 1. As expected, the carbonyl values increased and the iodine values decreased relative to the duration of heating. No differences were noted in saponification values, nor in ester contents. These data will serve as reference information for the various fractions isolated from the thermally oxidized oils.

Table 2 shows the yield (wt %) of the fractions from the thermally oxidized oils after heating based on the amount of samples put on the fractionating columns. The acylglycerol monomers (fraction I) decreased substantially with heating and these values were compensated for by increases of more polar materials. Figure 1 is an example of a TLC chromatogram of a thermally oxidized oil (70 hr heating), which had been fractionated by column chromatography on silica gel. Although fraction I contained a small amount of hydrocarbons near the solvent front (less than 0.2%), it was not further purified because they had no effect on hydrolysis in the preliminary experiments. Fraction I consisted of acylglycerol monomers, and fractions II-IV comprised the dimers, trimers and polymers, respectively (16,21). The results were similar to the observations of Paradis and Nawar (17) using heated corn oil.

Table 3 shows chemical properties for the fractions from the thermally oxidized oils as indicators of thermal oxidative and polymeric deterioration. This resulted in greatly reduced iodine values, and carbonyl values that increased considerably during heating. For fractions II, III and IV, there was a steady increase in saponification values during heating, which was more evident with higher polarity. Regarding total ester bonds, no significant differences were observed among the monomers, dimers and trimers. However, the amounts in the polymer fractions showed significant decreases, ca. 60 μmol for the 70 hr of heating, and ca. 90 μmol for the 100 hr of heating.

The changing patterns for the hydrolysis of the unheated oil, and the heated oils by pancreatic lipase are shown in Figure 2. Comparable values for the heated oil fractions are in Figure 3. The results represent the ratio of the free fatty acids released (AV) during the enzy-

TABLE 1

Analytical Data for Fresh and Thermally Oxidized Sunflower Oils[a]

Property	F[b]	Heating period (hr) 50	70	100
Peroxide value (meq/kg)	2.4[e]	5.7[d]	2.1[e]	0.5[f]
Carbonyl value (meq/kg)	3.0[d]	105.4[e]	113.5[e]	156.7[f]
Iodine value	137.3[d]	115.7[e]	102.5[e]	87.3[f]
Saponification value	178.6[d]	179.5[d]	181.0[d]	182.1[d]
Ester content (μmol/100 mg lipid)	338.8[d]	334.4[d]	331.3[d]	325.9[d]
Relative ester content (%)[c]	(100.0)	(98.7)	(97.8)	(96.2)

[a]Each value is an average of 5 determinations.
[b]F = unheated oil.
[c]Expressed as triolein, based on 338.8 μmol/100 mg lipid.
[d-f]Values without the same superscript are significantly different ($P<0.05$).

TABLE 2

Yield (%) of Fractions from Thermally Oxidized Oils[a]

Fraction	Effuent solvent[b]	Heating period (hr)[c] 50	70	100
I	20% IPE in HEX	70.4±2.2[d]	60.5±2.1[e]	49.4±1.9[f]
II	60% IPE in HEX	10.1±0.3[d]	13.6±0.5[e]	14.5±0.5[e]
III	IPE	9.6±0.3[d]	13.1±0.5[e,f]	15.5±0.6[f]
IV	DEE	8.3±0.3[d]	9.9±0.4[d]	15.9±0.6[f]

[a]Mean ± SEM (n=5).
[b]IPE = isopropyl ether; HEX = n-hexane; DEE = diethyl ether.
[c]The recoveries of the oils were 98.4, 97.1 and 95.3% for 50, 70 and 100 hr, respectively.
[d-f]Values without the same superscript are significantly different ($P<0.05$).

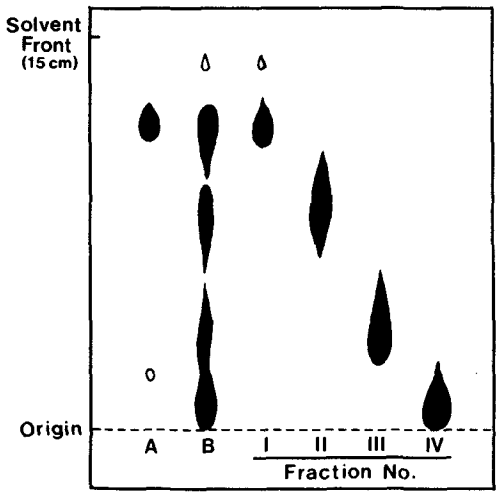

FIG. 1. Thin layer chromatogram for the thermally oxidized oil at 70 hr of heating. The lipids were developed with petroleum ether/diethyl ether/acetic acid (70:30:2, v/v/v) and detected with iodine vapor. A = unheated oil, B = heated oil, I-IV = fractionated compounds (see Materials and Methods).

matic reaction relative to the respective saponification values (SV) according to Iwai et al. (22). After 30 min incubation, the unheated oil was more than 30% hydrolyzed. However, for the heated oils, the values decreased to 23, 18 and 13%, respectively, for the 3 periods of heating. As shown in Figure 3, each fraction obtained from the thermally oxidized oils was hydrolyzed to a considerable degree in the initial 5 min of incubation. Regardless of the heating period, the monomers were hydrolyzed at least as well as the unheated oil. The other 3 fractions (dimers, trimers and polymers) were less well hydrolyzed as the heating period was extended. For example, after 70 hr of heating, the total hydrolysis for the dimers was ca. 50% that of the monomers, whereas hydrolysis for the trimers was ca. 33% that of the monomers. The hydrolysis rates for the trimers and polymers being relatively lower remained almost constant throughout the incubation.

DISCUSSION

It has been known for many years that heating of oils produces compounds which are more difficult to hydrolyze with lipase (13,23). The formation of these compounds in a vegetable oil depends on its degree of unsaturation and the temperature to which it is exposed (24). Sunflower oil heated at a temperature for domestic frying was separated by means of column chromatography into 4 fractions (monomers, dimers, trimers and polymers). The enzymatic hydrolyses of the fractionated products were studied in relation to the chemical properties.

Relatively high initial reaction rates are characteristic for the in vitro system used, but no real differences in the hydrolysis of the monomers was observed among the heating periods (Fig. 3), and they were hydrolyzed at least as well as the unheated oil. Although the monomer fractions isolated after heating showed some changes in their chemical properties (Tables 1 and 3), this amount of oxidation did not affect their hydrolyses by pancreatic lipase. On the other hand, there were decreases in hydrolysis for the dimers and trimers proportional to the heating period (Fig. 3). For all 3 of these fractions, the ester contents remained unchanged regardless of the severity of heating, but the chemical properties of the dimers and trimers showed more evidence of oxidative deterioration which was reflected in reduced hydrolysis. Eisenhauer et al. (25) indicated that thermal polymerization involves 2 or more molecules. With the monomers, it is possible to hydrolyze

TABLE 3

Analytical Data for Fractions from Thermally Oxidized Oils[a]

Fraction	Property	Heating period (hr) 50	70	100
I	Carbonyl value (meq/kg)	8.1[c]	23.2[d]	26.5[d]
	Iodine value	116.8[c]	105.7[c,d]	93.6[e,f]
	Saponification value	178.0[c]	180.1[c]	180.7[c]
	Ester content (μmol/100 mg lipid)	338.8[c]	336.7[c]	336.2[c]
	Relative ester content (%)[b]	(100.0)	(99.3)	(99.2)
II	Carbonyl value (meq/kg)	84.7[e]	113.5[e,f]	142.3[f]
	Iodine value	101.2[d,e]	87.8[f,g]	79.8[f,g]
	Saponification value	180.5[c]	187.4[e,d]	190.2[c,d]
	Ester content (μmol/100 mg lipid)	336.7[c]	334.6[c]	334.1[c]
	Relative ester content (%)[b]	(99.3)	(98.8)	(98.6)
III	Carbonyl value (meq/kg)	143.7[f]	183.5[g]	225.2[h]
	Iodine value	96.4[d,e]	90.2[e,f]	74.6[g]
	Saponification value	182.0[c]	192.3[c,d]	198.6[d,e]
	Ester content (μmol/100 mg lipid)	335.2[c]	334.2[c]	332.7[c]
	Relative ester content (%)[b]	(98.9)	(98.6)	(98.2)
IV	Carbonyl value (meq/kg)	236.8[h]	282.9[i]	312.6[i]
	Iodine value	90.2[e,f]	89.3[e,f]	70.1[g]
	Saponification value	190.7[c,d]	208.4[e,f]	215.8[f]
	Ester content (μmol/100 mg lipid)	290.4[d]	277.8[d,e]	247.8[e]
	Relative ester content (%)[b]	(85.7)	(82.0)	(72.6)

[a]Each value is an average of 5 determinations.
[b]Expressed as triolein, based on 338.8 μmol/100 mg lipid.
[c-i]Values without the same superscript are significantly different ($P<0.05$).

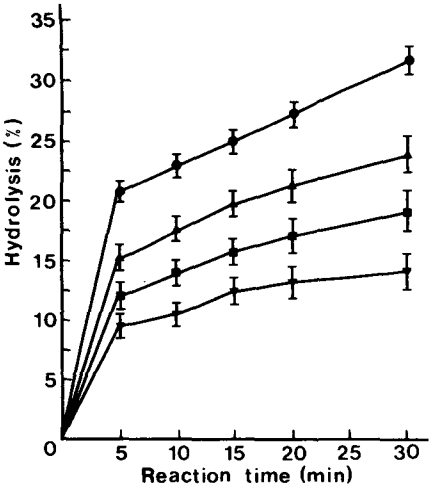

FIG. 2. Time course for the hydrolysis of thermally oxidized oils by pancreatic lipase. ● = unheated oil; ▲, ■ and ▼ = 50, 70 and 100 hr heated oils, respectively. Hydrolysis (%) = AV/SV × 100. Each value is an average of 5 determinations.

2 out of 3 of the ester bonds readily, whereas with the dimers and trimers which are larger, due to a number of different chemical entities and C-C linkages, internal ester groups would not be available for hydrolysis, so still only a maximum of 2 exposed ester groups on the primary carbons could be hydrolyzed.

The decrease in ester bonds for the polymers was substantial, and unique among the fractions. Fraction IV was more polar and complicated, and contained thermal degradation products capable of inactivating the enzyme (26). Table 4 shows hydrolyses (%) of the original oils by pancreatic lipase taken from Figure 2. Theoretical percentages of hydrolyses were calculated from the yield data in Table 2 and the hydrolysis value for each fraction as shown in Figure 3 at selected times of 5, 15 and 30 min. These values also are presented in Table 4 for comparison with the experimental values. The hydrolysis percentages calculated were always higher than those determined with the original heated oils. These oils contained numerous oxidation and decomposition prod-

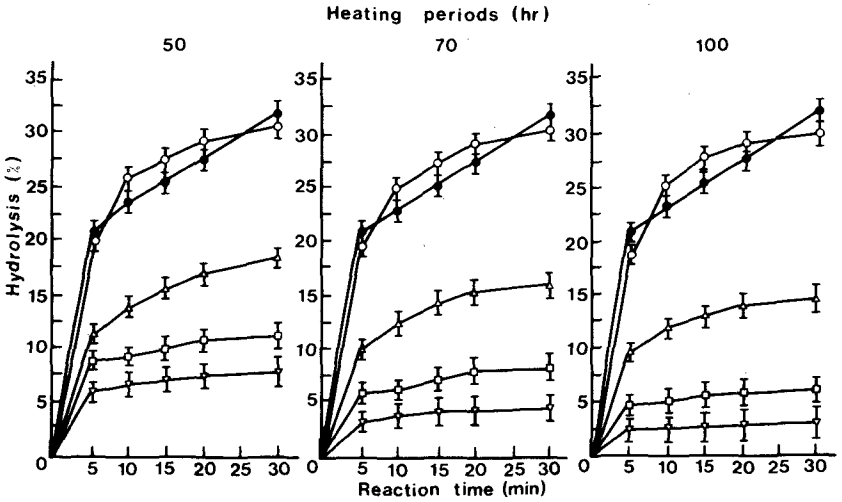

FIG. 3. Time course for the hydrolysis of fractionated oils by pancreatic lipase. • = unheated oil; ○, △, □, and ▽ = monomer, dimer, trimer and polymer, respectively. (See Fig. 2 for additional information.)

TABLE 4

Percent Hydrolysis by Pancreatic Lipase of Thermally Oxidized Oils[a]

Heating Period (hr)	Reaction time (min)					
	5		15		30	
	Found[b]	Calculated[c]	Found	Calculated	Found	Calculated
50	15.2±0.7[d,e]	15.9±0.6[d]	20.6±0.9[d]	21.5±0.8[d]	23.5±1.1[d,e]	24.7±1.1[d]
70	12.3±0.6[e,f]	12.8±0.5[e,f]	16.8±0.8[e,f]	18.9±0.7[d,e]	18.9±0.9[f,g]	21.6±0.9[e,f]
100	9.3±0.4[g]	10.4±0.4[f,g]	12.3±0.6[h]	15.5±0.5[f,g]	13.7±0.7[h]	17.2±0.6[g]

[a]Mean ± SEM (n=5).
[b]Values obtained by pancreatic lipase using thermally oxidized oils as shown in Fig. 2.
[c]Calculated from data in Table 2 and the hydrolysis (%) of each fractionated compund as shown in Fig. 3.
[d-h]Values in each column without the same superscript are significantly different ($P<0.05$).

ucts formed during the heating process, and some are capable of inactivating the enzyme (26). The recoveries of the oils after column chromatography were ca. 98% at 50 hr of the heating, 97% at 70 hr and 95% at 100 hr (Table 2). The more polar products tended to be absorbed on the silica gel, and not eluted with the solvents (27) resulting in the lower recovery with longer heating. Therefore, the fractionated products, especially the monomers and dimers, were more purified than the original heated oils and relatively free of lipase inhibitors. Also, the greater part of the loss of recovery would be from the polymer fractions. The experimental hydrolyses values were somewhat depressed for the heated oils (Table 4), because the enzyme inhibitors were present in the whole unfractionated samples. In the case of the theoretical values, the inhibition would be significant only for fractions III and IV so the higher composite values calculated in Table 4 indicate that at least the monomers (fraction I) were not inhibited, and are most responsible for the greater total hydrolysis.

The enzymatic hydrolysis of the fractions from the thermally oxidized oils was reduced both by longer exposure to frying temperatures, as well as by increased polarity among the fractions. The latter is associated with changes in the composition of the acylglycerol compounds due to accumulation of oxidized and polymerized components.

ACKNOWLEDGMENTS

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A Prostaglandin-Like Fatty Acid from a Species in the Cyperaceae

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ABSTRACT

A C_{20} cyclic trihydroxy unsaturated fatty acid was isolated and characterized as a representative member of a group of oxygenated fatty acids from the aquatic sedge, *Eleocharis microcarpa*, Torr. Characterization of the compound as 11-hydroxy-14-(3,5-dihydroxy-2-methylcyclopentyl)-tetradec-9-ene-12-yneoic acid was accomplished by various chemical and spectral methods.
Lipids 18:000-000, 1983.

INTRODUCTION

In the course of our investigation of the allelopathic relationship between the fresh water sedge, *Eleocharis microcarpa*, Torr., and blue-green algae (Cyanochloronta), a relatively large number of hydroxy and hydroxy keto free fatty acids were isolated. These substances appear to occur naturally in *E. microcarpa* and are implicated as the allelopathic agents (1).

One of the compounds was investigated more thoroughly than the others and was characterized as 11-hydroxy-14-(3,5-dihydroxy-2-methylcyclopentyl)-tetradec-9-ene-12-yneoic acid. In view of its similarity to prostaglandins, to prostaglandin-like compounds produced enzymatically from other plants (2), and the importance of lipid peroxidation in general (3, 4), the occurrence should be of interest to other investigators. This class of compounds may have significance to algal succession (5).

MATERIALS AND METHODS

Plant Material

E. microcarpa, was collected from ponds located near Hattiesburg, Mississippi, and was extracted fresh or fresh frozen. A voucher specimen is on file in the University of Southern Mississippi herbarium.

Bioassays

Bioassays were used to follow activity against blue-green algae through each step of isolation and final purification. It was found that using sensitivity disks on agar plates sprayed with the challenge organism *Anabaena flos-aqua* gave results within 36 hr. The diameter of the zone of inhibition gave a rough measure of specific activity.

Chemicals

All solvents used in this investigation, except for high performance liquid chromatography (HPLC), were purchased as reagent grade and distilled in glass prior to use. HPLC grade was used for retention time studies. All solvent mixture ratios were v/v. Trimethylsilane derivatives were prepared with DMB Sil Prep (Applied Science Laboratories, State College, PA). Diazomethane used in making methyl esters was generated from Diazald, N-methyl-N-nitroso-*p*-toluenesulfonamide (Aldrich Chemical Company, Milwaukee, WI). 2-Thiobarbituric acid (TBA) and prostaglandins PGA_1 and PGE_1 were purchased from Sigma Chemical Company (St. Louis, MO).

Extraction

Wet *E. microcarpa* (typically 2 kg) was refluxed in 2 l of distilled water for ca. 1 hr and then allowed to cool. After Filtering, the resulting liquid was acidified to a pH of 2 with conc HCL and extracted 3 times with 200-ml portions of chloroform. The chloroform layers were combined and dried with anhydrous sodium sulfate. The chloroform was removed under reduced pressure. This procedure yielded 0.2 g of waxy yellow semisolid material.

Column Chromatography

Two cm glass columns of 80-200 mesh silica gel (50 g) were used. A 0.5-g portion of the crude extract was eluted with 300 ml of each chloroform (fraction I), 1:1 chloroform/acetone (fraction II), acetone (fraction III) and methanol (fraction IV).

Analytical and Preparative Thin Layer Chromatography (TLC)

Further purification of fraction II by TLC was difficult because of the large number of compounds and their functional similarity. Four solvent systems were employed for both

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analytical and preparative TLC: system I, 85:15:2, chloroform/methanol/water; system II, 20:1, chloroform/methanol; system III, 60:40:2, hexane/ethyl ether/acetic acid; and, system IV, 50:50:2, hexane/ethyl ether/formic acid. Analytical TLC was performed on precoated sheets of Silica Gel 60, F-254, 0.2 mm, on aluminum backing purchased from Brinkmann Instruments, Inc. (Westbury, NY).

Preparative TLC was done in 2 stages: first, solvent system I was used; second, after isolation, each band was chromatographed again using system IV. Separation procedures are summarized in Figure 1. The scheme was developed after much trial and error and the use of bioassay results for guidance. For simplicity, only R_f values for bands separated by rechromatography of band 4 are shown. All preparative plates were made in these laboratories using Brinkmann Silica Gel 60 with F-254 indicator at a thickness of 0.5 mm on 20 x 20 cm glass plates. From 15 to 20 mg of material to be separated were applied to each plate. Bands were visualized under UV light, scraped from the plates and eluted with 2:1 chloroform/methanol. All chemical and spectral studies were performed on freshly purified material.

Analytical HPLC

Retention times of components in fraction II were compared with prostaglandins PGA_1 and PGE_1. The comparison was made on an Alltech C_{18} column, 25 cm x 4.6 mm, 5 μ using a linear gradient of H_2O/methanol starting with 2:1 v/v and ending with 100% methanol. The pH of the water component was adjusted to 3.6 with acetic acid to retard ionization of the free acids. A Laboratory Data Control UV detector set at 280 nm was used to monitor eluted components.

Derivatization and Tests

Methyl esters were prepared with diazomethane, using methods described in the literature. Trimethylsilyl ether and ester derivatives were prepared by reacting ca. 1 mg of sample with 1 ml of DMF Sil Prep under anhydrous conditions for a minimum of 12 hr. Shorter time periods did not produce complete silylation. Ozonolysis was accomplished by passing ozone through 1-mg samples dissolved in chloroform followed by reduction with 5 mg of triphenylphosphine at room temperature overnight. Procedures for the TBA test (5) and for ketones using 2,4-dinitrophenylhydrazine are

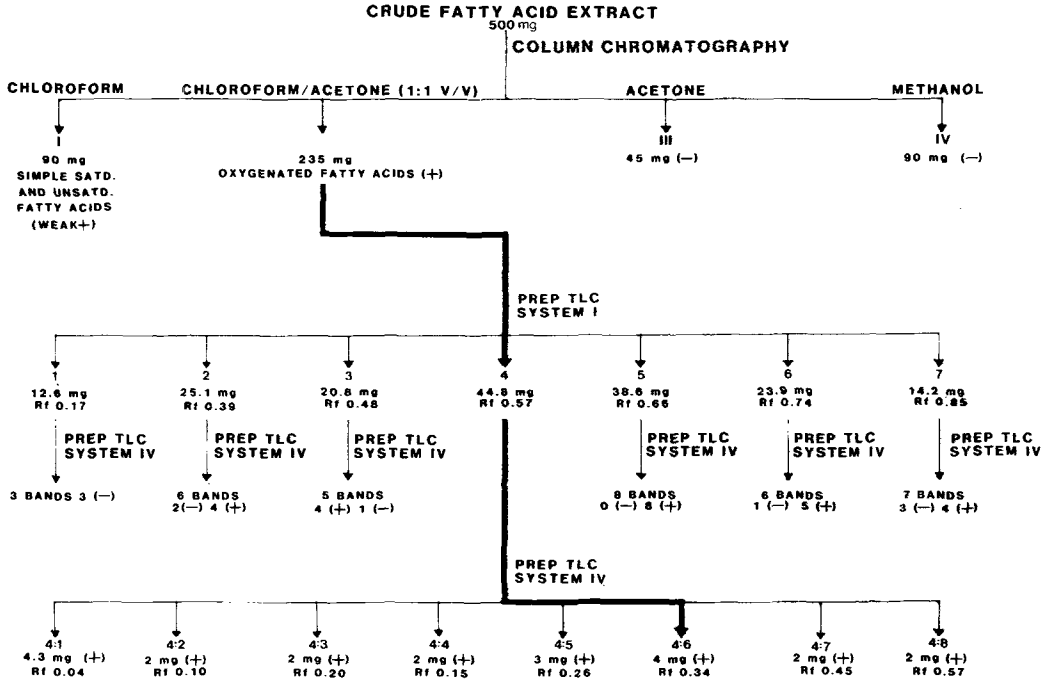

FIG. 1. Column and preparative TLC. Bioassays are shown in parentheses. (+) denotes at least a minimum detectable zone of inhibition around paper disc.

described in the literature.

Spectral Analysis

Infrared spectra were taken next on a Perkin Elmer 567 spectrophotomer and ultraviolet spectra with a Varian Cary 17 spectrophotometer. Low resolution mass spectra (MS) were obtained with a Dupont 2149 mass spectrometer. When gas chromatography (GLC) effluents of silylated derivatives were scanned, this instrument was connected to a Varian 2740 equipped with 6 ft × ⅛ in. stainless steel column packed with 3% Dexsil 300 on Chromosorb W AW. High resolution spectra were done at the Florida State University High Resolution Mass Spectroscopy Laboratory on an AEI MS-9. Both spectrometers were operated at an electron potential of 70 eV. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a JEOL FX90Q Fourier transform spectrometer.

RESULTS AND DISCUSSION

We chose to examine the oxygenated fatty acids in *E. microcarpa* because of bioassay data and previous reports (6,7) that suggested these materials may be natural algal inhibitors. Extraction of plant material with boiling water and partitioning into chloroform resulted in reasonable yields of free fatty acids that were relatively free of pigments and of other substances tending to interfere with column and TLC.

Fraction II, from column chromatography, gave typical carboxylic IR absorption (8) between 2850 cm^{-1} and 2350 cm^{-1} and at 1710 cm^{-1}. A positive test with 2,4-DNP and a strong IR absorption at 3400 cm^{-1}, which was unchanged by esterification, confirmed that this mixture contained keto and hydroxy free fatty acids. A positive test on fraction II with TBA indicated also the presence of endoperoxides or, at least, compounds which decompose under conditions of the test to give malonaldehyde (3,9).

Fraction II gave 43 bands in the final stage of preparative TLC. Thirty-three bands had definite activity. IR spectra are strikingly similar, exhibiting the same major features as noted for the unseparated fraction II. UV spectra are also similar: absorption at 275 nm and 220 nm, with the latter being strongest. Considering the ease with which polyunsaturated fatty acids can undergo autoxidation, the isolation and separation procedures were modified to minimize exposure to air and light. Band patterns remained unchanged. It therefore appears that these substances are produced by *E. microcarpa*.

Extraction of pond water from which *E. microcarpa* was gathered, followed by the same chromatographic procedures shown in the separation scheme produced many bands in common to those purified from the plant. Commonality was shown also by HPLC. Pond water concentrations of constituents equivalent to fraction II were ca. 0.5 ppm.

The results of the TBA test, IR and chemical evidence suggested that some components of fraction II may be similar to the prostaglandins. Relative retention times of PGA_1 and PGE_1 were compared with components of fraction II by HPLC. Figure 2 shows that elution occurs in the less polar half of the chromatogram.

STRUCTURAL STUDIES

The compound chosen for structural studies, I, (4:6 shown in Fig. 1), was roughly in the middle of those bands having good activity. The HPLC retention time is shown in Figure 2. IR (film) 3400 (broad), 2850 (broad) tailing to 2350, 2925 (s), 2850 (s), 1710 (s) cm^{-1}. IR (methyl ester) (film) 3400 (broad), 2925 (s), 2850 (s), 1735 (s) cm^{-1}. Little difference between I and its methyl ester was noted in the 3400 cm^{-1} region, but the broad absorption between 2850 and 2350 cm^{-1} was eliminated by esterification which confirmed the presence of

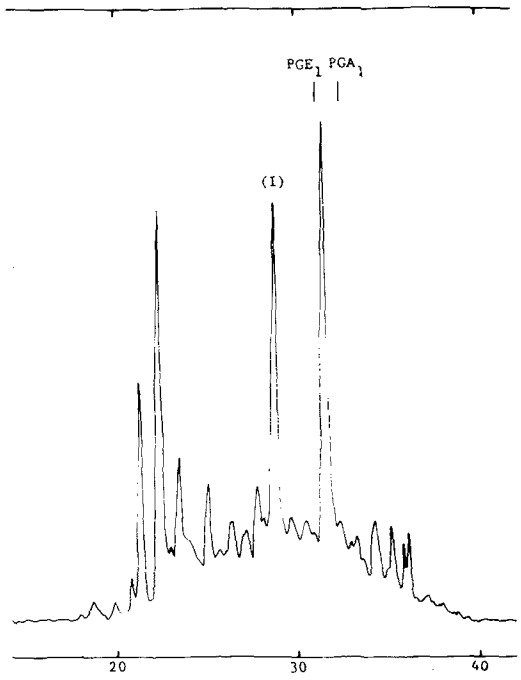

FIG. 2. Analytical HPLC of fraction II. Retention times of PGE_1 and PGA_1 are shown by vertical lines.

a carboxyl group in I. Also, the carbonyl absorption was shifted to 1735 cm^{-1}, which is normal for an isolated ester group. Multiple hydroxyl groups are indicated by the large absorption of the ester 3400 cm^{-1}. We used the method of Albro and Fishbein (10), by which the ratio of the intensity of the 3400 cm^{-1} absorption to that of the ester carbonyl is compared with known esters having different numbers of hydroxyl groups; we calculated the ester of I to have 3 such groups.

Low resolution mass spectrometry (MS) of I produced a complex pattern (Fig. 3) and had several metastable ions that aided in assigning fragmentation pathways. High resolution MS produced empirical formulas for all ions over 69 m/e. Maximum m/e was 316.2045 with a calculated formula of $C_{20}H_{28}O_3$ and 7 rings and double bonds (R+D). Since 3 hydroxyl groups and at least one carboxyl group were indicated from chemical and spectral data and since high molecular weight secondary alcohols can be expected to dehydrate readily on evaporation (11), 2 water molecules were assumed to be lost prior to detection. The trimethylsilyl derivative of I, by GC-MS, showed ion clusters centered at 498 and 572. Since these derivatives give losses of ca. 73 amu (12-14), a 352 MW for I was considered probable.

Ions containing 2 oxygen atoms and one (R+D) corresponding to the end of the molecule having the carboxylic acid group were found as follows: m/e 73, $C_3H_5O_2$; m/e 87, $C_4H_7O_2$; m/e 115, $C_6H_{11}O_2$; m/e 129, $C_7H_{13}O_2$; and m/e 143, $C_8H_{15}O_2$. This series of ions denoted the presence of a group of seven methylenes adjacent to the carboxyl group. The odd electron ion m/e 156, $C_9H_{16}O_2$ was significant when considered in conjunction with m/e 169, $C_{10}H_{17}O_2$, 2 (R+D) in indicating the presence of a double bond between carbons 9 and 10. Ion m/e 199, $C_{11}H_{19}O_3$, 2 (R+D) represents the addition of CH_2O to m/e 169. This addition indicates the presence of a hydroxyl group on carbon 11. Ions at m/e 211, $C_{12}H_{19}O_3$; 3 (R+D) and m/e 223, $C_{13}H_{19}O_3$; 4 (R+D) suggest a triple bond between carbons 12 and 13. The next higher mass ion which contains 3 oxygens is m/e 301, $C_{19}H_{25}O_3$; 7 (R+D) representing the addition of C_6H_6 and 3 (R+D). There are very few possible structures for a C_6H_6 neutral fragment. Since the spectrum shows no ions contaiing 3 oxygen atoms between m/e 223 and 301, the 78 amu loss (C_6H_6) was probably due to a methyl substituted cyclopentadiene. The ion at m/e 301 is also the most abundant high mass ion and is obviously due to loss of a methyl group from the parent ion.

The Δ^9 double bond was confirmed by GC-MS of ozonolysis products from the ester of I which produced 9-oxymethyl nonanoate. Other ozonolysis products were not identified. The above evidence suggests II (see Fig. 4) for the dehydrated 316 MW compound detected by MS.

The 90 MHz ^{1}H-NMR ($CHCl_3$-d) of I, 0.87 ppm (m,3H,$CHC\underline{H}_3$), 1.32.1.53, 1.78 (broad t, 16 H,$nC\underline{H}_2$), 2.14 (s,2H,$CHC\underline{H}_2$), 2.33

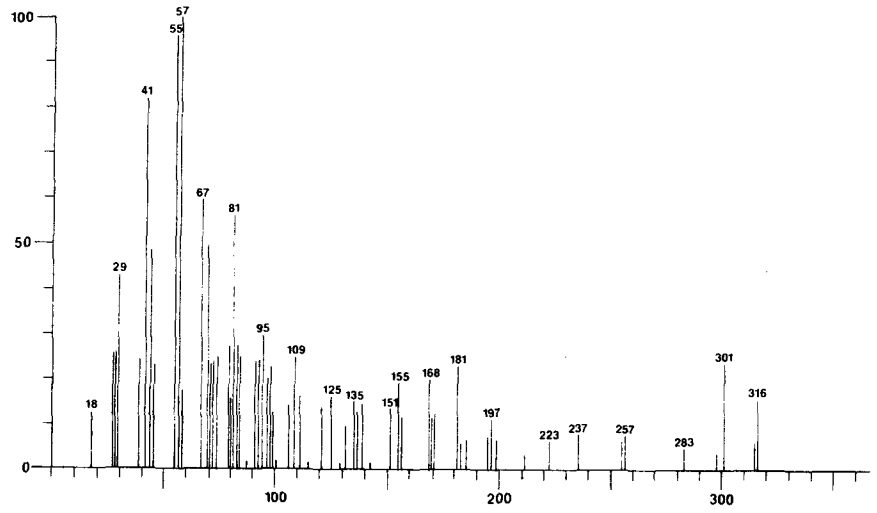

FIG. 3. MS fragmentation pattern of I.

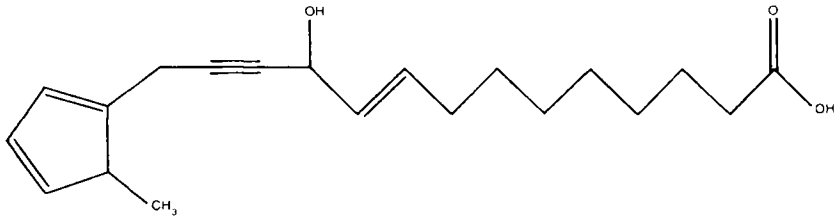

FIG. 4. Structure of II.

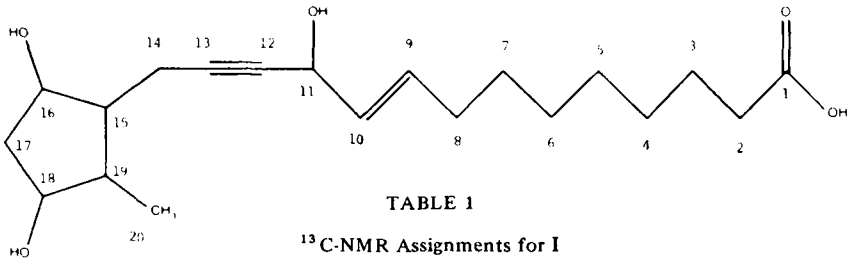

TABLE 1

^{13}C-NMR Assignments for I

Carbon no.	Chemical shifts (ppm)	Carbon no.	Chemical shifts (ppm)
1	162.2	11	56.0
2	34.1	12	82.1
3	24.7	13	83.5
4	29.0	14	29.7
5	29.7	15	52.0
6	29.7	16	77.2
7	24.6	17	41.1
8	34.0	18	78.4
9	116.0	19	43.9
10	132.3	20	14.3

(s,2H,C<u>H</u>$_2$COOH), 2.90 (broad, 1 H, O<u>H</u> non H-bonding), 3.70 (broad, 2H,CH$_2$C<u>H</u> O<u>H</u> CH), 4.70 (d,1H,CC<u>H</u> OH CH), 5.42 (broad m, 2H, nC<u>H</u>C<u>H</u>, 7.42 (broad m, 4H,OH), confirms most features indicated by MS. Additionally, the broad absorption at 7.41 ppm shows 4 replaceable protons.

The ^{13}C-NMR spectrum (Table 1) was obtained with 64,000 scans and produced shifts for 20 carbons. We were particularly interested in confirming the existence of the 2 acetylenic carbons indicated by MS and needed information about the ring substitution pattern. Cooper and Fried (15) correlated chemical shifts for several prostaglandin analogs, including acetylenic carbons at approximately the same relative positions. Their acetylenic carbon absorptions averaged 82 and 85 ppm, very close to those obtained for carbons 12 and 13 of I. The 1,2,3,5 substitution pattern of the ring also appears to be confirmed. Cooper and Fried determined shifts for carbons equivalent to C_{16}, C_{17}, and C_{18} in I to be ca. 73, 42 and 78 ppm, respectively. Dehydration of I would be expected to produce the cyclopentadienyl ring indicated in II. We conclude that I has the structure shown in Table 1.

As stated eralier, I is representative of many components in fraction II. In addition, HPLC cochromatography of fraction II with PGA_1 and PGE_1 shows similarity. These components of *E. microcarpa*, therefore, may have physiological properties other than inhibition of blue-green algae.

Further studies are in progress. HPLC is more convenient than TLC and has shown the potential of providing larger amounts for more detailed studies. We have preliminary evidence that many of the same oxygenated fatty acids occur in other aquatic plants. They may, in fact, be widespread in such plants and may partially explain diversity and succession in aquatic systems.

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A Comparison of the Oleaginous Yeast, *Candida curvata*, Grown on Different Carbon Sources in Continuous and Batch Culture

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ABSTRACT

The oleaginous yeast, *Candida curvata* D, was grown in both batch and continuous culture on 5 different carbon sources to compare the efficiency of fat production from the various substrates. Maximum lipid accumulation occurred in batch culture with xylose as the carbon source on nitrogen-limited medium reaching a level of 49% (w/w) of the biomass, but this was reduced to 37% at the optimum dilution rate (D = 0.05/hr) in a chemostat. Both the highest biomass and lipid yields were attained in continuous culture with lactose as the sole carbon source at a dilution rate of D = 0.04/hr, giving an efficiency of substrate conversion of 60 g of biomass and 18.6 g lipid per 100 g lactose utilized. The relative proportions of the major fatty acids (16:0, 18:0, 18:1, 18:2) in the lipid were found to vary considerably in batch culture and in continuous culture under carbon-limited conditions. However, on nitrogen-limited media in the chemostat, the fatty acid composition remained relatively constant over the whole range of dilution rates employed. Lipid from xylose-grown cells contained the greatest percentage of stearic acid (18:0) 15% and the lowest linoleic acid (18:2) 4%, whereas lipid from ethanol-grown cells contained elevated levels of oleic acid (18:1) 51% and decreased palmitic acid (16:0) 25%.

Lipids 18:000-000, 1983.

INTRODUCTION

Candida curvata was first described by Hammond and colleagues (1) as an oleaginous yeast capable of efficient conversion of whey permeate, i.e., lactose, to oil. As with most previous work on lipid accumulation (see refs. 2,3), the evaluation of the potential of *C. curvata* was established using batch culture (1,4). However, continuous culture would probably be a more efficient and cost-effective means of cultivating a yeast on a large scale (5) besides being able to give close control over the physiological state of the organism. Under the steady-state conditions of continuous culture, a constant composition of the cells is produced: this includes not only the total amount of lipid within the cells but the fatty acyl moieties of the lipid (6). As a steady state can be maintained indefinitely, the product from the fermenter also remains unchanged. Thus, besides being the method of yeast cultivation in many commercial processes, continuous culture is the ideal laboratory method for making unequivocal comparisons of the same organism grown under different conditions as all conditions, including the growth rate, can be accurately controlled.

The application of continuous culture to lipid accumulation has been examined in this laboratory with respect to *Candida* 107 (6,7), *Rhodotorula gracilis* (8) and *Lipomyces starkeyi* (9). In view of the likely commercial importance of *C. curvata*, we have, with the kind permission of Professor E. G. Hammond, examined the growth of this yeast in continuous culture. This paper reports a comparison of the yeast grown on 5 different carbon sources under both batch and continuous culture; batch culture being used to illustrate the inherent difficulties in attempting to obtain constant cell compositions under different growth conditions. The carbon sources were selected on the basis of what may be reasonable choices in any large-scale process: sucrose, lactose, glucose, xylose and ethanol. They, thus, represented 2 disaccharides, a hexose, a pentose and a C_2 compound.

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METHODS

The yeast, *C. curvata* D, used throughout this study, was kindly supplied by Professor E. G. Hammond, Iowa State University, Ames, Iowa.

Media

The nitrogen-limited media used for lipid accumulation contained (g/l): NH_4Cl, 0.5; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4.7H_2O$, 1.5; yeast extract, 1.5; $CaCl_2.6H_2O$, 0.1; $FeCl_3.6H_2O$, 0.008; $ZnSO_4.7H_2O$, 0.0001. The carbon sources were added to a concentration of 30 g/l. The carbon-limited media contained the same as above except that NH_4Cl was at 3.0 g/l and carbon source 10 g/l. The medium was adjusted to pH 5.5 with HCl be-

fore sterilization. The medium was sterilized by membrane filtration (pore size 0.25 μm), collected in sterile 20-liter aspirators and kept for 3 days at room temperature to ensure sterility before use.

Continuous Culture Operations

A one-liter (working volume) chemostat was used (LHE 500 series II, LH Engineering, Stoke Poges, Bucks, England) for all experiments. pH was maintained at 5.5 by the automatic addition of NaOH; temperature was controlled at 30 C. Addition of antifoam was maintained at preset intervals using an appropriate metering pump and timing clock. Incoming air was passed through 2 fiberglass filters connected in series; the aeration rate was maintained at 1 vol air/vol medium/min. The volume within the fermenter was kept constant by using an overflow weir (5 mm diameter). The assembled vessel was sterilized by autoclaving at 121 C for 45 min. The chemostat was erected and filled with 1000 ml of sterile medium to which was added a 2% (v/v) inoculum of *C. curvata* D. Agitation was by means of flat bladed impellers and operated at 500 rpm. The fermenter was used for batch culture by switching off the medium pump and run without pH control for 96 hr.

Samples of 50-100 ml were removed for analysis through an air-lock device into a sterile screw-top bottle. Steady-state conditions were maintained for at least 6 complete changes of medium in the vessel. Duplicate samples, taken every other day, were analyzed for yeast dry weight, percent lipid (w/w) and residual NH_4^+ and carbon source in the culture filtrates until steady-state samples were in agreement. NH_4^+ in culture filtrates was determined by the method of Chaney and Marbach (10). Glucose was estimated using the glucose oxidase-peroxidase method (Boehringer-Mannheim). Sucrose and xylose were estimated using methods based on Herbert et al. (11). Lactose and ethanol were determined using standard reagent kits from Boehringer-Mannheim GmbH.

Dry Weight Determination

Samples (10 ml) were centrifuged at 5000 × g for 5 min in preweighed, dried tubes and washed twice with 10 ml of distilled water. The pellets were dried at 80 C over P_2O_5 in a vacuum oven until constant weight (ca. 48 hr).

Lipid Estimation

Total lipid was determined by a method based on that of Folch et al. (12): lipid was extracted from freeze-dried cells with chloroform/methanol (2:1), filtered and washed with 0.9% naCl and distilled water. The extract was dried over anhydrous $MgSO_4$ and evaporated to dryness using a rotary evaporator. The lipid was redissolved in diethyl ether and transferred to a preweighed vial. The ether was removed in a stream of nitrogen and dried in a vacuum dessicator for 1 hr. This method is not the same as that advocated by Moon and Hammond (4) for maximum lipid extraction from this yeast but it is a procedure which we have found to be convenient and reproducible.

Fatty Acid Analysis of the Lipid

Fatty acid methyl esters were prepared using sodium methoxide (13). The total lipid sample was dissolved in hexane to give a final concentration of 75 mg/ml. One hundred μl sample was removed and added to 2 ml methanol, followed by 1 ml sodium methoxide and heated 60 C for 15 min. The contents were cooled and acidified with 10% H_2SO_4 in methanol using bromothymol blue as indicator. Two ml hexane were added and mixed and the upper layer removed for analysis using gas chromatography.

Gas Liquid Chromatography (GLC)

Samples were analyzed in a Pye Unicam series 104 gas chromatograph (Cambridge, England). Samples were separated on a glass column (1.5 m × 4 mm) pretreated with dichloromethyl silane and packed with 5% diethyleneglycol succinate on Chromosorb 6 W H-P (100-200 mesh). The carrier gas was nitrogen at a flow rate of 50 ml/min and the column was held at 185 C. Peaks were identified by comparison of their retention times with those of authentic fatty acid methyl standards.

RESULTS

The growth of *C. curvata* in batch culture and continuous culture on glucose, sucrose, lactose, xylose and ethanol is shown in Figure 1 (a-o). The patterns observed were similar to those observed previously with oleaginous yeasts in both batch (1,4,14) and continuous culture (6-9).

In batch culture (Fig. 1, a-e), with a high C:N ratio to ensure high lipid accumulation, the nitrogen was consumed after ca. 30 hr growth in each case (not shown). The amount of cellular lipid then increased from ca. 10% of the biomass to between 30 and 35% after 90 hr as the carbon continued to be assimilated and metabolized to lipid. The efficiencies of the conversions of carbon source into both biomass and lipid are summarized in Table 1. Lactose was

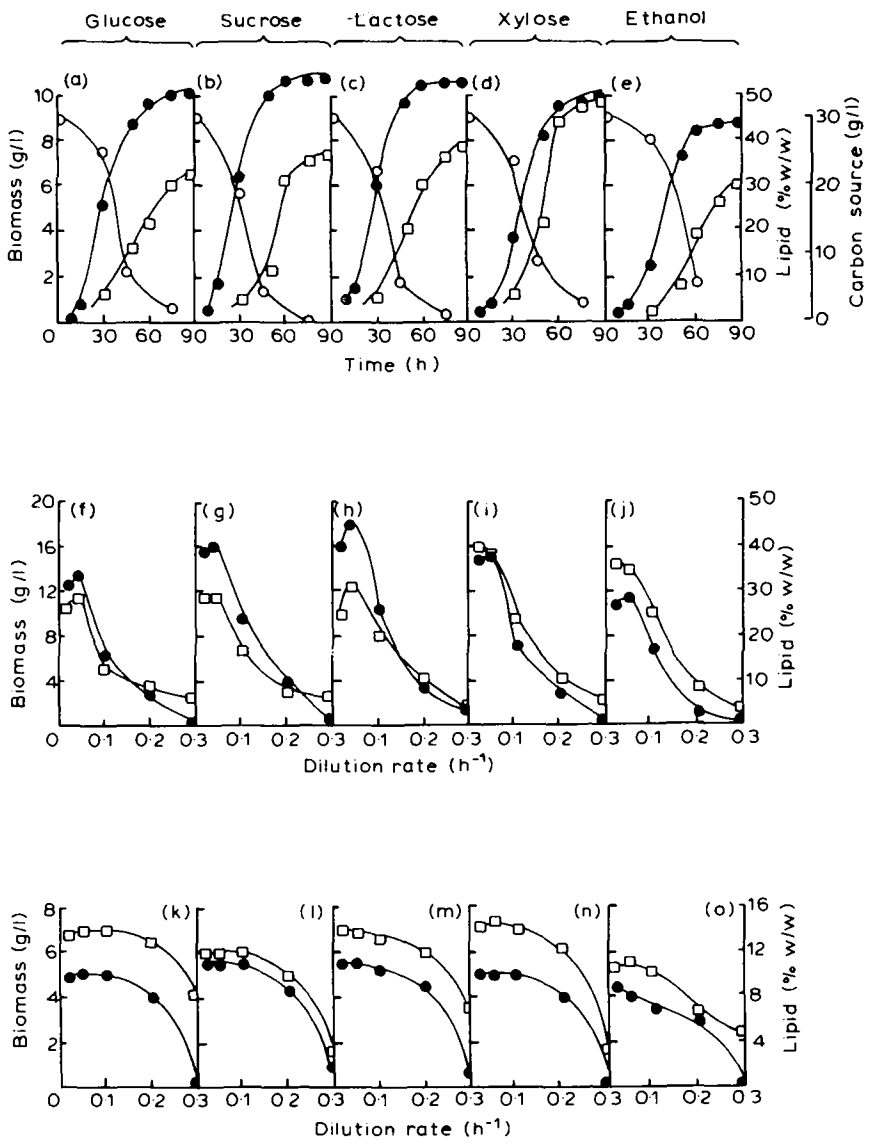

FIG. 1. Lipid and biomass production by *C. curvata* growing on glucose, sucrose, lactose, xylose and ethanol: • biomass (g/l); □ lipid % of biomass; ○ carbon source (g/l). Figs. a-e, growth in batch culture; f-j, growth in continuous culture under nitrogen-limited conditions; k-o, grown in continuous culture under carbon-limited conditions.

the most effective carbon source for biomass production; xylose was the most efficient for lipid production. Ethanol was the least efficiently utilized carbon source for both biomass and lipid production.

In continuous cultures, under carbon-limited conditions (Fig. 1, k-o), lipid content of the cells did not exceed 15% of the biomass. Beyond the critical dilution rate (i.e., at the point beyond which wash-out of the cells begins to occur), the lipid content of the cells declined abruptly with all 5 substrates. Under these growth conditions, there was insufficient carbon available to allow lipid accumulation to occur. However, under nitrogen-limited conditions (Fig. 1, f-i), sufficient excess carbon was available to allow lipid accumulation and in all 5 cases maximum lipid accumulation occurred at a dilution rate of 0.04-0.05/hr. Wash-out of the cells was complete at a dilution rate (=

TABLE 1

Lipid Production by *C. curvata* Grown in Batch Culture for 90 hr[a]

Carbon source (30 g/1)	Biomass (g/1)	Lipid (% w/w)	Substrate utilized (g/1)	Biomass yield (g/100 g substrate utilized)	Lipid yield (g/100 g substrate utilized)
Glucose	10.2	33.2	28.3	36.0	11.9
Sucrose	11.2	37.4	28.0	40.0	14.8
Lactose	12.5	39.2	29.6	42.2	16.5
Xylose	9.9	48.6	27.3	36.3	17.4
Ethanol	8.5	30.1	25.5	33.3	10.0

[a]Culture conditions as described in Materials and Methods. Biomass (10 ml) and lipid (100 ml) samples were removed and treated in duplicate. Carbon sources were assayed as in Methods.

TABLE 2

Efficiency of Lipid Production by *C. curvata* Grown in Continuous Culture on Various Carbon Sources under Nitrogen-Limited Conditions

	Carbon source (30 g/1)				
	Glucose	Sucrose	Lactose	Xylose	Ethanol
Dilution rate (per hr)	0.04	0.04	0.04	0.05	0.05
Residence time (hr)	25	25	25	20	20
Substrate utilized (g/1)	29.8	29.6	29.8	29	30
Biomass (g/1)	13.5	16	18	15	11.5
Rate of biomass synthesis (g/1/hr)	0.54	0.63	0.72	0.75	0.58
Biomass yield (g biomass/100 g substrate)	45	53	60	51	38
Lipid (% of biomass w/w)	29	28	31	37	35
Total lipid produced (g/1)	3.94	4.54	5.6	5.5	4.0
Rate of lipid synthesis (g/1/hr)	0.16	0.18	0.22	0.27	0.2
Lipid yield (g lipid/100 g substrate)	13.1	15.1	18.6	18.3	13.3
Specific rate lipid production (g lipid/100 biomass/hr)	0.012	0.011	0.0124	0.018	0.017

Analyses carried out as given in Table 1.

maximum specific growth rate) of 0.30/hr. Table 2 summarizes the data for the efficiencies of conversions of carbon source to biomass and lipid in the 5 cases. The most efficiently used carbon source for biomass was, as with batch culture, lactose. But in contrast with batch culture results (Table 1), lactose was also the most effective substrate for lipid production. Xylose, however, was just slightly less efficient and, within the limits of experimental error, should probably be regarded as equally good as lactose.

One of the advantages of continuous culture over batch cultivation methods can be seen from a comparison of Tables 1 and 2. With all 5 substrates, the efficiencies of conversion of carbon to both biomass and to lipid were higher with the former method of growth. The steady states engendered by the chemostat allow the cell to achieve optimum dynamics of its biochemistry and this is reflected by the cell achieving maximum growth (i.e., cell yield) per mole of substrate utilized. It can also be seen from Tables 1 and 2 that the productivity (g product per liter fermenter volume per unit time) of the continuous procedures were up to 5 times higher than the batch culture methods: with lactose, for example, the rate of biomass production was 0.72 g/l/hr in continuous culture but was $12.5 \div 90 = 0.14$ g/l/hr in batch culture. These figures become even more in favor of continuous culture if one adds to the fermentation time of the batch culture (i.e., 90 hr) a period for "turn around" of the fermenter (say 24 hr for cleaning, resterilizing and medium addition). The rate of lipid production (g/l/hr) also can be shown to be 4 to 5 times faster in continuous culture than in batch.

The non-steady state of a batch culture is shown when successive samples of the cells are taken during growth as often considerable variations in cell composition can be seen (see also ref. 4). It was, therefore, no surprise to see vari-

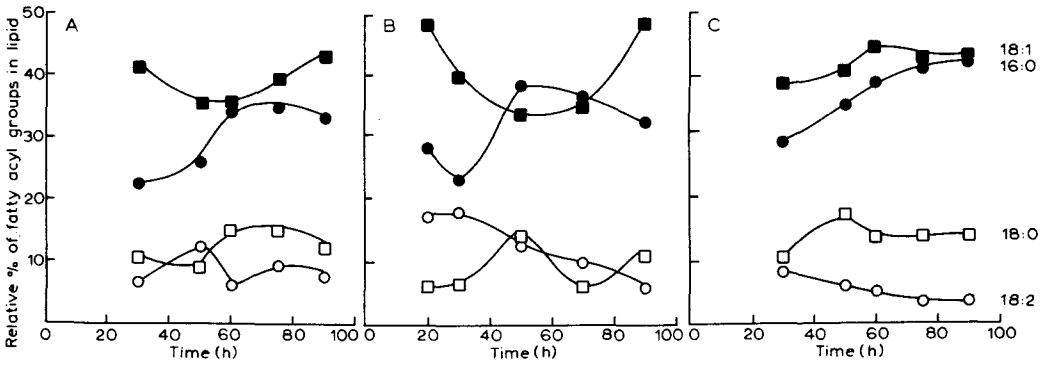

FIG. 2. Fatty acyl composition (relative % w/w) of lipid of *C. curvata* grown in batch culture with: A, glucose; B, lactose; C, xylose. • = 16:0; □ = 18:0, ■ = 18:1; ○ = 18:2. Traces of 14:0 and 16:1 were seen in most cases.

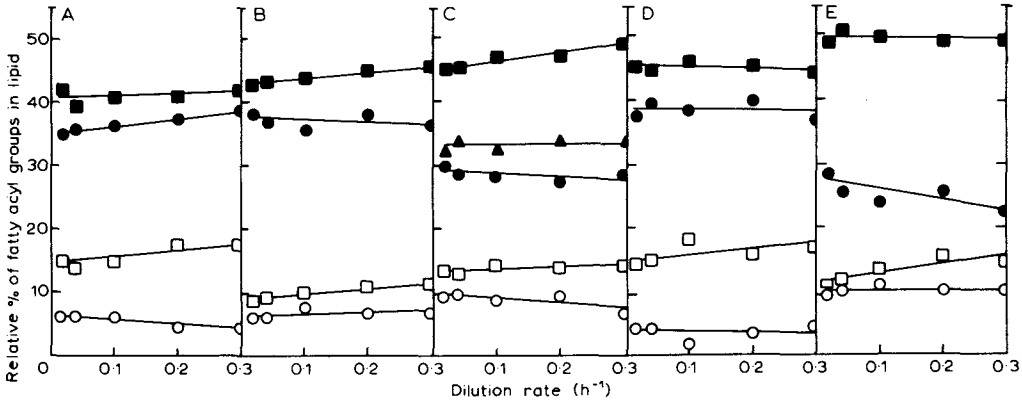

FIG. 3. Fatty acyl composition (relative % w/w) of lipid of *C. curvata* grown in continuous culture under nitrogen-limiting conditions with: A, glucose; B, sucrose; C, lactose; D, xylose; E, ethanol. • = 16:0, □ = 18:0; ■ = 18:1; ○ = 18:2. Traces of 14:0 and 16:1 were seen in most cases.

ations in the fatty acyl composition of the cell lipid of *C. curvata* when grown in batch culture on glucose, lactose and xylose (Fig. 2). (Data for the fatty acyl groups from sucrose- and ethanol-grown cultures was not obtained except after 96 hr–see Table 3.) With each substrate, oleic acid (18:1) was the major fatty acid, although palmitic acid (16:0) was almost as abundant. Linoleic acid (18:2) and stearic acid (18:0) were the only other 2 major fatty acids, although traces of myristic acid (14:0) and palmitoleic acid (16:1) were seen in most analyses. Stearic acid was almost never higher than 15% of the total fatty acids. A comparison of the final fatty acyl composition taken at the end of growth (96 hr) for the yeast grown on all 5 substrates is given in Table 3. This showed that xylose produced the oil with the lowest degree of unsaturation (Δ/mole–ref. 15) while ethanol produced the one with the highest degree of unsaturation. The oils, though, in all 5 cases were not widely different.

In continuous culture, the composition of the fatty acyl groups remains constant under any one set of growth conditions (6). Thus, samples of *C. curvata* taken from the chemostat running with each of the 5 substrates at the same dilution rate can be accurately compared. Thus, at the dilution rate where maximum lipid accumulation occurred (i.e., 0.04/hr under nitrogen-limited conditions, see Fig. 1, f-i), the fatty acyl groups from the 5 cultures showed differences which could be asserted to be slight but significant (see Table 3). As with the batch cultures, xylose produced the lipid with the lowest degree of unsaturation and ethanol the one with the highest. The oils produced from glucose and sucrose were similar to each other as were those from lactose and ethanol.

When the specific growth rate (= dilution

TABLE 3

Fatty Acid Composition of Lipid from *C. curvata* Grown on Different Carbon Sources

Carbon source	Batch culture[a]					Continuous culture[b]				
	16:0	18:0	18:1	18:2	Δ/mol[c]	16:0	18:0	18:1	18:2	Δ/mol[c]
Glucose	33.0	12.0	42.9	7.3	0.59	35.8	13.7	39.7	6.4	0.55
Sucrose	32.4	11.3	42.0	6.7	0.57	36.6	9.6	43.5	5.9	0.57
Lactose	32.5	11.0	49.0	6.0	0.63	28.0	13.2	46.9	10.8	0.69
Xylose	41.2	14.0	43.0	3.5	0.51	29.5	15.0	44.5	3.9	0.52
Ethanol	26.5	12.5	49.0	8.9	0.69	25.7	12.0	51.2	10.0	0.72

[a]Samples after 96 hr growth.
[b]Samples from dilution rate = 0.04/hr.
[c]Δ/Mol = degree of unsaturation (ref. 15).

rate) was changed in each chemostat, there was almost no change in lipid composition when cultures were run with nitrogen-limiting medium. Figure 3 shows the remarkable constancy of the fatty acyl groups in going from a very slow specific growth rate, D = 0.02/hr, to the very fastest one, D = 0.3/hr. However, when the fatty acids of the lactose culture grown under carbon-limited conditions were examined, their composition changed quite markedly in going from the slowest specific growth rate to the highest one (Fig. 4); the relative proportion of palmitic acid decreased by nearly 50% while that of oleic acid increased by over 50%; linoleic acid decreased to a very low amount at the high dilution rates while stearic acid reached a maximal level. In spite of these large changes, the degree of unsaturation of the total fatty acids remained unchanged throughout the whole range of dilution rates.

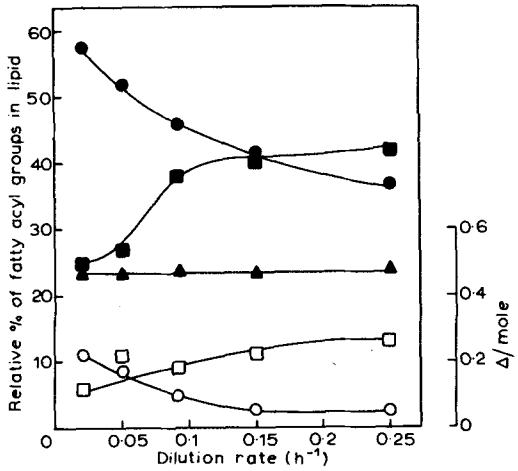

FIG. 4. Fatty acyl composition of *C. curvata* grown on lactose under carbon-limited conditions. ○ = 16:0; □ = 18:0; ■ = 18:1; ○ = 18:2; ▲ = Δ/mole–see ref. 15.

DISCUSSION

C. curvata is an oleaginous yeast of some potential. Its efficient conversion of lactose to oil has already been amply recorded by Hammond and associates (1,4,16) and in this paper we have shown that it is almost as equally efficient in converting other substrates–glucose, sucrose, xylose and ethanol–into lipid and biomass. The efficient utilization of xylose is noteworthy in view of the current interest in this material as a substrate for microbial fermentation process (17). Xylose is readily available in large quantities from the chemical hydrolysis of hemicellulose which, along with cellulose, is the most abundant renewable resource in nature. Ethanol was the poorest of the 5 substrates tested in producing lipid and this can probably be attributed to it inducing expression of the enzyme isocitrate lyase. Such enzyme activity would decrease the amount of isocitrate acid, and thence citric acid, which was accumulated. This usually occurs by isocitrate dehydrogenase being the sole enzyme for isocitrate dissimilation and it being dependent upon AMP for activity (18). As AMP is at a low concentration in oleaginous yeasts, a mechanism is provided which leads to the accumulation of citrate which is then the immediate precursor, by the action of ATP:citrate lyase, of acetyl-CoA. Ethanol, by inducing an alternative route for isocitrate metabolism, is therefore not a good substrate for achieving high lipid accumulation in spite of it appearing to be, at first inspection, an excellent substrate to promote lipid accumulation as it would be expected to be readily metabolized (via acetaldehyde and acetate) to acetyl-CoA. Clearly, though, this is not sufficient metabolic reason to engender high lipid levels. However, not all yeasts show this loss of

efficiency; *R. gracilis* has been reported to have only slightly less better yields of biomass and lipid in changing from glucose to ethanol (19).

The biomass yield of 42 g cells/100 g substrate for the yeast grown on lactose in this present work was slightly less than the value of 46% calculable from the data reported by Moon and Hammond (4). However, our lipid yields were substantially less than theirs: 16.5% as compared to their value of 27%. This could be due to the differences in lipid extraction techniques but is probably attributable to their use of whey or whey permeate which contains many nutrients besides lactose and which must be supposed to be beneficial to lipid production.

Although there was a slight decrease in the percentage of lipid in the biomass in going from batch culture to continuous culture, the efficiency of utilization of the substrate increased considerably with all 5 substrates tested and thus resulted in much higher biomass and lipid yields.

The merits of continuous culture were also reflected in the production of a product of unvarying composition. This is an obvious advantage of continuous culture if considering any commercial development. The relative content of the major fatty acids in the lipid produced from the hexoses was ca. palmitic (36%), stearic (13%), oleic (44%) and linoleic (6%), which is similar to that reported for *Candida* 107 (6) but markedly different to that of *R. glutinis* (8). The ability of this yeast to produce a good quality oil from a wide selection of substrates indicates the potential economic viability of a process utilizing such a yeast. The profitability of this process obviously depends on the utilization of a substrate of little intrinsic value to produce an oil of a much higher value. The use of continuous culture techniques to minimize operating costs should be of considerable relevance to the overall economics of the process.

ACKNOWLEDGMENT

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Biochemical Activities During Lipid Accumulation in *Candida curvata*

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ABSTRACT

Intracellular and extracellular concentrations of citrate and the specific activities of ten different enzymes in *Candida curvata* D were examined in relation to lipid biosynthesis in batch and continuous culture. Citrate was found to accumulate prior to lipid production and declined markedly as lipid accumulated in batch culture. The cells excreted citrate as the culture became nitrogen-limiting after 30 hr of growth, but little more was expelled after 40 hr when lipid accumulation was more marked. In continuous culture, only low levels of citrate were detected at the lower dilution rates and citrate was completely absent from both the cells and medium above a dilution rate of 0.1/hr. The activity of malic enzyme, malate dehydrogenase and ATP:citrate lyase increased in batch culture on lipid accumulated and, in continuous culture, both malic enzyme and ATP:citrate lyase varied in parallel with the specific rate of lipid synthesis which increased with increasing dilution rate. Activity of malate dehydrogenase, citrate synthase and glucose-6-phosphate dehydrogenase decreased with increasing dilution rate. The regulatory significance of these enzymes in lipid accumulation by *C. curvata* is discussed. *Lipids* 18:000-000, 1983.

INTRODUCTION

Candida curvata is a rapidly growing oleaginous yeast (1,2) which can readily convert a number of different substrates into lipid (3). Such biochemical studies that have been carried out with oleaginous yeasts, and which have included *C. curvata*, have indicated it to be a typical yeast of this type (4,5). However, in view of the likely commercial importance of this yeast in being able to grow on dairy wastes of whey and whey permeate (1,2,6), we have now examined the intermediary metabolism of this yeast in more detail.

Of principal concern to us has been the role of citric acid in lipid accumulation. In the metabolism of carboyhydrates to lipid, it is necessary for carbon to enter the mitochondrion and, although acetyl-CoA is formed therein, it cannot exit from this organelle (7). Consequently, citrate is used as the means of transporting carbon back into the cytoplasm (5). Citrate is cleaved by the cytoplasmic enzyme ATP:citrate lyase, which is only found in oleaginous yeasts (4), to generate acetyl-CoA and this is then used as the precursor for fatty acid biosynthesis. Thus, observations concerning the concentrations of citric acid during lipogenesis should be very revealing about the possible rate-limiting step of the overall process. Such observations, though, need careful interpretation and additional knowledge is, therefore, simultaneously needed concerning the activities of various enzymes of intermediary metabolism in this yeast. This paper reports the results of such a study. We have used both batch and continuous modes of cultivation of the yeast for, although we have already argued (3) the advantages of the latter method over the former in achieving the most efficient and economical conditions for lipid production, batch culture methods allow one to see fluctuations in metabolites and enzyme activities which would not be apparent in the steady-state conditions of a chemostat.

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METHODS

The yeast *C. curvata* strain D was obtained from Professor E. G. Hammond, Iowa State University, Ames, Iowa. The yeast was cultivated on a glucose/salts medium at pH 5.5 containing 30 g glucose/l and 0.5 g NH_4Cl/l as nitrogen-limiting media (3). Details of batch and continuous culture apparatus, cell dry weight and lipid determinations have been described previously (3).

Preparation of Intracellular Extracts

The culture samples (30 ml; ca. 200 mg dry wt) were filtered through prewashed cellulose acetate membrane filters (47 mm diameter, 0.45 μm) and washed twice with 5 ml of ice-cold buffer containing 40 mM Tris/HCl pH 7.0, 0.6 M sorbitol and 0.1 mM EDTA and once with 5 ml ice-cold distilled water. The filtration was carried out under suction so that the whole procedure took less than 70 sec. The filtered cells were transferred, on the filter, to 5 ml ice-cold $HClO_4$ (30% v/v) and left to extract for 1 hr. The suspension was neutralized with 1 M

KOH and the resulting $KClO_4$ and precipitated protein removed by centrifugation at 10,000 g for 5 min. The clear supernatant was retained for metabolite assay.

Preparation of Cell-Free Extracts

Cells were harvested by centrifugation at 5,000 g for 5 min and washed twice in a buffer containing 50 mM Tris/HCl, pH 7.8, 1 mM $MgCl_2$ and 1 mM dithiothreitol. The cells were resuspended in the above buffer and disrupted by one passage through a precooled French cell. The extract was centrifuged at 40,000 g for 30 min, lipid filtered off and the supernatant retained at 0 C for enzyme assays.

Assays

Citrate was determined according to Dagley (8). Protein was estimated by the method of Bradford (9). Glucose-6-phosphate dehydrogenase (E.C.1.1.1.4.9) was assayed according to Noltman et al. (10). Pyruvate dehydrogenase complex (E.C.1.2.41) was assayed by the method of Young et al. (12). NAD^+ and $NADP^+$ dependent isocitrate dehydrogenase (E.C.1.1.-1.4.1) were assayed according to Kornberg (13). Citrate synthase (E.C.4.1.3.7) was assayed according to Srere et al. (14) as described by Boulton and Ratledge (15). ATP:citrate lyase (E.C.4.1.3.8) was assayed according to Srere (16). Aconitase (E.C.4.2.1.3) was assayed by the method of Anfinsen (17). NAD^+-dependent malate dehydrogenase (E.C.1.1.1.3.7) was assayed according to Englard and Siegal (18). Malic enzyme (E.C.1.1.1.40) was assayed according to Hsu and Lardy (19).

RESULTS

The patterns of lipid accumulation by *C. curvata* D in batch culture and continuous culture have been reported in the preceding paper (3). Figure 1 shows the relation between the intracellular and extracellular concentrations of citrate and lipid production in batch culture. Citrate built up over the first 40 hr of growth, during which time the lipid concentration constituted less than 10% of the biomass but, as the lipid increased over the next 20 hr, the concentration of citrate in the intracellular pool steadily decreased to 15% of the peak value. No significant changes in citrate level were observed after 70 hr. The excretion of citrate into the external medium did not begin until after 30 hr, by which time NH_4^+ in the medium had become exhausted (3). By 40 hr, 80% of the total external citrate accumulated had been excreted, with only a small build-up continuing over the remaining 60 hr.

To relate these observations to the metabolism of the cell, we followed the activities of the various enzymes which have been implicated in the flow of carbon into lipid (20,21), see Figure 5. Figure 2 shows the patterns of activity of 10 different enzymes, indicated as either mitochondrial (M) or cytosolic (C) from previous work (5), during batch growth on glucose. Five of the enzymes showed very little variation in activity at any stage during the culture, whereas the other 5, namely, pyruvate carboxylase, glucose-6-phosphate dehydrogenase, malic enzyme, malate dehydrogenase and ATP: citrate lyase, all showed an increase in specific activity as the cultures progressed—i.e., as lipid

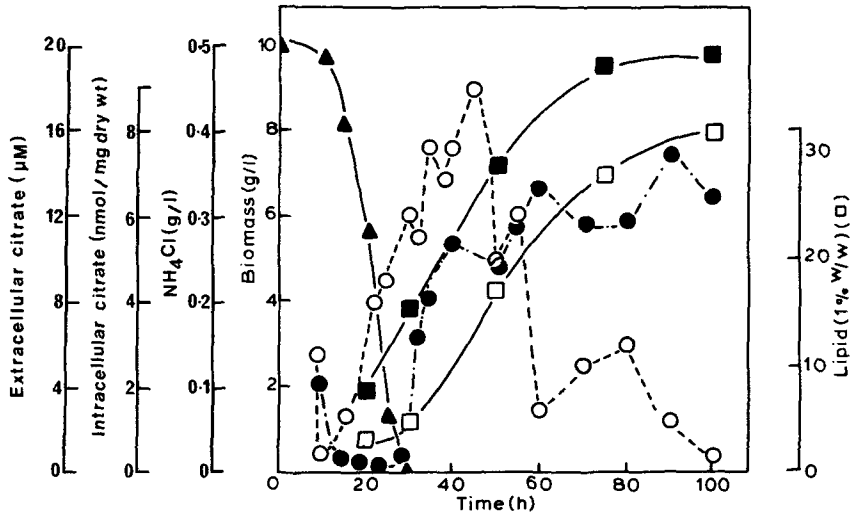

FIG. 1. Concentrations of intracellular and extracellular citrate in *C. curvata* growing on glucose in batch culture. Biomass, ■; NH_4 concentration in culture medium, ▲; % lipid in biomass, □; intracellular citrate concentration, ○; extracellular citrate, ●.

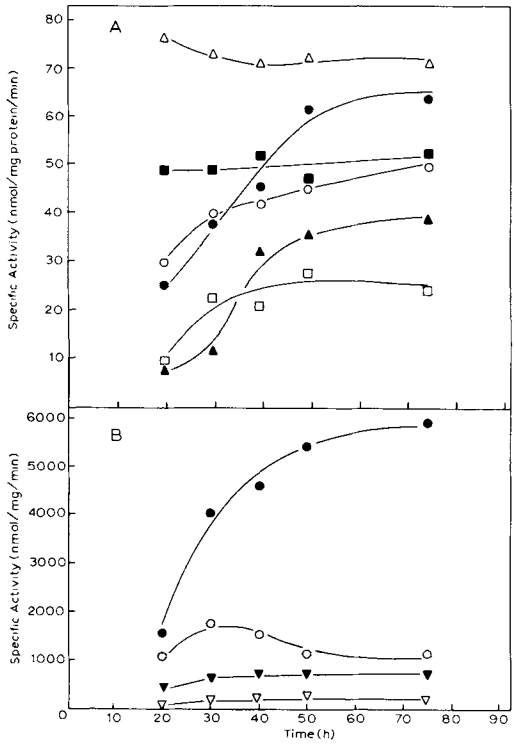

FIG. 2. Enzyme activities of *C. curvata* during batch growth on glucose; M = mitochondrial, C = cytosolic. A: malic enzyme (C), •; pyruvate carboxylase (M), ○; pyruvate dehydrogenase (M), ■; NAD^+-isocitrate dehydrogenase (M), □; ATP:citrate lyase (C), ▲; aconitase (M), △. B: NAD^+-malate dehydrogenase (M and C), •; $NADP^+$-isocitrate dehydrogenase (C), ▽; citrate synthase (M), ○; glucose-6-phosphate dehydrogenase (C), ▼.

gradually accumulated. The most prominent increases, however, were observed with the latter 3 enzymes which showed 3-fold, 4-fold and 5-fold increases, respectively. Dialysis of extracts indicated that increases in specific activities were not due to the presence of low molecular weight effectors in the extract and thus were more likely to be due to increased synthesis of the active enzyme. It, therefore, appears that activities of malic enzyme, ATP:citrate lyase and malate dehydrogenase increase as the amount of accumulated lipid increases in batch culture, indicating their correlation with lipid biosynthesis.

A characteristic of batch culture is the ever-changing concentration of nutrients and metabolites as well as the growth rate of the cells. This can be used to advantage, as we have already seen, in that the changes which occur in cell composition can be correlated to changes in the metabolic activity of the cells allowing various hypotheses to be advanced. However, with continuous culture techniques, the growth rate of the cells is controlled by the rate at which the limiting nutrient is supplied to the culture (i.e., the dilution rate). Thus, a series of steady states of different growth rates can be created and it then becomes possible to correlate changes in cell composition to the metabolic activities of the cell without the uncertainty that comes when one is dealing with transient states. Although there will be different patterns of changes in cells in going from batch culture to continuous culture, one should nevertheless be able to draw the same conclusions in both cases (22-24).

Figure 3 shows the concentrations of citrate as attained in continuous culture in relation to the specific rate of lipid synthesis over the range of growth rates followed. The intracellular citrate content was highest at the lowest dilution rate, D = 0.02/hr, and declined sharply until no further citrate could be detected at D 0.1/hr. The excretion of citrate into the medium followed the same pattern as that of the intracellular citrate but declined even more dramatically, so that only at the 2 lowest dilution rates could it be detected. The decrease in citrate concentrations correlated with the simultaneous decrease in lipid accumulated (see also ref. 3) but not with the specific rate of lipid synthesis which increases with increasing dilution rate. The marked disappearance of citrate from both the cells and external medium may be explained by the higher activities of ATP:citrate lyase, the enzyme which cleaves citrate, at these dilution rates.

Figure 4 shows the activities of the same 10 enzymes as previously examined in batch culture determined in a chemostat culture over a range of growth rates. The NAD^+:isocitrate dehydrogenase (mitochondrial), pyruvate carboxylase, aconitase and pyruvate dehydrogenase showed little or no variation at any dilution rate whereas the activity of the $NADP^+$:isocitrate dehydrogenase (cytosolic), citrate synthase, glucose-6-phosphate dehydrogenase and, more markedly, malate dehydrogenase all decreased as the dilution rate increased. However, the activities of both malic enzyme and ATP:citrate lyase increased 3-fold when the dilution rate was varied from 0.03 to 0.2/hr.

DISCUSSION

Our current understanding of the metabolism of glucose into lipid is summarized in Figure 5 where it will be seen that citrate is a key intermediate of the process. The gradual

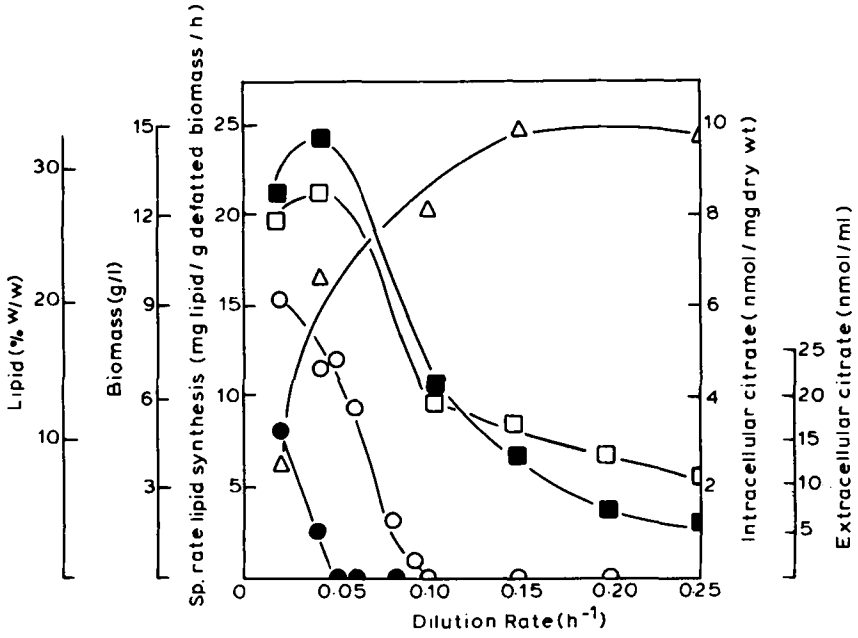

FIG. 3. Intracellular (○) and extracellular (●) citrate concentrations during continuous culture of *C. curvata* on glucose with NH_4^+-limiting medium. Biomass, ■; % lipid in biomass, □; specific rate of lipid biosynthesis, △.

intracellular build up of citrate during growth of an oleaginous yeast, such as *C. curvata*, reflects the sequence of changes in metabolism as nitrogen becomes limiting within the cell. Under these conditions, we have already shown (21) that the intracellular concentration of AMP decreases, that of ATP increases, and the activity of the mitochondrial NAD^+-dependent isocitrate dehydrogenase, which has an absolute requirement for AMP (21), also declines. The accumulating isocitrate equilibrates, via aconitase, to citrate which consequently builds up in the mitochondria. This citrate is then transported across the mitochondrial membrane, in exchange for L-malate (5), and is then cleaved to produce acetyl-CoA and oxaloacetate.

This hypothesis, therefore, explains the observed changes in concentrations of both intracellular and extracellular citrate during growth. The rapid excretion of citrate into the medium between 30 and 40 hr indicates that intramitochondrial citrate first passes into the cytosol where, because it builds up here, the rate of metabolism of citrate must be slower than the rate of efflux from the mitochondria. Thus, the excess citrate passes out of the cell. After this 10 hr period, only a small amount of citrate is expelled which indicates that the rate of intracellular citrate utilization is now accelerating—this, we presume, is by ATP:citrate lyase whose increase in activity was also observed at this time. This would then account for the subsequent depletion of the citrate pool and the plateau in the increase of the extracellular citrate concentration.

The significance of these controls is again illustrated by the profiles observed in continuous culture where citrate concentration and ATP:citrate lyase activity showed an inverse relationship as the dilution rate increased. These results, in part, i.e., from a dilution rate of 0.02-0.05/hr, support the conclusion reached with another oleaginous yeast, *L. starkeyi*, that ATP:citrate lyase is the rate-limiting step in lipid biosynthesis (2,25). However, from a dilution rate of 0.05/hr to wash-out ($D = 0.25$/hr), citrate was undetectable, either intra- or extracellularly, even though ATP:citrate lyase activity was undiminished and the specific rate of lipid biosynthesis continued to increase. This would then indicate that the rate of supply of citrate to the ATP:citrate lyase, or a prior reaction, is now the limiting reaction in lipid biosynthesis. What aspect is limiting in the supply of citrate is difficult to say, as the production of citrate in the cytosol (see Fig. 5) will be in-

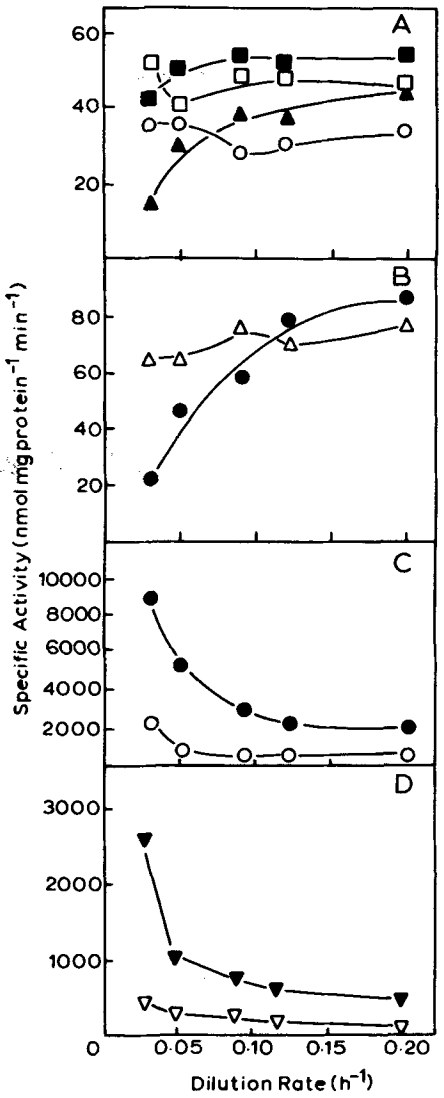

FIG. 4. Enzyme activities of *C. curvata* during continuous culture of *C. curvata* on glucose with NH_4^+-limiting medium; M= mitochondrial, C = cytosolic. A: pyruvate dehydrogenase (M), ■; NAD^+-isocitrate dehydrogenase (M), □; ATP:citrate lyase (C), ▲; pyruvate carboxylase (M), ○; B: malic enzyme (C), ●; aconitase (M), △; C: malate dehydrogenase (M and C), ●; citrate synthase (M), ○; D: glucose-6-phosphate dehydrogenase (C), ▼; $NADP^+$-isocitrate dehydrogenase (C), ▽.

fluenced by a number of separate activities (5).

In batch culture, the specific activity of ATP:citrate lyase did indeed increase after nitrogen had become depleted and citrate concentrations fell. However, it is difficult to assess accurately the possible control of ATP:citrate lyase in vivo from crude extracts due to the large dilution and disruption of cellular compartments during preparation. A valid comparison of specific activities can still be made, however, on the basis that all activities measured throughout the experiments are relative under the conditions of the preparation used. At present, it is not possible to separate mitochondrial and cytosolic fractions rapidly from yeast cells to enable an accurate analysis of the distribution of metabolite levels, such as citrate, between the 2 compartments. Consequently, it is difficult to determine the degree of regulation of either mitochondrial citrate efflux or ATP:citrate lyase activity during lipid accumulation.

Two other enzymes also showed significant changes during lipid accumulation—namely, the NAD^+:malate dehydrogenase and malic enzyme. Both enzymes follow ATP:citrate lyase in the sequence of events following the metabolism of cytosolic citrate, see Figure 5. Malate dehydrogenase exists as at least 2 isoenzymes, a mitochondrial one and a cytosolic one, both of which have a role in regenerating intramitochondrial oxaloacetate for the malate-citrate shuttle. The specific activity of malate dehydrogenase measured is the sum of all the isoenzymes so the observed increase in activity during batch culture may be due to the synthesis of one or all of the various isoenzymes.

The significance of the changes in the activity of malic enzyme is uncertain. Like that of ATP:citrate lyase, the activity of this enzyme paralleled changes in the specific rate of lipid synthesis and increased as lipid increased in batch culture. The enzyme in yeast is reported to function in the direction of pyruvate production (26) with concomitant NADPH formation. Both the pyruvate and NADPH formed would stimulate lipid production but the subsequent utilization of malate would deprive the citrate-malate translocase of its substrate, thus preventing citrate efflux to the cytosol (5). Work is currently in progress to determine the extent and significance of this competition in lipid accumulation.

It can be concluded from this study with *C. curvata* D that, as the available nitrogen becomes exhausted, synthesis of other cell constituents decreases and lipid production predominates. However, it is also evident from our results that cellular metabolism changes over a period of time to promote more efficient lipid production and this is reflected by the increased synthesis of those enzymes which chanel carbon into lipid.

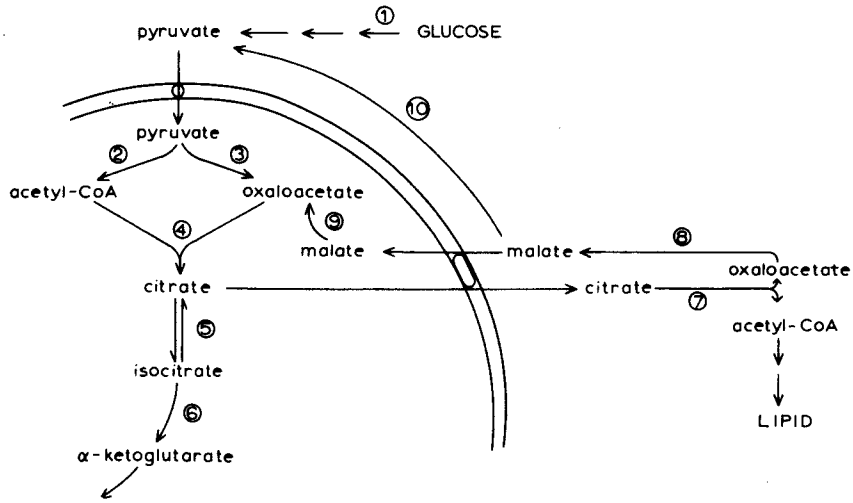

FIG. 5. Flow of carbon from glucose into lipid in *C. curvata*. Enzymes: 1, glucose-6-phosphate dehydrogemase; 2, pyruvate dehydrogenase; 3, pyruvate carboxylase; 4, citrate synthase; 5, aconitase; 6, NAD^+:isocitrate dehydrogenase; 7, ATP:citrate lyase; 8, cytosolic NAD^+:malate dehydrogenase; 9, mitochondrial NAD^+:malate dehydrogenase; 10, malic enzyme.

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METHODS

Evaluation of the Rapid Micromethod for Ultracentrifugal Separation of Labeled Plasma Lipoproteins

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ABSTRACT

The fractionations of plasma lipoproteins by 2 methods were compared to evaluate the rapid separation (Airfuge®) method for lipoprotein distribution studies. When [^{125}I] labeled very low density, low density, and high density lipoproteins (VLDL, LDL, HDL), were separately centrifuged in buffers at d = 1.006, 1.06 or 1.2 g/ml by the conventional ultracentrifuge and the Airfuge®, separations of the fractions in the Airfuge® were incomplete at both 5 C and 24 C, especially at d = 1.006. [^{3}H] Benzo (a)pyrene, when added to plasma, associates with the plasma proteins and lipoproteins, especially LDL. Compared to the standard techniques, the Airfuge® method greatly overestimated its distribution into VLDL. The distribution of [^{3}H] vitamin D_3 into the VLDL plus LDL fraction was also overestimated by the Airfuge® procedure. It is concluded that caution should be observed in quantitative studies of lipoproteins in the Airfuge®. A careful comparison of the distribution into or fractionation of lipoproteins by the 2 methods should always precede any quantitative determinations involving the Airfuge®. *Lipids* 18:xxx-xxx, 1983.

The standard method for fractionation and isolation of the plasma lipoproteins is a time-consuming procedure (1) which renders it impractical for the clinical laboratory. The development of a rapid and relatively inexpensive method for this fractionation from small amounts of plasma has simplified the procedure for quantitating lipoprotein cholesterol (2). It involves the use of an air-driven centrifuge (Beckman Airfuge®). Although the method as originally described was said to be satisfactory for clinical laboratory separations (2), more recently published articles have suggested that the precision was not sufficient to estimate cholesterol in each lipoprotein fraction, and several modified procedures have been published (3-5). The Airfuge® has also been used in studies on the structure and interaction of the apolipoproteins (6,7).

Major obstacles in metabolic studies with labeled lipoproteins have been the quantity of blood required during repeated venipunctures as well as the centrifugation time required for lipoprotein fractionation. Since the Airfuge® method requires only 2-3 hr of centrifugation and less than 1.5 ml plasma for the separation of the 3 major lipoprotein classes, its use could be valuable in lipoprotein research. In the study reported here, the method has been evaluated for use in the separation of very low density, low density, and high density lipoproteins (VLDL, LDL, HLD) which were radiolabeled in the apolipoprotein portion with ^{125}I or which were labeled with a [^{3}H] lipophilic compound. The hydrophobic chemical, [^{3}H] benzo(a)pyrene, which is known to partition mainly into the plasma lipoproteins (8), and the fat-soluble vitamin, [^{3}H] vitamin D_3, were incubated with plasma which was then fractionated by centrifugation in the Airfuge® and in the conventional ultracentrifuge.

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MATERIALS AND METHODS

VLDL (d = 1.006 g/ml), LDL (d = 1.02-1.06 g/ml), and HDL (d = 1.06-1.21 g/ml) were isolated from plasma containing 0.02% sodium azide by sequential flotation during differential density ultracentrifugation at 5 C (9). Each lipoprotein fraction was radiolabeled with ^{125}I by the iodine monochloride method (10). After extensive dialysis to remove free ^{125}I, 0.5 ml of each labeled lipoprotein fraction was mixed individually with 5-ml aliquots of human plasma. Less than 5% of the counts were associated with the lipid portion of the molecules. Each sample was then divided into 3 portions; one was dialyzed against a KBr buffer (0.05 M phosphate) at density d = 1.006 g/ml, another at d = 1.06 and the third at d = 1.2. The densities of the buffers were checked by pycnometry. Samples were then centrifuged in the Airfuge® at room temperature at 160,000 × g for 1, 2, 3 or 4 hr. The contents of each tube were fractionated at ambient temperature by aspiration of 20-μl portions from top to bottom. The radioactivity in each aliquot was quantitated in a Model 5500 gamma counter (Beckman Instruments, Palo Alto, CA). The percentage of the total radioactivity present in each fraction was calculated; the radioacitivity in the

top 80 μl was summed to estimate the flotation of the lipoprotein(s). Samples for the ultracentrifuge were prepared in the same way, i.e., each labeled lipoprotein was mixed with 30 ml plasma and adjusted to the appropriate density by dialysis in the buffers as described above. Samples were centrifuged at 40,000 rpm for 20 hr at 5 C in a Sorvall OTD-2 ultracentrifuge in a T865 rotor (Sorvall, Newtown, CT). Samples were fractionated by aspiration of 2-ml aliquots from top to bottom; 50-μl aliquots were counted for gamma radioactivity. The percentage of the counts at the top was calculated by summing the upper 6 ml.

In another series of experiments, [^{125}I]VLDL, [^{125}I] LDL and [^{125}I] HDL were each further purified before centrifugation. These samples were separately applied to an Ultragel A-4 column (45 x 2 cm) (LKB, Gaithersburg, MD), which provided separation of the lipoprotein fractions. Samples were eluted with a 0.15 M NaCl, 0.05 M PO_4 buffer. The 1.5 ml fractions were monitored by absorbance at 280 nm and quantitation of [^{125}I] radioactivity. The peak fractions were pooled and checked by SDS-PAGE for the characteristic protein bands of each lipoprotein and for possible proteolysis (11). Protein was determined by the method of Lowry et al. (12). Each labeled lipoprotein was divided into portions and dialyzed against phosphate-buffered KBr at d = 1.006, 1.06 or 1.2, as described above. For the centrifugation experiments, 3 μl of the labeled lipoprotein was layered on top of 150 μl KBr buffer at the appropriate density, e.g., [^{125}I] VLDL at d = 1.006 was layered on buffer at d = 1.006 and [^{125}I] VLDL dialyzed at d = 1.06 was overlayered on buffer at d = 1.06. In separate experiments in the Airfuge®, duplicate samples were overlayered and underlayered to determine whether this procedure affected the flotation of the lipoproteins. Samples were centrifuged in the Airfuge® for 3 hr and fractionated as described above. Concurrently, 0.6 ml of the same labeled lipoprotein was overlayered on 29 ml of buffer at the appropriate density and ultracentrifuged as described above. Comparisons of the separation patterns in Airfuge® centrifugation at different temperatures were also performed in a subsequent set of experiments. Each labeled lipoprotein was centrifuged at each density at 5 C and at room temperature as described above. For the 5 C spins, the Airfuge® was placed in a cold room and allowed to reach 5 C before operation.

In a third series of experiments, [G-^{3}H] benzo(a)pyrene (Amersham, Arlington Heights, IL) was adsorbed to glass beads in a vial by evaporation of the solvent under N_2 (13). In a typical experiment, 0.1 mCi [^{3}H]benzo(a) pyrene was incubated at 37 C for 4 hr with 10 ml plasma. Aliquots of the plasma were then dialyzed against KBr buffers at d = 1.006, 1.06 and 1.2 g/ml. Two-ml aliquots were mixed with 28 ml buffer, spun in the ultracentrifuge and fractionated as described above. Each fraction was mixed with 15 ml Aquasol (New England Nuclear, Boston, MA) and the tritium radioactivity was quantitated in a scintillation spectrometer (Beckman Instruments). For the Airfuge® centrifugations, 175-μl aliquots were centrifuged for 2 and 3 hr and fractionated, as described above. Each fraction was counted in 15 ml Aquasol.

Incorporation of [1α, 2α (n)-^{3}H] vitamin D_3 (Amersham) into plasma was performed exactly as described for [^{3}H] benzo(a)pyrene. Centrifugation experiments were conducted as described above. Centrifugation was also performed at both room temperature and 5 C with isolated LDL and HDL which had been separately labeled with [^{3}H] vitamin D_3. Each sample was dialyzed against KBr buffers of d = 1.006, 1.06 and 1.2 and 25 μl of the sample was then overlayered on 150 μl buffer of the appropriate density. Duplicate samples were underlayered. Samples were then centrifuged for 3 hr as described above.

RESULTS

Centrifugation in the Airfuge® for 3 hr yielded better separation of [^{125}I] LDL in plasma than 1 and 2 hr (Table 1), but no further improvements occurred with 4 or more hours of centrifugation. Table 1 shows the percentage of radioactivity present in the floating fraction(s) of the Airfuge® tubes compared to that in the top of the ultracentrifuge tubes. Estimates of the [^{125}I] LDL separation by flotation at plasma d = 1.06 g/ml were equivalent in the 2 methods. Although only 53% of the counts were present in the top fraction at d = 1.06 in the ultracentrifuge, the majority of the remaining counts were present in the next 2 aliquots. Separation of [^{125}I] labeled lipoproteins is not as sharp as that of unlabled samples because of the tendency of these labeled compounds to adhere slightly to the centrifuge tubes. Approximately 5% of the radioactivity remained at the top of the Airfuge® tube compared to 1.5% in the conventional centrifugation tube at d = 1.006; at d = 1.2, only ca. 60% of the radioactivity was present at the top of the Airfuge® tube, compared to 87% in the ultracentrifuge.

When isolated [^{125}I] labeled LDL, VLDL and HDL were separately centrifuged in phosphate-buffered KBr solutions at d = 1.006, 1.06 or 1.2, greater differences were noted in the frac-

TABLE 1

Fractionation of [^{125}I] Low Density Lipoprotein from Plasma at Different Densities

Time of centrifugation (hrs)		d = 1.006	d = 1.06	d = 1.2
Ultracentrifuge[a]	20	1.5%	53%	87%
Airfuge®	1	9 (7-11)[b]	52 (44-58)	43 (38-51)
	2	6 (5-8)	56 (48-63)	45 (35-51)
	3	4 (3-5)	51 (45-57)	59 (54-66)

[a]The total number of radioactive counts/min in each ultracentrifuge tube (30 ml) was ca. 230,000. The number in each Airfuge® tube averaged 7,000 in one experiment and 30,000 in another. Values represent the average percent of the total counts in top fraction of each tube as described in Methods; each average is based on 4 tubes.

[b]Range of values.

TABLE 2

Comparison of the Distribution Percentage of [^{125}I] Labeled Lipoproteins in Buffer in the Airfuge® and Ultracentrifuge

	d = 1.006		d = 1.06		d = 1.2	
	A[a]	U	A	U	A	U
[^{125}I] VLDL	52, 54	74, 71	76, 81	79, 78	88, 71	80, 80
[^{125}I] LDL	41, 42	1, 1	72, 76	81, 86	83, 74	87, 93
[^{125}I] HDL	41, 42	1, <1	7, 6	4, 3	45, 46	32, 46

[a]A = Percent of the total counts in top fraction of Airfuge® tubes; U = percent of the total counts in top fraction of ultracentrifuge tubes. Individual values are shown. Each Airfuge® tube contained an average of 70,000 radioactive counts/min. The ultracentrifuge tubes contained ca. 400,000 counts each.

tionation patterns from the 2 methods (Table 2). VLDL was underestimated at d = 1.006 in the Airfuge®, while LDL and HDL failed to sediment properly through the buffer at this density. Also, LDL flotations in the Airfuge® at d = 1.06 and 1.2 were slightly less than expected; HDL fractionations at these 2 densities compared favorably with that in the ultracentrifuge. One of the major problems in this experiment was the adhesiveness of the [^{125}I] lipoproteins to the cellulose propionate tubes. About 20% of the total radioactivity in the VLDL and LDL samples and 5% in the HDL remained in the tubes after removal of the fluid contents. As can be seen from Table 3, small differences occurred in the Airfuge® fractionations obtained at room temperature (24 C) and at 5 C in the LDL samples only. No differences in distribution were found whether samples were overlayered or underlayered.

Data in Table 4 indicate that fractionation of [^{3}H] benzo(a)pyrene-containing lipoproteins by the Airfuge® method at d = 1.006 resulted in overestimation of the amount of the compound in VLDL. At d = 1.06, both VLDL and LDL should float during centrifugation and at d = 1.2, the combined lipoproteins (VLDL, LDL and HDL) should float. Compared to ultracentrifugation, the Airfuge® method resulted in a slight overestimate of benzo(a)pyrene distribution into the plasma lipoproteins.

Partitioning differences also were found when [^{3}H] vitamin D_3 was incubated with plasma and centrifuged by the 2 methods. Again, an overestimation of the amount of labeled vitamin D_3 in VLDL at d = 1.006 and in VLDL and LDL at d = 1.06 occurred (Table 5). Furthermore, data from ultracentrifugation indicated that almost ¾ of this labeled compound in plasma partitioned into the total lipoprotein fraction; data from the Airfuge® experiments, however, indicated that less than half partitioned into lipoproteins. Data in Table 6 indicate that the estimation by the Airfuge® of labeled vitamin D_3 in the d = 1.006 fraction was high at both 24 C and 5 C, although there is a substantial difference in the estimates at the 2 temperatures. The distributions of this label at d = 1.06 and 1.2 did not differ in Airfuge® centrifugation at the two temperatures.

TABLE 3

Comparison of the Distribution Percentage of [^{125}I] Labeled Lipoproteins in Buffer in the Airfuge® at 2 Temperatures

	d = 1.006		d = 1.06		d = 1.2	
	24 C	5 C	24 C	5 C	24 C	5 C
[^{125}I] VLDL	52, 50[a]	53, 51	70, 63	62, 64	72, 78	73, 67
[^{125}I] LDL	38, 47	21, 29	72, 72	85, 82	74, 86	88, 80
[^{125}I] HDL	41, 36	41, 41	15, 13	19, 18	51, 46	50, 47

[a]Percent of total counts in the top fraction of the Airfuge® tube. Individual values are shown.

TABLE 4

Comparison of the Distribution of [^{3}H] Benzo(a)pyrene into Plasma Lipoproteins Using 2 Methods of Centrifugation

	Percent distribution into top fraction	
	Airfuge®	Ultracentrifuge
d = 1.006[a]	46[b]	20
d = 1.06	52	47
d = 1.2	57	52

[a]At d = 1.006, the top fraction should contain only VLDL; at d = 1.06, LDL + VLDL; at d = 1.2, HDL + LDL + VLDL.

[b]The values are averages of duplicates; no numbers varied from the average more than 2 percentage points. Each Airfuge® tube contained an average of 75,000 cpm and each centrifuge tube, 2 X 10^6 cpm.

TABLE 5

Comparison of the Distribution of [^{3}H] Vitamin D_3 into Plasma Lipoproteins Using 2 Methods of Centrifugation

	Percent distribution into top fraction	
	Airfuge®	Ultracentrifuge
d = 1.006	22[a]	4, 4
d = 1.06	37, 33	15, 17
d = 1.2	45, 43	72, 73

[a]Individual values are shown. Each tube contained an average of 5,500 counts/min.

DISCUSSION

The conventional ultracentrifugal procedures for separating lipoproteins are time-consuming, relatively expensive and require large volumes of plasma. The air-driven tabletop ultracentrifuge micromethod was developed for estimation of VLDL, LDL and HDL cholesterol in clinical laboratories (2). The present study was undertaken to evaluate the utility of this rapid method for estimating the degree of partitioning of labeled hydrophobic compounds into the various plasma lipoproteins.

The Airfuge® method compared favorably with ultracentrifugation in the flotation of LDL in plasma at d = 1.06 but was less satisfactory at d = 1.2. Fractionation of [^{125}I] labeled VLDL or HDL added separately to plasma led to equivocal results after centrifugation in both the Airfuge® and centrifuge, as the apoproteins of these 2 fractions can exchange in the presence of plasma (data not shown). Therefore, some of the radioactive counts originally associated with the apoproteins of VLDL, for instance, become associated and sediment with unlabeled HDL, resulting in spuriously high counts in the infranate. Centrifugation of each [^{125}I] lipoprotein individually in buffers of appropriate densities, rather than plasma, alleviated this problem and permitted the evaluation and comparison of the 2 methods. The Airfuge® method proved unsatisfactory for flotation of VLDL and sedimentation of LDL and HDL at d = 1.006; for instance, if [^{125}I] LDL

TABLE 6

Comparison of the Distribution Percentage of [^{3}H] Vitamin D_3-Associated Lipoproteins in Buffer in the Airfuge® at 2 Temperatures

	d = 1.006		d = 1.06		d = 1.2	
	24 C	5 C	24 C	5 C	24 C	5 C
[^{3}H] Vitamin D_3-LDL	52, 50[a]	53, 51	70, 63	62, 64	72, 78	73, 67
[^{3}H] Vitamin D_3-HDL	35, 33	18, 17	25, 24	20, 21	71, 70	61, 70

[a]Percent of total counts in the top fraction of the Airfuge® tube. Individual values are shown.

and [^{125}I] VLDL had been present in the same sample, the amount of VLDL would have been much overestimated from the radioactive counts in the top fractions. The differences noted were not due to the difference in temperature of spinning as inadequate sedimentation of LDL and HDL occurred in the Airfuge® at both 24 C and 5 C. This observation is in agreement with the findings of Kupke (5) and Widhalm et al. (3); Stroble and Widhalm (14) have ascribed the lack of precision in VLDL separation to slight vibrations of the rotor, resulting in mixing of the tube contents. Our findings, however, suggest that at d = 1.006, LDL and HDL simply are not sedimented properly in the Airfuge® and this results in an incomplete fractionation. If rotor vibration caused mixing, the amount of radioactivity in the supernate after centrifuging [^{125}I] HDL at d = 1.06 would be much higher. Instead, at d = 1.06 and 1.2, centrifugation of each lipoprotein species yielded similar distributions with both the Airfuge® and ultracentrifuge (Table 2). Although tube slicing may have improved the overall precision, as suggested by several investigators, it is unlikely that results from Airfuge® centrifugation at d = 1.006 would have been altered.

The addition of labeled hydrophobic compounds to plasma followed by centrifugation provided an entirely different approach; rather than having a discrete label on only one lipoprotein species, the labeled benzo(a)pyrene and vitamin D_3 associated to some extent with all 3 lipoprotein groups. The Airfuge® method proved less than satisfactory in determining the amount of distribution of hydrophobic compounds into the plasma lipoproteins. Very little of the difference may be ascribed to different temperatures of centrifugation in the two methods, since Airfuge® centrifugation at 5 C resulted in little improvement in fractionations (Table 6). We concluded from these experiments that the percent distribution into VLDL was always overestimated; that if the majority of the compound associated with LDL, as benzo (a)pyrene, its distribution into that fraction was slightly overestimated; and that if its major association was with HDL, as vitamin D_3, its distribution into LDL is greatly overestimated. In short, it is not possible to assess accurately the percent distribution of a labeled hydrophobic compound into the individual lipoprotein classes by the Airfuge® method.

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Digitonin-Precipitable Sterols as a Measure of Cholesterol Biosynthesis: Contradictory Results

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ABSTRACT

In experiments with 4 different types of cells, we evaluated the cholesterogenic activity by incorporation of ^{14}C-acetic acid into cholesterol and digitonin-precipitable sterols. In every case, the cholesterogenesis appeared considerably faster when expressed as digitonid than when expressed as real cholesterol production, and sometimes the data obtained by the 2 methods were contradictory. Detailed analysis of both digitonid components and nonprecipitable radioactive metabolites showed that a very variable fraction of methyl sterols (including bifunctional methyl sterols) co-precipitates with the C-27 sterols. In cholesterol regulation studies and particularly when the cells exhibit a low cholesterogenesis, the digitonin method is unsuitable and can lead to erroneous interpretations.

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INTRODUCTION

Measurement of cholesterol biosynthesis from labeled precursors as digitonin-precipitable sterols is a standard method for studying cholesterol regulation (1-6). Cholesterol and its C-27 precursors are assumed to be the main components of digitonids; however, 4-4'-dimethyl sterols, which are metabolites of the cholesterol biosynthetic pathway, are also precipitated to some extent by digitonin, as was recently confirmed by R.J. Cenedella (7).

In the case of highly cholesterogenic cells, this simple and rapid method may be satisfactory, but when the analyzed mixture contains low cholesterol levels and many labeled cholesterol metabolites, the data could lead to erroneous interpretation.

In this study, we compare the results obtained by measuring the cholesterol biosynthesis from ^{14}C-acetic acid in several types of cells using 2 different methods: as digitonid and by evaluation of authentified ^{14}C-labeled cholesterol.

MATERIALS AND METHODS

Materials

[2-^{14}C] Sodium acetate (45-55 mCi/mM) was from C.E.A., reference samples of sterols from Steraloids, digitonin from Fluka, culture medium RPMI-1640, DEM, MEM from Flow laboratories. All silica gel plates were from Merck. Radioactivity was scanned on a Berthold scanner model LB 2760; GLC analyses were performed on a Carlo Erba chromatograph, model 2300, and mass spectra on a LKB 2091 gas chromatograph-mass spectrometer (GC-MS Department, University I, Montpellier).

Methods

Cell culture. Human peripheral blood lymphocytes from voluntary normal donors were isolated as described in reference 8 and cultured in RPMI-1640 supplemented with antibiotic (gentamicin: 80 μg/ml) and 20% lipoprotein-free AB serum according to Tabacik et al. (9) at a concentration of 4×10^6 cells/ml medium. Lymphocytes from leukemic hamster (L_2C cells) were isolated and cultured in RPMI-1640 according to Philippot et al. (3). Human genital skin fibroblasts from children were cultured in monolayer in DEM supplemented with 10% fecal calf serum (10). The human mammary carcinoma cell line MCF_7 was from Dr. Lipman (National Cancer Institute, Bethesda, MD). The cells were cultured in monolayers in MEM supplemented with 5% fecal calf serum.

Incubation with [2-^{14}C]sodium acetate. Before ^{14}C-acetate incorporation, all the cells were separated from serum-supplemented culture medium and placed in culture medium without serum (5×10^7 lymphocytes/ml medium in plastic tubes; monolayer culture: 3 ml medium in 6 cm diameter petri dishes). Twenty μl [2-^{14}C] sodium acetate solution (20 μC) was added per ml culture medium and cells incubated at 37 C for various lengths of time (lymphocytes under gentle stirring; cells in monolayer under air + 5% CO_2 stream).

After incubation, the cells were separated from culture medium, washed 3 times with PBS and transferred into glass tubes with 2 ml of 10% sodium hydroxide. After 1 hr of saponification at 85-90 C, the total ^{14}C-incorporation into the cell was measured (on an aliquot) and the nonsaponifiable material was extracted with petroleum ether.

Lipid analysis. Half of the nonsaponifiable

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Abbreviations: TLC: thin layer chromatography; GLC: gas liquid chromatography; 4,4'-dimethyl sterols or C-29 sterols: cholesten-4,4' dimethyl-3β-ols; 4α-methyl sterols or C-28 sterols: cholesten-4α-methyl-3β-ols; and HMG CoA: 3-hydroxy-3-methyl-glutaryl coenzyme A.

material was precipitated with digitonin according to the standard method (11); the remainder was analyzed according to the method described in reference 12. We also analyzed, in the same way, the sterols recovered from the digitonid and in several cases, the supernatant from digitonid precipitation.

Briefly, the nonsaponifiable material was fractionated by TLC in system I into the C-27 sterol zone, C-28 sterol + aliphatic alcohol zone, C-29 + C-30 sterol zone, front zone. The 3 sterol zones were reduced with BH_4Na in order to separate the bifunctional methyl sterols (4,4′-dimethyl or 4,4′,14α-trimethyl-cholestenols, 32 (or 31)-al) as more polar diols (TLC in system I).

C-27 sterol analysis: the C-27 sterol zone was acetylated (pyridine + acetic anhydride) and the acetate mixture (supplemented with 10 μg lathosterol acetate) spotted on a silica gel-NO_3Ag impregnated plate. The plate was developed in system II. Preparation of plates: glass precoated Silica Gel G plates were dipped in a 10% aqueous NO_3Ag solution, then activated for 2 hr at 120 C.

The C-29 + C-30 sterol zone was analyzed on the same kind of NO_3Ag-impregnated glass silica gel plate in system III.

The C-28 sterol + aliphatic alcohol zone was oxidized with chromic anhydride dissolved in acetic acid and the aliphatic alcohols were evaluated as aliphatic acids in system I.

The front zone was fractionated on a silica gel plate in system IV with the reference samples: squalene, oxido-squalene, lanosteryl stearate as methylsterol ester, cholest-7-en-3-one as sterone.

The sterone zone was reduced with BH_4Na (20 hr in aqueous dioxan at room temperature) and the sterols obtained analyzed on TLC in system I, then as acetates in system III and by GLC (of the acetates): capillary column, OV 101; oven temperature, 260 C; injector 300 C; He gas flow, 0.8 kg/cm^2; H_2, 0.5 kg/cm^2. Mass spectra of the acetates: electron energy, 70 eV; trap current, 50 A; tension acceleration, 3500 V; ion source temperature, 260 C, and molecular separation temperature, 240 C.

The methyl sterol ester zone was reduced with $AlLiH_4$ and the sterol mixture obtained purified by TLC in system I; the sterol zone eluted from system I with pure ethyl ether was divided into 2 parts: one part was analyzed in system III and the other by GLC and MS (same conditions as above).

System I: silica gel, hexane/ether/acetic acid, 50:50:0.5, 2.5 hr, continuous run at room temperature.

System II: glass precoated (+ NO_3Ag) plate, carbon tetrachloride/benzene, 90:10, 20 hr, continuous run at 4 C.

System III: glass precoated (+ NO_3Ag) plate, carbon tetrachloride/benzene, 95:5, continuous run at 4 C.

System IV: silica gel plate; one run in hexane/ethyl acetate, 97:3.

Digitonin precipitation (11). After enrichment of the nonsaponifiable material by cholesterol (1 mg or 0.25 mg in 200 μl acetone/diethyl ether, 1:1), the sterols were precipitated by addition of 1 ml digitonin solution (0.5% in 90% ethanol). After one night at room temperature, the precipitate was centrifuged and washed 3 times successively with 0.5 ml of ethanol/diethyl ether, 1:1; acetone/diethyl ether, 1:1; and diethyl ether. After decomposition of the digitonid with pyridine (200 μl), the radioactivity of the digitonin-precipitable sterols was counted and the mixture analyzed in the same way as the nonsaponifiable material.

$$\% \text{ precipitated methyl sterols from nonsaponifiable material} = \frac{\text{dpm methyl sterols from digitonin}}{\text{dpm methyl sterols from nonsaponifiables}} \times 100$$

Radioactivity was counted in a Packard scintillation counter (mode 1 TRI-CARB 460 CD) in a toluene solution of 2,5-diphenyl oxazole. Proteins were measured according to Bradford (13).

RESULTS AND DISCUSSION

Cholesterol Biosynthesis in Normal Human Lymphocytes

It is well known that native lymphocytes manifest a low lipid metabolism which can be stimulated by culture in a poor lipid medium (8). In a previous study (7), we followed the stimulation of cholesterol biosynthesis by culturing cells for several days in a lipid-free medium. The biosynthesis was evaluated by incorporation of radioactive acetic acid into digitonin-precipitatable sterols and into cholesterol itself (as shown in Fig. 1). Maximum stimulation would be at ca. 44 hr according to the digitonin-precipitation method, whereas cholesterol production reached an acute maximum after 66 hr of culture. This experiment, which was designed to allow the choice of optimum time of stimulation, could lead to erroneous interpretation, if based on digitonid evaluation only.

As the specificity of the digitonid method mainly depends upon the precipitation of methyl sterols (which is incomplete), we analyzed the precipitated sterols after two times of stimulations (20 hr and 66 hr), and determined

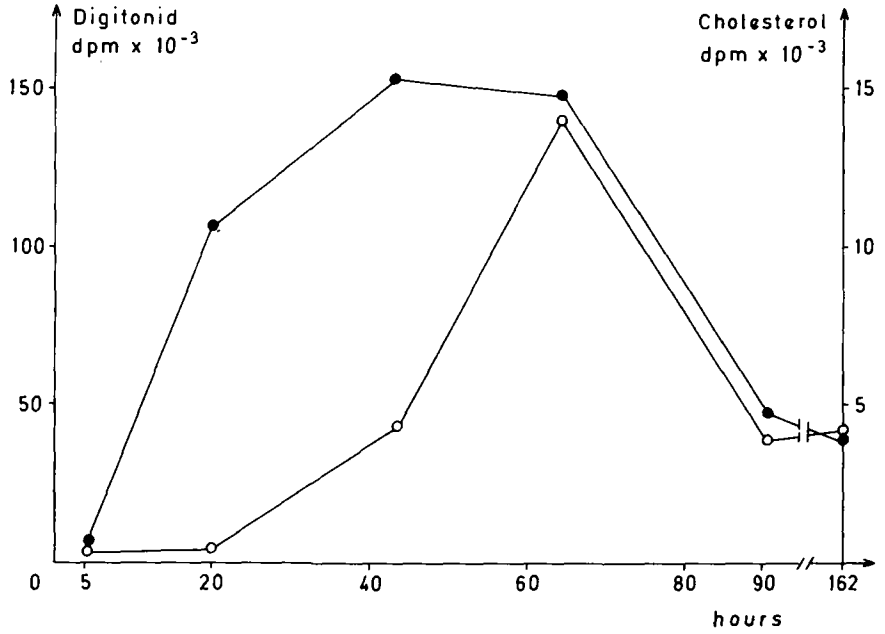

FIG. 1. Experiment 1: cholesterol biosynthesis in normal human lymphocytes was stimulated by culture in the presence of lipoprotein-free serum for 0 to 162 hr, and ^{14}C-acetic acid incorporation by 5 X 10^7 cells into cholesterol and digitonin-precipitable sterols was measured after different times of culture: —•—•— dpm X 10^{-3} digitonid; —o—o— dpm X 10^{-3} cholesterol.

at each time the percentage of methyl sterols precipitated by digitonin from nonsaponifiable material.

In experiment 1, the amount of nonradioactive cholesterol added for precipitation with digitonin was 1 mg, but in all further experiments, we added only 0.25 mg of cholesterol in order to facilitate the analysis at the state of TLC.

Table 1 shows composition of digitonids: according to a previous study (12), we isolated, after BH_4Na reduction of the sterol zone (from chromatography in system I), a polar fraction which was identified as a mixture of a lanosten-3β, 32-diols. These diols arose from methyl sterols bearing a carbonyl function at C-32, which are metabolites of lanosterol demethylation, and are noted in Table 1 as "bifunctional methyl sterols." If we compare the cholesterogenesis expressed as cholesterol or digitonid after 20 hr and 66 hr culture, the following observations can be made: in experiment 2, the stimulation was 26 times higher after 66 hr than after 20 hr, but according to the digitonid method, it was only 6 times higher; in experiment 3, the lack of agreement between the 2 methods is more evident in that after 66 hr culture the cells produced 40 times more cholesterol than after 20 hr, but the digitonid level was only 1.2 times higher; in experiment 4, the digitonin-precipitation method led to a contradictory result – a stimulation lower after 66 hr than after 20 hr, whereas the cholesterol biosynthesis was higher (as expected).

In Table 2, we can see the percentage of methyl sterols which have precipitated by digitonin from the nonsaponifiable material: this value varies from 24 to 87%.

Cholesterol Biosynthesis in L_2C Cells

Table 3 shows the composition of the sterols precipitated and the percentage of methyl sterols which have precipitated from the nonsaponifiable material (Tabacik et al., unpublished results). This percentage is considerably lower than in the case of normal human lymphocytes and varies only from 1 to 12.8%, although the composition of the cholesterol metabolites was apparently similar for the 2 types of cells and the amount of added nonradioactive cholesterol was the same (0.25 mg). If the precipitation of the 2 kinds of methyl sterols (monofunctional

TABLE 1

Composition (%) of Digitonin-Precipitable Material after Incorporation of ^{14}C-Acetic Acid by Stimulated Human Lymphocytes

Experiment no.	1		2		3		4	5
Stimulation time (hr)	20	66	20	66	20	66	66	66
% C-29, C-30 Sterols	4.5	1.9	35.9	11.5	5.3	2.1	1.2	5
C-28 Sterols + *n*-fatty alcohols	12.0	6.3	4.8	6.2	3.5 2.8	2.3 1.9	1.7 1.4	2.7 2.2
C-27 Sterols	33.1	58.3	8.8	41.1	13.3	50.7	63.0	44.5
(Cholesterol)	(2.4)	(11.2)	(1.2)	(22.1)	(0.7)	(8.0)	(12.1)	(8.1)
Bifunctional methyl sterols	50.3	33.50	50.5	47.1	75.0	42.9	32.5	45.5
dpm X 10^{-3} nonsaponifiable material/5 X 10^7 cells	94.4	267.5	834	696	126.7	72.5	233.9	93.6
dpm X 10^{-3} digitonid/5 X 10^7 cells	32.4	190.0	475.8	581.0	101.2	36.0	153.9	45.4
dpm X 10^{-3} cholesterol/5 X 10^7 cells	0.8	20.8	2.7	107.5	1.3	3.0	20	2.6

Cholesterol biosynthesis in normal human lymphocytes was stimulated by culture in the presence of lipoprotein-free serum for 20 or 66 hr, and ^{14}C-incorporation into nonsaponifiable material, digitonin-precipitable material and cholesterol were measured after 4 hr incubation. At each incubation time, both total nonsaponifiable material and digitonin-precipitable material were analyzed and the percentage of methyl sterols precipitated from nonsaponifiable material into the digitonid was calculated.

and bifunctional) is examined in more detail, it is apparent that the bifunctional precipitates more eaasily than the monofunctional.

Figure 2 shows the yield of cholesterol and digitonin-precipitable sterols biosynthesized from the whole radioactivity incorporated by the cell. We observed that these 2 parameters are almost equivalent only after a long incubation time with the precursor. If we used an intermediary incubation time, e.g., 4 hr (a time which is often used in cholesterol regulation studies), the cholesterol produced by the cells was still 3 times lower than the digitonin-precipitable sterols.

Cholesterol Biosynthesis in MCF$_7$ Cells

In the nonsaponifiable material, we idenfified a large amount (10-30%) of aliphatic alcohols (14); a fraction of these (4-16%) was recovered in the digitonid from which we also isolated bifunctional methyl-sterols (see Table 4).

The percentage of total methyl sterols precipitated from the nonsaponifiable material varies from 36.6% to 41.4% and until the 6th hour of incubation there is a considerable disparity between the 2 tested methods for cholesterol evaluation. If the precipitation of the 2 kinds of methyl sterols is examined in more detail (as in the case of L_2C cells), it is again apparent that the bifunctional precipitates more easily than the monofunctional. Figure 2 shows the yield of cholesterol and digitonin-precipitable sterols from the total radioactivity incorporated into the cell between 0 and 24 hr incubation. After 4 hr incubation, "cholesterol biosynthesis" evaluated as digitonid was still twice as high as when measured by direct quantification of the final product.

Cholesterol Biosynthesis in Human Fibroblasts

The cholesterol biosynthesis was evaluated from ^{14}C-acetate after various incubation times (15). We found very small amounts of radioactive cholesterol in total nonsaponifiable material, and this very low cholesterogenic activity was also confirmed by a low digitonin precipitation (Table 5). However, the cholesterogenic activity, when evaluated as digitonid, was already maximal after 8 hr, whereas the cholesterol yield was considerably lower (10 times) and still increasing (see Fig. 2).

At every incubation time, nonprecipitable lipids (supernatant of digitonid) represent ca. 80% of nonsaponifiable material. These lipids are generally assumed to be composed of squalene and oxido-squalene. According to this widely acknowledged concept, one could conclude that these compounds accumulate in nonsaponifiable material and that a post-HMG CoA regulation of the process occurs between squalene and oxido-squalene. We analyzed these nonpolar compounds (see Table 6) and found that the main components were triterpenic metabolites, sterones and esters of methyl sterols (compounds very difficult to saponify),

TABLE 2

Percentage of Methyl Sterols Precipitated with Digitonin from Nonsaponifiable Material After Incorporation of ^{14}C-Acetic Acid by Human Lymphocytes

Incubation time (hr)	2						4							
Experiment no.	1						2		3		4		5	6
Time of stimulation (hr)	6	20	44	66	91	162	20	66	20	66	20	66	66	66
dpm X 10^{-3} methyl sterols from nonsaponifiable material	6.3	52.8	41.7	21.4	8.1	5.9	274.4	370.4	99.4	20.6	29.4	21.3	262.3	107.2
dpm X 10^{-3} methyl sterols from digitonid	1.5	23.4	17.9	13.7	5.2	1.9	79.5	185.1	71.7	8.5	18.2	18.5	127.7	76.5
% precipitated methyl sterols	24	44	43	64	65	33	29	50	72	41	62	87	49	71

Cholesterol biosynthesis in normal human lymphocytes was stimulated by culture in the presence of lipoprotein-free serum for 20 or 66 hr, and ^{14}C-incorporation ino nonsaponifiable material, digitonin-precipitable material and cholesterol were measured after 4 hr incubation. At each incubation time, both total nonsaponifiable material and digitonin-precipitable material were analyzed and the percentage of methyl sterols precipitated from nonsaponifiable material into the digitonid was calculated.

$$\text{\% methyl sterols precipitated (from nonsaponifiable materials)} = \frac{\text{dpm methyl sterols from digitonid}}{\text{dpm methyl sterols from nonsaponifiable material}} \times 100$$

TABLE 3

Composition (%) of Digitonin-Precipitable Material after Incorporation of ^{14}C-Acetic Acid by L_2C Cells

Incubation time (hr)	0.25	0.5	1	2	4	24
	%					
C-29, C-30 Sterols	0	0	0.6	2.0	2.0	2.7
C-28 Sterols + *n*-fatty alcohols	1.8	1.8	3.2	1.5	4.1	8.7
C-27 Sterols	85.7	85.4	78.0	85.5	84.4	86.6
(Cholesterol)	9.6)	(15.0)	(12.0)	(22.2)	(22.2)	(29.3)
Bifunctional methyl sterols	12.5	12.8	18.2	11.0	9.5	0
dpm X 10^{-3} nonsaponifiable material/5 X 10^7 cells	149.8	357.9	485.5	642.1	783.8	1934.3
dpm X 10^{-3} digitonid/5 X 10^7 cells	59.3	144.3	195.0	258.8	374.6	597.7
dpm X 10^{-3} cholesterol/5 X 10^7 cells	5.7	21.6	23.4	42.7	83.9	566.9
dpm X 10^{-3} methyl sterols/ 5 X 10^7 cells from nonsaponifiable material						
monofunctional	22.2	142.3	169.9	205.5	233.8	745.7
bifunctional	43.7	97.1	162.7	202.9	258.3	142.4
dpm X 10^{-3} methyl sterols/ 5 X 10^7 cells from digitonid						
monofunctional	1.1	2.6	7.4	9.0	22.9	68.1
bifunctional	7.4	18.5	35.5	28.5	26.1	0
% precipitated methyl sterols[a]						
monofunctional	1.9	1.8	4.4	4.5	10.2	9.1
bifunctional	16.9	19.0	21.8	14.0	10.1	0
% total precipitated methyl sterols (mono- + bifunctional)	12.8	8.8	7.9	4.5	4.2	1.0

^{14}C-Acetic acid incorporation by L_2C cells into nonsaponfiable material and digitonin-precipitable material were measured and the composition (%) of each mixture determined according to the experimental procedure described in the text. From these data, we calculated the percentage of methyl sterols which were precipitated with digitonin from the nonsaponifiable material.

[a]See Table 2 or Materials and Methods.

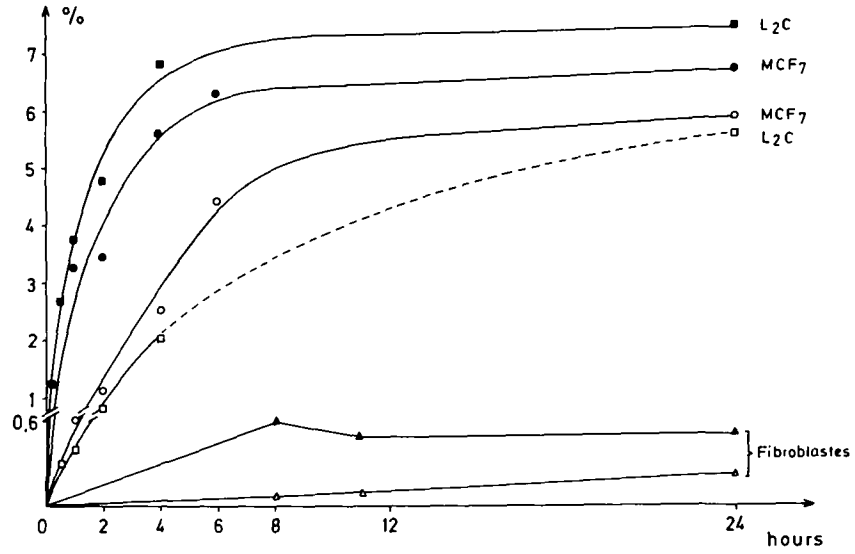

FIG. 2. ^{14}C-Acetic acid incorporation into cells, nonsaponifiable material, digitonin-precipitable material and cholesterol were measured after 0 to 24 hr incubation at 37 C in RPMI. From these data, we calculated the transformation of ^{14}C-acetic acid into digitonin-precipitable material and cholesterol by the cells.

whereas squalene was only a minor constituent. This would support a regulation at the lanosterol demethylation step rather than at squalene epoxidation.

The percentage of free methyl sterols precipitated by digitonin from nonsaponifiable material varied over 3 incubations (see Table 5). We have not looked for the presence of bifunctional methyl sterols, because of the small amount of labeled material (low digitonid precipitation).

CONCLUSION

From the experiments described above with 4 different types of cells and in agreement with early work, we can see that a fraction of the methyl sterols present in the nonsaponifiable material always co-precipitates with the C-27 sterols. This fraction is very variable, even though the amount of cholesterol added for digitonin precipitation (0.25 mg) is constant. The analyzed mixture contains microquantities of methyl sterols in the presence of a constant high amount of cholesterol. Our results agree with those of Cenedella (7) who observed, in similar cases, the co-precipitation of a large proportion of methyl sterols, but we are unable to explain why this proportion is so variable. The differences lie perhaps in the fact that the mixtures we analyzed were very complex, including all possible metabolites of the lanosterol demethylation (bifunctional methyl sterols, sterones, methyl sterols esters, etc.) which can bind one another (12). Our results show that the precipitated methyl sterols are mainly bifunctional methyl sterols. It is thus impossible to predict what proportion of the methyl sterols present in the total extract would be found together with C-27 sterols in the digitonid.

The supernatant from digitonin precipitation is generally considered to consist essentially of acyclic metabolites of cholesterol biosynthesis, i.e., squalene and oxido-squalene. In our experiments, these 2 compounds were always minor constituents of the nonprecipitable metabolites which could include high amounts of unexpected compounds such as aliphatic alcohols (Table 4), sterones and methyl sterol esters (Table 6).

In conclusion, our results indicate that digitonin precipitation is not a suitable method for measuring kinetics of cholesterol biosynthesis or for regulation studies, especially with low cholesterogenic cells. This conclusion since based on observations with 4 different types of cells could represent a general situation, mainly for

TABLE 4

Composition (%) of Digitonin-Precipitable Material after Incorporation of ^{14}C-Acetic Acid by MCF_7 Cells

Incubation time (hr)	1	2	4	6	24
	%				
C-29, C-30 Sterols	2.4	1.4	1.2	1.2	0.2
n-Fatty alcohols	4.3	3.5	2.4	1.9	0.5
C-27 Sterols	54.1	66.9	74.9	77.7	90.7
(Cholesterol)	(10.8)	(19.0)	(27.8)	(37.3)	(69.3)
Bifunctional methyl sterols	39.2	28.2	21.5	19.2	8.6
dpm X 10^{-3} nonsaponifiable material/mg P	303.6	281.2	559.9	729.5	2906.5
dpm X 10^{-3} digitonid/mg P	129.9	133	280.5	396.9	1918.3
dpm X 10^{-3} cholesterol/mg P	14.0	25.2	78.1	147.9	1329.2
dpm X 10^{-3} methyl sterols/ mg P from nonsaponifiable material					
monofunctional	44.9	26.5	37.1	70.0	296.4
bifunctional	101.3	80.9	104.2	116.9	194.3
dpm X 10^{-3} methyl sterols/mg P from digitonid					
monofunctional	3.1	1.8	3.4	4.7	38.3
bifunctional	50.9	37.5	60.3	76.2	164.9
% precipitated methyl sterols[a]					
monofunctional	6.9	6.7	9.2	6.7	12.9
bifunctional	50.2	46.3	57.8	65.2	84.6
% total precipitated methyl sterols	39.0	36.6	45.1	43.3	41.4

^{14}C-Acetic acid incorporation by MCF_7 cells into nonsaponifiable material and digitonin-precipitable material were measured and the composition (%) of each mixture determined according to the experimental procedure described in the text. From these data, we calculated the percentage of methyl sterols which were precipitated with digitonin from the nonsaponifiable material.

[a]See Table 2 or Materials and Methods.

TABLE 5

Composition (%) of Digitonin-Precipitable Material after Incorporation of ^{14}C-Acetic Acid by Human Genital Skin Fibroblasts

Incubation time (hr)	8	11	23
	%		
C-28, C-29, C-30 sterols	14.6	19.10	10.6
C-27 sterols	85.4	80.90	89.4
(cholesterol)	(12.5)	(20.40)	(42.0)
dpm X 10^{-3} nonsaponifiable material/mg P	51.0	59.8	105.4
dpm X 10^{-3} digitonid/mg P	6.9	6.6	9.9
dpm X 10^{-3} cholesterol/mg P	0.87	1.35	4.19
dpm X 10^{-3} free methyl sterols/mg P from nonsaponifiable material	14.67	13.82	14.73
dpm X 10^{-3} free methyl sterols/ mg P from digitonid	1.01	1.26	3.56
% precipitated methyl sterols[a]	6.9	9.11	24.2

^{14}C-Acetic acid incorporation by human genital skin fibroblasts into digitonin-precipitable material and digitonin-nonprecipitable material were measured and the composition of each mixture determined according to the experimental procedure described in the text. From these data, we calculated the percentage of methyl sterols which were precipitated with digitonin from the nonsaponifiable material.

[a]See Table 2 or Materials and Methods.

TABLE 6

Composition (%) of Supernatant from Digitonid after Incorporation of ^{14}C-Acetic Acid by Human Genital Skin Fibroblasts

Incubation time (hr)		8	11	23
% nonpolar compounds	squalene	2.3	1.2	1.3
	methyl sterol esters	23.1	25.7	21.1
	sterones	70.2	71.0	74.0
C-28, C-29, C-30 sterols		4.4	2.1	3.6

^{14}C-Acetic acid incorporation by human genital skin fibroblasts into digitonin-precipitable material and digitonin-nonprecipitable material were measured and the composition of each mixture determined according to the experimental procedure described in the text. From these data, we calculated the percentage of methyl sterols which were precipitated with digitonin from the nonsaponifiable material.

pulse-labeling studies with radioactive precursors.

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Detection and Determination of Lipase (Acylglycerol Hydrolase) Activity from Various Sources

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ABSTRACT

Methods for the detection and determination of lipases (acylglycerol hydrolases) and preparation of assays are reviewed including substrates, conditions and screening. Some newer methods for the determination of lipase activity are discussed. Several of these are: (a) titrimetry, (b) colorimetry of Cu soaps of free fatty acids (FFA), (c) colorimetry of chromophores in the acyl chain of FFA or in glycerol, (d) radioassay, (e) gas liquid chromatography, (f) enzymatic treatment of FFA and measurment of the resulting products, and (g) direct immunological determination of the lipase. Examples and sensitivities are given and advantages and disadvantages are described.
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Lipases or acylglycerol acylhydrolases (EC 3.1.1.3) (1) are defined for our purposes as enzymes which hydrolyze esters of long-chain aliphatic acids from glycerol at oil/water interfaces. Phospholipids and cholesteryl esters are not included as substrates, although there are lipases which will hydrolyze acylglycerols and phospholipids or cholesteryl esters. Emulsion globules, fat bodies or lipoprotein particles usually provide the interface and these have been termed the supersubstrate (2). Lipases have the important physiological role of preparing the fatty acids of water-insoluble TG for absorption into and transport through membranes by converting the TG to the more polar DG, MG, FFA and glycerol. With the exception of glycerol, these products are absorbed as mixed micelles from the lumen of the small intestine (3) or by lateral diffusion via a continuous water layer extending into the target cell (4). The FFA are transported in blood bound to albumin. Another important physiological role of lipases is to provide FFA for activation to CoA esters so that the acids can be oxidized, converted to prostaglandins or otherwise metabolized.

Lipases have many industrial uses (5). For example, the enzymes assist in the production of the characteristic flavors of Italian and mold-ripened cheeses and occasionally cause spoilage, even at -20 C. In raw bovine milk, activation of milk lipoprotein lipase can result in the rapid accumulation of 4:0, 6:0, 8:0 fatty acids, etc., to the point that these acids can be detected organoleptically. The flavor and odor of the acids are so overwhelmingly unpleasant that the milk must be discarded. Because the monetary value of the discarded milk can be substantial, several of the methods available for determination of lipase activity have been developed for the dairy industry (6).

Recently, various lipases, alone or in conjunction with other enzymes, have been widely used as hydrolytic reagents in the manual (7) and automated (8) determination of TG. The hydrolytic system must be able to digest completely all of the acylglycerols present to glycerol. The glycerol is determined by coupled enzymatic reactions and gives an indirect estimate of the acylglycerol content of the material, usually blood serum, being analyzed.

Some newer applications of lipases which have potential are hydrolysis of TG under mild conditions (9), synthesis of new TG by interesterification (10) and hydrolysis of other compounds. An example of the last is the preparation of ℓ-3-chloro-2-methyl propanol from the racemic propionate esters (11). The last synthesis is extraordinary because it is largely stereospecific, but uses pancreatic lipase which does not ordinarily have this property. Another ingenious application is the development of a sensor for neutral lipids based on an immobilized lipase and a flow-through pH electrode (12). The apparatus was employed to determine the neutral lipid content of serum.

For any of these applications, the enzyme being studied must first be identified as a lipase and then the activity be determined under nearly optimal conditions. Suitable methods for analysis of activity are prerequisites for monitoring purification and identification of specificity (13). The last major review of methods for the determination of lipase activity appeared in 1974 (14). Several extremely sensitive and novel analyses for lipase specificity have since been published. In this paper, I will review these analyses as well as procedures for detection of lipases. This paper should there-

Abbreviations: TG, triacylglycerols; DG, diacylglycerols; MG, monoacylglycerols; FFA, free fatty acids. Trioleoylglycerol is 18:0-18:1-18:1, etc.

fore be considered a summary of newer methods and not a comprehensive description. We have recently discussed the determination of lipase specificity (13).

UNITS OF ACTIVITY

A variety of units have been reported, but some authors have used the preferred IUPAC-IUB units (U) (15); μmol FFA released/min related to the volume of digestion mixture being tested or to the amount of substrate transformed. Others give activity in terms of 16:0 or 18:1, usually when these acids are employed to prepare a standard curve. Specific activity μmol of FFA released/min/mg protein is seldom seen. It would be helpful if all who investigate lipases report activity as U or preferably as specific activity. The latter must be given if the activities of lipase preparations from a variety of sources are to be meaningfully compared or if purification is being done.

DETECTION AND DETERMINATION OF LIPASE ACTIVITY

Lipolytic activity is generally signified by an accumulation of FFA and disappearance of TG when a tissue or fluid is incubated or stored. The presence of a lipase is then indicated. An example of this is the occurrence of the events above in germinating seeds. The lipase can be intrinsic or adventitious as in the growth of a microorganism in fatty foods. The conditions of detection, i.e., temperature, pH, presence of cations and fatty acid acceptors, will provide information required for the design of a suitable assay system. Beyond these parameters, it is necessary to assure that the definition of a lipase is met, but this may be implicit in detection. Since lipolytic activity may be very weak in circumstances where the enzymes are diffuse, activity may not be noticed unless extremely sensitive methods of detection are applied. These will be discussed later.

Substrates

In some studies, the source of the lipase also provides the substrate, e.g., adipocytes, but in most cases an external substrate is provided. Trioleoylglycerol (18:1-18:1-18:1) is an ideal substrate for lipases. If it is hydrolyzed in an emulsion by an enzyme, the definition of a lipase is fulfilled. In addition, it is liquid at the usual assay temperatures and is therefore easily emulsified. Solid substrates are digested, but much more slowly. Olive oil is a good, inexpensive substitute for 18:1-18:1-18:1. It contains over 70% 18:1. Commercial oils contain impurities which can be removed by passage of the oil through neutral alumina in hexane/ethyl ether (9:1, v/v) (16). Tributyrylglycerol (4:0-4:0-4:0) and all other TG soluble in the solvent mixture can be similarly purified, but the alumina will not retain compounds less polar than TG.

Tributyrylglycerol is a convenient substrate because it can be dispersed in water by shaking or stirring without the addition of emulsifiers. It is useful in lipase screening tests and in the quantitation of lipolytic activity by continuous automatic titration. Investigators must understand that 4:0-4:0-4:0 does not meet the definition of a substrate for lipases. Although most lipases will hydrolyze this substrate, so will some esterases and "lipase" activity should be verified with 18:1-18:1-18:1.

Many investigators have employed chromogenic substrates, such as fatty acid esters of 4-methylcoumarin (umbelliferone). Esterases release an alcohol (phenol, eosin, umbelliferone, naphthol, etc.) from these chromogens which is determined directly by fluorimetry. The method is very sensitive, but phenolic esters appear to be unsuitable for assays of lipases. Umbelliferone caprylate yielded only 0.2% of the activity obtained with 18:1-18:1-18:1 (17). Some of the esters hydrolyze spontaneously at pH 8.0, the optimum for many lipases. Umbelliferone esters are partially soluble in water and methyl cellosolve is added to improve solubility. Nevertheless, these substrates are still applied to the assay of lipase activity. Doouijwaard-Kloosterziel and Wouters (18) evaluated some properties of the lipase of *Geotrimchum candidum* with umbelliferyl esters. Roy (19) investigated lipases from *Pseudomonas fluorescens* and *Serratia marcescens* with the assay. Mattey and Morgan did the same with *Candida lipolytica* (20). However, Severson et al. (21) obtained higher activities with methylumbelliferone stearate dispersed with glycerol and lecithin than with similarly dispersed trioleoylglycerol when assaying pigeon adipose tissue ester hydrolase activity. There was little hydrolysis of methylumbelliferone stearate in the absence of lecithin. Activity with ethanolic trioleoylglycerol was 2-4 times higher than either dispersed substrates. Apparently, umbelliferyl esters are acceptable substrates if properly dispersed.

The Tweens (esterified polyoxyethylene derivatives of sorbitan) were substrates for various microorganisms (22,23) but are not suitable as substrates. They are heterogeneous, do not resemble TG and are hydrolyzed by esterases. Although not good substrates, the Tweens are useful emulsifiers. Obviously, their

use as emulsifiers will cause confusion.

Others have prepared acylglycerols esterified with fatty acids which contained a colored probe. The fatty acid can be measured spectrophotometerically either directly or after treatment with a color-forming reagent. For example, trinitrophenyl-ω-aminolauroyl TG (24) and 2,3 dimercapto-1-propanol tributyrylglycerol (25,26) have been utilized for assay of lipase activity. Cox and Horrocks (27) employed 16:0 and 10:0 analogs of 1-mercapto-2,3-propanediol as substrates for MG the lipase of *Rhizopus delemar*.

Suitable substrates for determination of DG and MG lipases can be synthesized as described by Jensen and Pitas (28) and Buchnea (29). With 2-MG and 1,2 (2,3)-DG, acyl migration to 1 (3) MG or 1,3-DG must be avoided or minimized or the results may be confounded. Precautions are discussed in reference 13.

Conditions

The pH optima of most lipases lie between 7 and 9, although there are exceptions, primarily the seed lipases. Phosphate, NH_4OH-HCl and tris have been the traditional buffers. The Good series, a group of zwitterionic buffers, is good for the pH range of 6-8 (30). Ferguson et al. (31) have described 5 new zwitterionic buffers with pKa between 6.9 and 7.9, which appeared to be equal to or better than other buffers available. To my knowledge, they have not been used for lipases. These are available commercially as MOPSO, DIPSO, etc., from the usual suppliers.

Lipases are active over a very wide temperature range: -20 C to 65 C, but the usual range is 30-45 C. Trioleoylglycerol and 4:0-4:0-4:0 will be liquid at these temperatures, a requirement for optimal activity.

Unless the substrates are lipoproteins, adipocytes, fat bodies, etc., they should be emulsified. Since the velocity of lipolysis is a function of the supersubstrate concentration, i.e., of the surface offered to the enzyme, the substrate should be dispersed in as fine an emulsion as possible. To prepare relatively stable emulsions, the substrate must be liquid (the buffer can be heated to melt the substrate) and emulsifiers, stabilizers or both added. Emulsifiers, such as sodium dodecyl sulfate, bile salts, phospholipids, and various nonionic detergents are examples. Several different types of emulsifiers should be checked because some will inhibit lipases, e.g., hepatic lipoprotein lipase is inhibited by sodium dodecyl sulfate (32). Stabilizers such as gum arabic (acacia) can maintain emulsions without the addition of surface-active agents. However, gum arabic contains calcium ions which can be removed if required (33). Emulsification is done with sonic energy or high speed mixers, such as a Waring Blendor. Since this is empirical, preparation must be identical with each batch. Severson et al. (21) achieved dispersion of 18:1-18:1-18:1 with the aid of glycerol plus lecithin followed by rapid mixing. The method of dispersion was that of Nilsson-Ehle and Schotz (34). The mixture should contain enough Na^+ or H^+ ions (usually as 0.1-1.0 M NaCl or supplied by the buffer) to suppress enzyme inhibition by interfacial charge effects. Calcium ions (0.02-0.1 M), Mg ions or bovine serum albumin are incorporated as fatty acid acceptors since accumulation of FFA usually inhibits lipases. With lipoprotein lipase, calcium ions had little effect on lipolysis of (35) chylomicrons, so the user must seek the optimal acceptor. The inhibitory effect of FFA and its reversal by fatty acid acceptors should be reinvestigated, since much of this work was done before purified lipase preparations became available. Another reason is that some lipases may be metalloenzymes, because some of the cations appear to enhance activity beyond their role as fatty acid acceptors. This could easily be tested by binding the FFA with an ion exchange resin or bovine serum albumin during digestion in the absence of cations.

The digestion should be terminated by the addition of acid to the incubation vessel. The acid inactivates the lipase, converts soaps to free acids and prevents emulsification during subsequent extraction. Acidification is a must, because Ca and Mg soaps of 16:0 and 18:0 are similar to limestone and will not be recovered unless the acids are free. The mixture is then extracted with organic solvents. The mixture can also be analyzed directly by addition of solvents and titration (36). However, the endpoint pH must be above 8.0 so that all the FFA are ionized and irreversible acceptors of FFA cannot be present.

The digestion mixture must be vigorously agitated during incubation to renew the surface constantly and remove FFA which can inhibit lipases. We use a water bath in which tubes, etc., can be agitated. Others have employed a dental amalgamator (Wig-L-Bug) or an orbital sander equipped with tube clamps in incubators. Whatever the nature of the shaker, the same size and shape of incubation vessel must be used to maintain the same surface-to-volume ratio throughout. If sequential studies are being done, separate vessels should be employed for the same reason.

Screening

Purified 4:0-4:0-4:0 mixed with nonnutrient agar and poured into a Petri dish is convenient for screening. Cultures of microorganisms can be added to any desired originally clear medium. Pieces of tissue can be placed on the surface of the agar or holes cut into it with a cork borer, and these filled with the fluid being investigated. The agar is opaque, but if a diffusible lipase is present, the substrate will be hydrolyzed to water-soluble 4:0 (and possibly 1-4:0) which leaves a clear ring around the source of the enzyme. We utilized this procedure to detect lipase activity in dental pulp (37). Visualization of the zone of clearing can be enhanced by the inclusion of the dye, Victoria blue (38) and $MgSO_4$ (22) or $CaCl_2$ (23) which form insoluble white soaps. These will appear as rings around the sites of application. Although suitable for screening, these methods are insensitive. Trioleoylglycerol can be substituted for 4:0-4:0-4:0 with the enhancement procedures. Screening with the aid of 4:0-4:0-4:0 may indicate lipolytic activity, but since this substrate is not, by definition, a substrate for lipases only, all positive results should be confirmed by hydrolysis of 18:1-18:1-18:1, or purified olive oil or 12:0-12:0-12:0.

Legakis and Papavassilliou (37) screened for lipases in microorganisms with thin layer chromatography (TLC). Cultures were grown in a medium containing 18:1-18:1-18:1, the medium was extracted with 0.5 ml of ethyl ether and 50 μl of the ether was potted on TLC plates. The plates were sprayed with a copper acetate reagent after development for visualization and the spots quantitated densitometrically. Estimates of the FFA produced could also be made visually. Bennett et al. (40) similarly observed the hydrolysis of corn oil by *Helminthosporium maydis*. This microorganism causes southern corn leaf blight. They employed 2 solvent systems for development of TLC plates. The first, benzene/ethyl ether/ethanol/acetic acid (50:40:2.0:0.2, v/v), was allowed to migrate 70% of the plate length. After drying in air, the plate was placed in the second solvent system of hexane/ethyl ether (94:6, v/v) and development allowed to the top. Benzene should be replaced by cyclohexane because it is extremely toxic and may be carcinogenic. In both of these procedures, the disappearance of TG as an indicator of lipolysis could be estimated.

DETERMINATION

The conditions of assay are available in the references below. This information plus the observations made during discovery of the lipase should provide sufficient data to prepare an assay medium for a new lipase. At this point, it is not necessary that optimal activity be achieved, only that it be reproducibly measurable. The conditions required for optimal activity will become apparent as the enzyme is fully characterized, also unnecessary for initial assay. The number of trials required for full assessment of pH, temperature, etc., can be greatly reduced by use of a statistical treatment, response surface methodology (41). The design minimizes the number of combinations which need to be tested, and provides values for the provision of the maximum response by several variables. So far as I know, it has not been applied to the characterization of lipases, but Smith et al. (42) employed the procedure to evaluate the effect of 3 variables upon the physical stability of milk fat emulsions.

In the methods to be described, the FFA usually are not separately recovered from the digestion mixture. If this is necessary, it can be done by selective extraction (43), trapping with an ion exchange resin (32,44) or arrestant column of basic silica gel (45), and thin layer (40, 46) and high performance liquid chromatography (47). Edwards-Webb (44) trapped extracted rumen FFA (4:0-18:3) on an ion exchange resin, eluted the acids and converted them to methyl esters with the eluting solvent in a sealed vial. The method avoids the problem of serious loss when analyzing short-chain acids. Dutta et al. (48) hydrolyzed TG with pancreatic lipase directly on a thin layer plate, then separated the digestion products. The FFA were recovered and quantitated by gas liquid chromatography (GLC). The method has the advantage of speed but reaction conditions would have to be determined for other lipases.

In the methods to be discussed, FFA are determined by (a) titrimetry, (b) conversion to Cu salts and colorimetry, (c) inclusion of a chromophore in the acyl chain, in glycerol or as a substitute for glycerol (umbelliferone) and spectrophotometry or fluorimetry, (d) use and measurement of radioactive fatty acids, (e) GLC, (f) treatment with enzymes and measurement of a product, and (g) immunological methods. The glycerol produced by lipolysis has been measured by enzymatic production of bioluminescence.

(a) Titrimetry. Titrimetry can be done directly on the assay mixture. Moskowitz et al. (36), in a study of *Mucor miehei* lipase, titrated to pH 9.5 after stopping the reaction by the addition of ethanol. The addition of ethanol also dissolves the fatty acids, in this case from olive oil. With direct titrations, it is imperative that the assay mixture does not contain Ca^{2+} or Mg^{2+}

which would remove fatty acids from the titration reaction, the endpoint be above pH 8.5 (6.5 for 4:0-4:0-4:0) to ensure complete ionization of the FFA and the substrate be 4:0-4:0-4:0, 18:1-18:1-18:1 or olive oil containing fatty acids which will dissolve readily in the aqueous ethanol. The Food Chemicals Codex has a procedure for this titration with 4:0-4:0-4:0 as the substrate (49). Direct titration has also been carried out continuously with a recording pH stat for pancreatic lipase (50) and adipocytes (51). The other type of titrimetry is nonaqueous titration of the digestion mixture in the extraction solvent with alcoholic base (52). Again, the endpoint must be above pH 8.5 to obtain complete ionization of the FFA.

(b) Conversion to Cu salts. Fatty acids can be converted to Cu soaps which are measured spectrophotometrically after reaction with chromogenic reagents. The method originally developed by Duncombe (53) has been refined and applied to plasma (54), serum (55), milk (56) and single oat grains (57). The procedure is sensitive. Hron and Menahan (54) readily detected 50 nmol of 16:0 and employed 50 µl of plasma. The correlation coefficient (n = 51) between the Cu soap method and GLC was 0.989 over a wide range of concentrations (55). The test can be applied to routine analysis. Shipe et al. (56) tested 200 milk samples/hr with the aid of an automatic sampler and a flow-through cell for the spectrophotometer. Bowyer et al. (58) described a semiautomated assay for FFA in serum employing Cu soaps. Sensitivity was improved 2-fold by Redding et al. (59) to the 20-400 nmol range.

(c) Inclusion of a chromophore. Gatt et al. (24) assayed lipases of rat brain microsomes, lyophilized rat bile, hog pancreas and *Rhizopus arrhizus delemar* with acylglycerol esters of trinitrophenylaminolauric acid. The free acid is measured spectrophotometrically. The method is sensitive; 0.012 absorbance unit is equivalent to 1 nmol of the 12:0 derivative, but the substrate is not commercially available. Directions for synthesis are given.

Rick and Hockeborn (25) and Furukawa et al. (26) determined lipolytic activity in serum with 2,3-dimercapto-1-propanol-tributyrylglycerol as the substrate. Lipolysis releases 2,3-dimercapto-1-propanol and 4:0. The thiol compound is coupled with 5,5′-dithio-*bis* (2-nitrobenzoate) and the absorbance determined. Fifty µl of serum was analyzed so the sensitivity is equivalent to the Cu soap methods. Cox and Horrocks (27) synthesized the 10:0 and 12:0 derivatives of 2,3-mercapto-1-propanol and assayed the MG lipase activity of *Rh. delemar*. Fifty nmol of released thiol was easily detected and directions for synthesis of the substrates are given. With this substrate, the chromogen is actually on the glycerol moiety.

The successful employment of umbelliferyl esters by Severson et al. (21) suggests that these compounds can be used as substrates for assay. The method is sensitive, 0.1-5 nmol, and the substrates are available and relatively inexpensive. Severson et al. (21) also checked activity with trioleoylglycerol; a precaution that I recommend.

(d) Use of Radioactive fatty acids. The activities of lipases from the following sources have recently been obtained with radioassays: postheparin plasma (32), chicken (43) and rat adipose tissue (60,61), mouse adipocytes (62), and rat (63) and human liver (64). Oleoylglycerols labeled with ^{14}C or ^{3}H were substrates and nanogram quantities of the product are detectable.

(e) Gas liquid chromatography. GLC has been applied to the quantitation of FFA in serum (55), plasma (65,66), and vegetable oils (67). Internal standards of 15:0 or 17:0 were employed to determine the absolute amounts of the FFA and the methylation procedures derivatized FFA only. The methods were capable of measuring 100 nmol of FFA in serum and plasma and 2 mg in the oils. Chapman (67) presents the calculations for conversion of GLC recorder peak areas to weight with 17:0 as the internal standard and summation of the individual FFA.

(f) Treatment with enzymes. Treatment of FFA with various enzymes results in products which are detectable at very low levels. Ulitzer and Heller (68) isolated mutants of a luminous bacterium, *Benecke harveyi*, which, via a luciferase, responds to as little as 1 pmol of 14:0 and 100-200 pmol of 16:0 or 18:1 with the production of light. The luminescence was measured in a photomultiplier photometer. They analyzed the activity of *Candida cylindracea* and porcine pancreatic lipases with the system at substrate levels of 100 µmol of 14:0-14:0-14:0 and 10 ng/ml of *C. cylindracea* lipases obtaining 160 pmol of 14:0 per minute. The method, although extremely sensitive, presents difficulties because the microorganism supplying the luciferase must be cultured. Commercially available luciferase (Boehringer-Mannheim) might be suitable.

Minzuno et al. (69) converted FFA to the CoA esters with a synthetase, oxidized the acyl CoA to a *trans* enoyl CoA and H_2O_2 using an oxidase and then oxidized the H_2O_2 with peroxidase to a chromogen. The range of detection was 0-200 nmol of 18:1.

When 18:2 is oxidized by lipoxygenese, an

18:2 hydroperoxide results. Proelss and Wright (70) oxidized Fe^{2+} to Fe^{3+} with the hydroperoxide and reacted the latter with thiocyanate to produce a red complex which was quantitatively tested. Griebel et al. (71) continuously monitored the oxygen consumption during the lipoxygenase reaction with a polarographic oxygen electrode. They added serum to a reaction mixture containing 18:2-18:2-18:2 and measured 0-12 μmol (0-520 μl lipase) of 18:2/1 of reaction mixture. As compared to a Cu soap method, they obtained a correlation coefficient of 0.98.

Miles et al. (72) reported on the direct determination of FFA in 2-5 μl of plasma eliminating extraction. The method is based on the quantitation of adenosine monophosphate produced by the formation of acyl-CoA with ATP and acyl CoA synthetase. Zero to 10 nmol of FFA were measured and the enzymes needed are commercially available.

Glycerol released by lipolysis from human fat cells was analyzed by bioluminescence (73). Glycerol-dependent ATP consumption was measured with a kit containing luciferon and luciferase and 0.5 μmol of glycerol/l was determined. The authors were able to assay the glycerol released from only 5,000-10,000 fat cells. There are many kits available for the enzymatic determination of serum TG in which glycerol is actually determined. Five of these have been evaluated for analysis of TG (74) and the information can be applied to the selection of a kit for the determination of glycerol produced by lipolysis.

(g) Immunological methods. Specific immunoassays have been developed for chicken adipose tissue lipoprotein (75) and human pancreatic (76) lipases. Cheung et al. (75) incubated antilipoprotein lipase immunoglobulins coupled to hydrophilic beads with the lipase and immunoglobulins labeled with ^{125}I were added. The labeled immunoglobulin was reacted with the antigen (lipase) associated with the immunoabsorbent. The quantity of lipoprotein lipase in the sample was proportional to the amount of radioactivity bound to the solid phase immunoabsorbent. The range of the assay was 0.1-1.1 ng of lipase. Bovine milk lipoprotein and pigeon and chicken liver lipases were not detected. The specific activity of the original purified enzyme preparation was 10,815 μeg FFA/hr/mg protein.

Grenner et al. (76) described an immunoassay (enzyme linked immunoabsorbent assay or ELISA) for human pancreatic lipase in plasma which followed the sandwich principle. Antibodies to pancreatic lipase (sp act, 2000 IU/mg) were raised in sheep and dogs. Peroxidase was conjugated to the canine antibodies. Serum, containing pancreatic lipase, was incubated with the sheep antibodies in a test tube, then the peroxidase conjugated antibodies added forming the sandwich. After this reaction, bound lipase was determined by the colorimetric estimation of peroxidase with H_2O_2 and *o*-phenylenediamine. Lipase concentrations in serum of between 3 and 300 μg/ℓ can be measured. One of the advantages of the method is that the user does not have to work with ^{125}I as in radioimmunoassay. This disadvantage of both methods is the requirement for a highly purified lipase but, with both, enzyme mass and activity are measured. In enzymology, enzymes have almost always been indirectly determined by their activity, not directly as above by mass.

Which method should be selected? The decision depends upon the user's requirements and resources. For most, a direct titration of the assay medium or of a nonaqueous extract will suffice. With the latter, a pH meter is not needed as indicators are satisfactory. The choice of procedure will also be influenced by the number of samples to be analyzed, the sensitivity desired and the availability of equipment and enzymes. The information I have provided should enable users to select a method which will suit their needs.

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COMMUNICATIONS

Effect of Age on Plasma Bile Acids and Lipid Components in the Rat

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ABSTRACT

With increasing age, total plasma bile acid contents increased in rats over a period of 11 months, and also total plasma cholesterol and carcass fat contents increased in the same manner. Plasma showing high bile acid levels at 11 months was found by means of high performance liquid chromatography to contain cholic acid as one of the major components, chenodeoxycholic acid and trace deoxycholic acid. These results suggest that there are close relationships between the plasma bile acids and age-dependent changes of lipid components in the rat.
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INTRODUCTION

It has been well established that food and nutrition play an important role in the etiology of hypercholesterolemia and atherosclerosis (cardiovascular disease) (1).

In this report, we examine the effect of age on plasma bile acids and lipid components in the rat.

MATERIALS AND METHODS

Weanling male rats of the Wistar-Imamichi strain (our Institute's strain) were housed in suspended cages with wire mesh bottoms. The animal room was kept at 22.0 ± 0.5 C and the relative humidity at 65 ± 5%. Room lighting consisted of 12-hr periods of light and dark. Rats weighing 50-60 g were randomly assigned to 1 of 3 groups, each containing 6 animals. Each group was fed the same diet. Composition of the diet is shown in Table 1. The diet and water were provided ad libitum. The rats of each group aged 3, 5 and 11 months were fasted for 16-20 hr and the body weights of the individual rats were measured before they were killed. The blood was collected by inferior vena cava with a heparinized syringe while the animals were unconscious (etherized). The blood was centrifuged and the plasma frozen at −20 C. The liver was removed and the liver weights of the individual rats were determined.

The liver and carcass were analyzed for total fat by the Soxhlet method using ethyl ether. Total plasma bile acid levels were determined by the method of Mashige et al. (2) and cholesterol by the method of Zak (3).

Bile acids in plasma were assayed by a rapid method based on high performance liquid chromatography (HPLC) (4). HPLC was performed on a Shimadzu LC-3A liquid chromatograph with a Shimadzu RF-510 fluorescence spectromonitor. We employed 2 chromatographic steps consisting of a preliminary purification of bile acids by ion-exchange chromatography on an Amberlite XAD-7 column (50 × 8 mm) and an analytical step by reversed-phase HPLC with

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TABLE 1

Composition of the Diet

	Percent
Wheat flour	44.0
Rice	24.0
Corn	13.0
Fish meal	4.0
Skim milk	3.0
Casein	2.0
Defatted soybean	2.0
Yeast powder	2.0
Salt mix[a]	3.9
Vitamin mix[b] (water-soluble)	0.08
Soy oil[c]	2.0
Contents	
Protein	15.8
Fat	4.3
Carbohydrate	67.9
Vitamins and minerals	12.0

[a]Composition in g/kg: $CaHPO_4 \cdot 2H_2O$, 355.6; $K_3C_6H_5O_7 \cdot H_2O$, 237; $CaCO_3$, 163.5; NaCl, 108; K_2HPO_4, 77.36; $MgCO_3$, 40.9; $FeC_6H_5O_7 \cdot 3H_2O$, 16.01; $MnSO_4$, 1.24; $CuSO_4 \cdot 5H_2O$, 0.178; $CoCl_2 \cdot 6H_2O$, 0.089; $K_2Al_2(SO)_4$, 0.089; $ZnCO_3$, 0.044; KI, 0.044; NaF, 0.0009.

[b]Composition in g/kg: choline chloride, 659; inositol, 263; nicotinic acid, 19.8; *para*-amino benzoic acid, 19.8; calcium pantothenate, 19.8; thiamine hydrochloride, 5.2; riboflavin, 5.2; pyridoxine-HCl, 5.2; folic acid, 2.6; biotin, 0.13; cyanocobalamin, 0.04.

[c]Soy oil (g) contained: retinyl acetate, 350 IU; cholecalciferol, 100 IU; α-tocopheryl acetate, 0.35 mg; menadione, 0.15 mg.

fluorometric detection (EX = 350 nm, EM = 460 nm, cell vol = 25 μl) using 3α-hydroxysteroid dehydrogenase as a post-column reaction enzyme. Zorbax™BP-ODS packed into a column (250 x 4 mm) was used as the packing material for HPLC with a mobile phase of 0.3% ammonium carbonate/acetonitrile (10:4, v/v). The standard mixture of cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid was separated satisfactorily. Calibration curves plotting the peak height of each bile acid were linear over their concentration ranges of 0-50 μmol/l. The recoveries of their bile acids were satisfactory (81.7-92.5%). The results with these methods were in fairly good agreement with the standard additions method.

RESULTS AND DISCUSSION

With increasing age (3-11 months), carcass fat contents increased in the same manner as body and liver weights (about 2 times); however, liver fat contents did not increase significantly. Changes in total plasma bile acids and cholesterol contents in the rat with regard to increasing age are shown in Figure 1. Total bile acids and cholesterol contents increased. Changes of total bile acids were essentially parallel to those of total cholesterol and carcass fat. These results indicate that both total cholesterol and bile acid levels in plasma have a good correlation under conditions in which rats become fat with increasing age.

Significantly higher total bile acid levels were observed in 11-month-old rats (Fig. 1). Figure 2 demonstrates good separation for the bile acids in the plasma. The plasma was found to contain cholic acid as one of the major components, chenodeoxycholic acid and trace deoxycholic acid. Uchida et al. (5) showed that the pattern of bile acids in fecal excretion was different from that in biliary excretion, and their data suggest that most bile acid, of which cholic acid is a major component, is reabsorbed and regulated by enterohepatic circulation. Their results and suggestions clearly seem to support our results.

It has been known that cholesterol levels in the plasma increase with advancing age in humans (6) and are higher in old animals (7). Cholesterol levels and metabolism in the body can be regulated by several mechanisms, including the synthesis, excretion and absorption of cholesterol and bile acids. The results of this study suggest that there are close relationships between plasma bile acids and age-dependent lipid components.

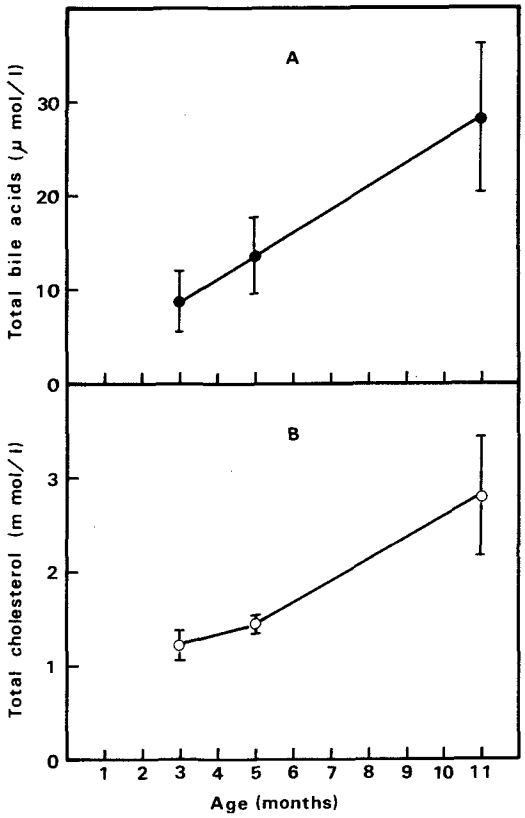

FIG. 1. Changes in total plasma bile acids (A) and cholesterol (B) contents in the rat with increasing age. Each point represents the mean ± SD for 6 animals.

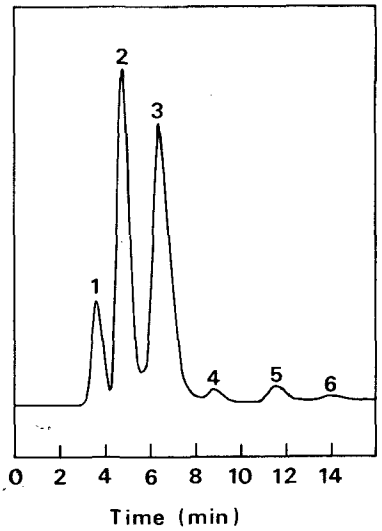

FIG. 2. Chromatogram of HPLC in the assay of bile acids in plasma of the rat indicated high total bile acid levels. Peaks: 1 = methanol; 2 = unknown; 3 = cholic acid (28.7 μmol/l); 4 = unknown; 5 = chenodeoxycholic acid (1.5 μmol/l); 6 = deoxycholic acid (trace).

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Fatty Acid Composition of Liver Lipids in Rats Fed Brominated Fatty Acids

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ABSTRACT

Feeding rats diets containing brominated corn oil or di- or tetrabromostearate as the monoglyceride produced changes in fatty acid composition of liver lipids. Those changes associated with the feeding of brominated corn oil or tetrabromostearate could be explained by the accumulation of triglyceride, and the changes associated with the feeding of dibromostearate could result from the proliferation of a membrane system. A unique response to the feeding of diets containing brominated corn oil is an increase in the level of γ-linolenic acid.

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Brominated oils have been used as food additives (1) and more recently as flame retardants (2). Only limited information is available concerning the toxicological properties of these products and even less is known of the toxicology of the brominated fatty acids themselves. In our laboratory, the response of rats fed brominated corn oil has been compared to that produced by feeding the monoglyceride of either dibromostearate or tetrabromostearate or a mixture of these two monoglycerides (3). The most pronounced changes were observed in heart and liver with brominated corn oil being more active than the brominated monoglycerides. The di- and tetrabromostearates varied in the effects they produced and in the manner they were distributed in the animal. Additional indices of the effects of these compounds have been derived from analysis of the fatty acid composition of liver lipids.

MATERIALS AND METHODS

Male Wistar rats (150 g) were fed semisynthetic rations in which the lipid component comprised: 10% corn oil, 8% corn oil + 2% brominated corn oil (BCO), 8% corn oil + 2% monoglyceride of dibromostearate (DBS), 8% corn oil + 2% monoglyceride of tetrabromostearate (TBS), and 8% corn oil + 2% mixture of brominated monoglycerides (BMG). The latter mixture was prepared to correspond to the brominated fatty acid composition of the BCO. The composition of the diets and the prodedures used to prepare the brominated components have been reported elsewhere (3). Zinc was added at a level of 6 mg/kg of diet as zinc acetate.

Animals were sacrificed after feeding the experimental diets for 35 days, the livers excised and held frozen until analyzed. Lipid was extracted with chloroform/methanol (4) and the methyl esters prepared using 5% hydrogen chloride in anhydrous methanol. Fatty acid analysis was carried out with an F&M Model 700 gas chromatograph fitted with a flame ionization detector and a LDC 308 integrator. The methyl esters were separated on a stainless steel capillary column (200 ft x 0.03 in.) coated with ethylene glycol succinate.

Thin layer chromatography was used to separate fatty acid esters on the basis of their degree of unsaturation with alumina as adsorbent and hexane as the developing solvent (3). Double bond location was established using ozonolysis (5,6) and the mass spectrum of an unknown fatty acid was obtained with a gas chromatograph-mass spectrometer (GC/MS) system (Varian 1200/ MAT CH-7 modified with a glass jet interface).

Analysis of variance was used to establish the effect of the dietary variables on levels of different fatty acids and the Newman and Keuls method (7) used to evaluate the difference among treatment means.

RESULTS AND DISCUSSION

The proportions of the major fatty acids in liver lipids are summarized in Table 1. Other polyunsaturated fatty acids (22:4, 22:5, 22:6) were detected but were present in smaller proportions (2% or less). Highly significant ($p<.01$) changes in the levels of 18:0, 18:1, 18:2 and 20:4 were associated with the dietary variables. These changes can be accounted for by either the accumulation of triglyceride or the proliferation of some membrane.

Stearate and arachidonate are common constituents of phospholipids, in particular, phosphatidylcholine (8). Consequently, membrane proliferation results in an increase in the pro-

TABLE 1

Fatty Acid Composition of Liver Lipids from Rats Ingesting Brominated Oils

Diet	Fatty acid (wt %)						
	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Corn oil	18.5±0.85	0.91±0.32	12.5±2.75^{a,b}	12.8±1.78^{a,b}	23.1±4.94^{b}	0.46±0.19^{a}	20.9±3.08^{b}
Brominated corn oil	19.7±1.61	1.81±0.62	10.6±1.59^{a}	18.5±3.48^{d}	20.8±1.02^{b}	4.18±1.41^{c}	17.3±3.30^{a}
Monoglyceride of dibromostearate	18.3±0.72	0.72±0.06	17.7±0.93^{c}	11.0±0.32^{a}	17.2±1.57^{a}	1.07±0.13^{a,b}	26.7±1.25^{c}
Monoglyceride of tetrabromostearate	19.4±0.20	1.11±0.23	10.8±1.36^{a}	16.2±2.28^{c,d}	22.6±1.78^{b}	1.50±0.43^{b}	19.2±0.50^{a,b}
Mixture of brominated monoglycerides	19.7±2.14	0.84±0.26	13.8±2.01^{b}	14.1±0.80^{b,c}	21.3±0.56^{b}	1.32±0.24^{a,b}	21.7±2.09^{b}

a,b,c,d Values with the same letter superscript not significantly different. $p<.05$.

portion of these fatty acids whereas triglyceride accumulation produces a decrease. Fatty acid changes associated with feeding BCO and TBS would be consistent with the accumulation of triglyceride as changes in crude lipid content (Table 2) also suggest. Feeding DBS increases the proportion of 18:0 and 20:4, suggesting the proliferation of a membrane system – most likely peroxisomes (3). In fact, the hepatomegaly and fatty acid changes observed with DBS are comparable to those observed with the hypocholesterolemic agent, chlorphenoxyisobutyrate (Tinsley, unpublished results), a substance known to induce proliferation of hepatic peroxisomes.

Fatty acid changes induced by the feeding of the monoglyceride mixture were intermediate between those produced by DBS and TBS. With an increase in crude lipid content, one might predict both triglyceride accumulation and membrane proliferation. The difference in response between BCO and an equivalent mixture of brominated acids fed as the monoglycerides is consistent with previous observations (3).

Another interesting observation is that the feeding of brominated fatty acids increases the proportion of γ-linolenic acid in liver lipids. This increase is most pronounced with the BCO and some inhibitory effect on the transformation of linoleate to arachidonic acid could be involved. Again, the brominated corn oil is producing a much greater effect than the brominated monoglycerides.

When fatty acid esters from livers of rats fed BCO were chromatographed on alumina, the gas chromatograph indicated the presence of substantial amounts of an unknown fatty acid in the fraction containing the polyunsaturated and brominated fatty acids. This fatty acid was not affected by procedures used for debromination (9). Mass spectral analysis also demonstrated that the compound was not

TABLE 2

Effect of Brominated Oils on Liver Size and Crude Lipid Content

Diet	Liver size (g/100 g body wt)	Crude lipid (mg/g liver)
Corn oil	4.08	44.1
Brominated corn oil	9.36	82.2
Monoglyceride of dibromostearate	7.10	49.9
Monoglyceride of tetrabromostearate	8.25	76.5
Mixture of brominated monoglycerides	7.84	68.0

brominated and that m/z and (m+1)/z were 292 and 293, respectively. This molecular weight corresponded to a linolenic acid methyl ester and the mass spectrum of the unknown component corresponded closely to that of a standard sample of the γ-linolenic acid ester. Retention times were also comparable. The presence of this isomer was confirmed by the identification of the appropriate C-6 aldehyde ester upon ozonolysis of the fraction containing the unknown component.

These data confirm previous observations of the toxicological effects of brominated fatty acids. Dibromostearate produced a response distinct from the tetrabromostearate, while the brominated corn oil produced a more pronounced effect than its constituent brominated acids fed as monoglycerides. Whether the latter effect is due to some additional component in the BCO or stems from a difference in the distribution of the fatty acids on the glycerol is not apparent at this time.

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Effect of Methylmercuric Chloride on Gangliosides of Mouse Neuroblastoma Cells in Culture

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ABSTRACT

The effect of methylmercuric chloride (CH_3HgCl) on the levels of gangliosides in mouse neuroblastoma cells (NBP_2) in culture was studied. The treatment of NB cells with low concentrations (0.1 μM and 0.2 μM) of CH_3HgCl, which did not affect the growth rate or morphology, caused an increase in the level of the GM3 ganglioside without changing the level of other gangliosides. The treatment of NB cells with higher concentrations (0.5 μM and 1 μM) of CH_3HgCl, which inhibited the growth of NB cells, caused a decrease in the level of GM3 and an increase in the level of GM2. These results show that alterations in the levels of specific gangliosides can be observed in cells which do not exhibit any detectable change in growth rate or morphology. This change may be associated with subtle changes in brain functions, including behavioral and psychological changes, after exposure to low concentrations of organic mercury.

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INTRODUCTION

It is well established that methylmercuric chloride (CH_3HgCl) causes a neurological disorder in humans which is referred to as Minimata Disease (1-2). CH_3HgCl accumulates in the central nervous system in rather large proportions after ingestion or after intravenous or intraperitoneal administration (3-6). The exact mechanisms of the effect of CH_3HgCl on nervous tissue are unknown, Using monolayer cultures of neuroblastoma (NBP_2) and glioma (C-6) as a model system, we have made several new observations. The most important of these include a marked alteration (increase and decrease) in the adenosine 3', 5'-cyclic monophosphate (cAMP) -dependent and -independent phosphorylation of cellular proteins in NB and glioma cells (7-8), an inhibition of PGE_1-sensitive adenylate cyclase in glioma cells but not in NB cells (9), an increase in the intracellular level of cAMP in both NB and glioma cells (10), and an alteration (increase and decrease) in the uptake of certain putative neurotransmitters in NB and glioma cells (11). We now report that the treatment of NB cells with low concentrations (0.1-1.0 μM) of CH_3HgCl causes an alteration in the levels of specific gangliosides.

MATERIALS AND METHODS

Tissue Culture

Mouse neuroblastoma clone (NBP_2), which has both tyrosine hydroxylase and choline acetyltransferase, was used in this study (12). NB cells were grown in F12 medium containing 10% a-γ-globulin newborn calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were maintained at 37 C in a humidified atmosphere of 5% CO_2. Cells (0.25×10^6 cells for control, 0.1 μM and 0.2 μM CH_3HgCl treatment; 0.5×10^6 cells for 0.5 μM and 1 μM CH_3HgCl treatment) were plated in Lux tissue culture dishes (100 mm). CH_3HgCl was added 24 hr after plating. The medium (20 ml) and CH_3HgCl were changed daily; the levels of gangliosides were determined after 3 days of treatment. CH_3HgCl inhibits the growth of NB cells (13-14). Therefore, different numbers of cells were plated, depending upon the concentration of CH_3HgCl, in order to obtain similar cell densities at the time of harvesting the cells for the assay of gangliosides. The daily change of medium was necessary in order to minimize the effect of acid pH of the medium, which has been reported to affect several cellular functions (15).

To determine the level of gangliosides, cells were washed 3 times with saline (0.9% NaCl), and then were removed in saline (1-2 ml) by a rubber policeman. The contents were lyophilized, weighed and then the levels of gangliosides were determined.

Ganglioside Analysis

Lipids were extracted from the lyophilized cells according to Suzuki (16). After reequilibrating, chloroform/methanol (2:1, v/v) and 0.2 vol of water were added (17). The resulting lower phase was washed by mixing with Folch's theoretical upper phase.

The gangliosides were purified by passage

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through a G25 Sephadex superfine column as described by Dreyfus et al. (18). Lipid-bound sialic acid determinations were performed by the resorcinol method (19).

The various ganglioside species were separated by high performance thin layer chromatography with the 3 successive solvent systems described by Harth et al. (20). Quantification was done by scanning (21).

RESULTS AND DISCUSSION

The lipid sialic acid contents of NBP_2 cells were similar to those reported for most of the other undifferentiated murine neuroblastoma clones (22-29). The ganglioside pattern of this NB clone (Fig. 1) is characterized by the complete absence of tri- and tetrasialoganglioside. Traces of the disialoganglioside GD1b could be observed in some extracts. However, NBP_2 cells differ from the other analyzed clones (22-29) by their low percentage of GM3 and GM2.

The treatment of NB cells with low concentrations of CH_3HgCl slightly decreased the total ganglioside content of the cells (Table 1). The Specific ganglioside, GM3, content increased by 60 and 36% of control at 0.1 and 0.2 μM CH_3HgCl, respectively; however, at higher concentrations, the level of GM3 decreased in a dose-dependent fashion (Table 1). The level of GM3 dropped to 31% of control in NB cells treated with 1 μM of CH_3HgCl. On the other hand, the level of GM2 did not change at lower concentrations of CH_3HgCl (0.1 and 0.2 μM); however, the level increased by 30 and 38% at concentrations of 0.5 and 1.0 μM CH_3HgCl, respectively.

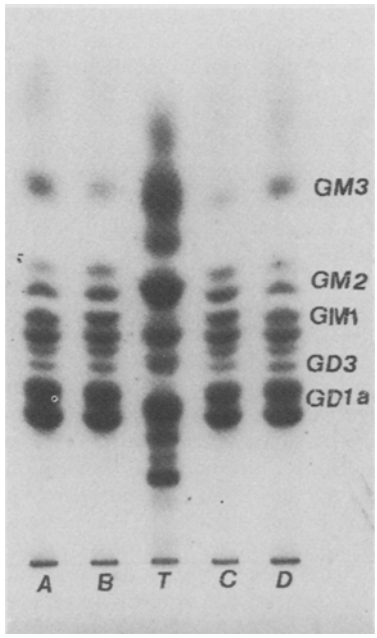

FIG. 1. Ganglioside pattern of neuroblastoma cells treated with methylmercuric chloride. Cells were grown in the presence of (A) 0.2, (B) 0.5, and (C) 1 μM methylmercuric chloride. (D) Control cells. In our experimental conditions, each ganglioside gave a double spot (see ref. 23, for the discussion of this phenomenon). The compound running faster than GM3 was not a ganglioside. It gave a faint blue spot with the resorcinol reagent. The same amount of lipid sialic acid was applied to the plate for the 4 samples. In order to show clearly the decrease of the GM3 gangliosides, this chromatography was overloaded. Markers gangliosides (T) were obtained from pig brain, Tay-Sachs disease (GM2) and glioma cells (GM3).

TABLE 1

Effect of Methylmercuric Chloride (CH_3HgCl) on the Levels of Gangliosides in Mouse Neuroblastoma (NBP_2) Cells in Culture

	Controls	Concentrations of CH_3HgCl 0.2 μM	0.5 μM	1 μM
Lipid neuraminic acid: (μg per mg dry weight)	0.55±0.004[a]	0.38±0.004	0.49±0.003	0.43±0.004
Gangliosides in % Total gangliosides				
GM3	6.8±0.8	9.3±0.3	4.2±0.4	2.1±0.3
GM2	9.9±1.7	9.9±2.3	12.9±0.6	13.7±0.5
GM1	21.0±3.0	20.4±1.6	22.9±2.0	22.2±2.0
GD3	11.5±1.6	11.8±3.0	10.0±2.0	12.7±2.3
GDla	51.4±1.4	48.6±3.1	50.0±2.0	49.3±1.6

[a]Standard deviation.

Cells were plated in Lux culture dishes and CH_3HgCl was added 24 hr after plating. CH_3HgCl and growth medium were changed and the levels of gangliosides were determined 3 days after treatment. Each value is the mean of 2 different sets of experiments done in duplicate. Gangliosides are named according to Svennerholm (43).

Few studies have been performed on the effect of heavy metals on brain lipids. It has been suggested that the main effect of heavy metals is the inhibition of the synthesis of myelin-specific galactolipids (30-33). However, a slight decrease of total brain gangliosides has been observed after administration of lead to newborn rats. This change was characterized by a slight increase of the simplest brain gangliosides at the expense of the higher polysialogangliosides (33).

The effect of CH_3HgCl on myelin galactolipids (31-33) shows that oligodendrocytes are one of the target cells for the effect of this metal compound on brain cells. This fits well with our previous study (13), which shows that glioma cells are more sensitive to CH_3HgCl than are NB cells for the criterion of growth inhibition (due to cell death and partial or complete inhibition of cell division). The present study shows that low concentrations of CH_3 HgCl (0.1 and 0.2 μM), which do not cause any detectable alterations in growth rate or morphology (8), increase the level of GM3. However, the concentration of CH_3HgCl (1 μM) which inhibits the growth of NB cells by 50% (13) markedly reduces the level of GM3, but increases the level of GM2. The mechanism of CH_3HgCl-induced changes in the levels of gangliosides is unknown.

The changes observed when the cells were grown in the highest CH_3HgCl concentrations may probably be attributed to two separate mechanisms: the decrease of GM3 might be the consequence of a decrease in the synthesis of this ganglioside. That only GM3 decrease in the mercury-treated cells suggests that the GM3 found in the membrane is different from the pool of GM3 used for the synthesis of GM2. This hypothesis is supported by data showing that the pool of gangliosides used for the synthesis is different from the pool which is deposited into cell membranes (34, see also for neutral glycolipids ref. 35, p. 315). However, we cannot exclude the possible interference of Hg^{2+} in the intracellular transport of GM3 or its incorporation into plasma membrane, the regulation of these 2 mechanisms being as yet totally unexplored.

Pathological studies have largely shown that an increase in a specific glycolipid is related to a decrease of the lysosomal enzyme involved in the catabolism of the accumulated glycolipid. GM2 is catabolized by a β-D-N-acetyl galactosaminidase. Kozik et al. (36-37) have observed that Hg^{2+} ions inhibit neuronal acid phosphatase but not alkaline phosphatase in intoxicated rats. Moreover, a notable inhibition of β-D-N-acetyl glucosaminidase was observed in *C. flexuosa* maintained in the presence of 5×10^{-8} M mercuric chloride, the concentration at which the growth of the hydroid (38) is only slightly inhibited.

Our earlier studies show that low concentrations of CH_3HgCl (0.1 and 0.2 μM) produce marked alterations in the cAMP-dependent and -independent phosphorylation of cellular proteins in NB cells (8). It remains to be established whether these changes in the phosphorylation of specific proteins could lead to alterations in the levels of gangliosides. In this respect, we have to mention the recent report of McLawhon et al. (39) showing that the stimulation of adenyl cyclase by opiates could be linked to the observed decrease of GM2 gangliosides in neuroblastoma cells.

Our present and previous (7-9) studies show that CH_3HgCl induces biochemical alterations in cells which do not exhibit any detectable change in growth rate or in morphology. Autoradiography has shown that methyl mercury distributes quite unevenly in the different areas of the brain (40). Berlin et al. (41) and Magos (42) reported mercury concentration of ca. 10 μg/g brain tissue in the animals intoxicated with methyl mercury. When employing mercury at 1 μM concentration, it may be that this concentration could be reached in the affected areas during chronic intoxication. Thus, our results may, in part, account for the subtle changes in brain functions, including behavioral and psychological, observed after exposure to low concentrations of organic mercury.

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Evidence that Chlorpromazine Inhibits Sterologenesis at Post-HMGCoA Reductase Sites in Rat Liver, in vitro

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ABSTRACT

The mechanism by which chlorpromazine inhibits cholesterogenesis in rat liver was investigated in vitro with the use of [^{14}C] acetate and [^{14}C] mevalonate as sterol precursors. Evidence was obtained that chlorpromazine blocks cholesterogenesis at multiple sites beyond HMGCoA reductase (β-hydroxy-β-methylglutarylCoA reductase, EC 1.1.1.34), the rate-limiting step. Squalene synthesis from both labeled acetate and mevalonate is reduced to a similar extent in the presence of chlorpromazine (29-36% at 0.5 mM). The data indicate that there is also an impairment of conversion of squalene to lanosterol, and of lanosterol to cholesterol. Overall inhibition of cholesterogenesis by chlorpromazine reached 65-75% at 0.5 mM and was concentration-dependent over the range 0.15-1.0 mM.
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INTRODUCTION

Chlorpromazine, a commonly used tranquilizer, has a number of interesting effects on sterol metabolism both in vivo and in vitro. The drug is mildly hypercholesterolemic in man (1,2) and the rabbit (3) but not in the rat (4,5). It is also an inhibitor of plasma LCAT (lecithin: cholesterol acyltransferase, EC 2.3.1.43) (5) and tissue ACAT (acylCoA: cholesterol acyltransferase, EC 2.3.1.26) (6-8). Paradoxically, chlorpromazine does not appear to affect hepatic cholesterogenesis in man (9) but does inhibit cholesterogenesis in arterial tissue (10), cultured cells (11), and in rat and pigeon liver (4,12,13). Studies reported to date with liver have investigated the effect of chlorpromazine on sterologenesis using labeled acetate as a precursor only (4,12,13). Since the studies employing labeled acetate measured only the incorporation of label into cholesterol or digitonin-precipitable products, no information was gathered on the possible site or sites of inhibition by chlorpromazine along the sterol biosynthetic pathway. In order to gain information on the site(s) of inhibition, the incorporation of [1-^{14}C] acetate and DL-[2-^{14}C] mevalonate into cholesterol, lanosterol, squalene and digitonin-precipitable sterols was studied in rat liver minces.

MATERIALS AND METHODS

Animals

Normal male rats (Upj:TUC(SD)spf, 225-250 g) were used in all experiments. The animals were individually caged with free access to food (Purina Chow) and water.

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Tissue Preparation and Incubation Procedures

The rats were killed by decapitation between 9 a.m. and 10 a.m. and the livers were immediately excised and rinsed in chilled 0.9% NaCl solution. One to four 500-mg pieces of liver were cut from the central region of the large lobe, placed on a watch glass on crushed ice and minced by hand using a scalpel blade (14). The minced tissue was transferred to 25-ml Erlenmeyer flasks containing 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, which contained either [1-^{14}C] acetic acid, sodium salt (0.66 μCi/ml, SA 56.0 Ci/mol) or DL-[2-^{14}C] mevalonic acid, dibenzylethylenediamine salt (0.57 μCi/ml, SA 47.0 Ci/mol). The isotopically labeled reagents were obtained from New England Nuclear Corp., Boston, MA. In all experiments except those described in Table 1 and Figure 1, paired tissue samples from each rat were incubated in the presence and absence of chlorpromazine·HCl (Sigma Chemical Corp., St. Louis, MO) which was added to the incubation flasks dissolved in 25 μl saline. In studies in Figure 1, 4 pieces of tissue were taken from each rat liver in order to establish a timecourse in autologous tissue. All incubations were performed at 37 C under ambient air for periods of 15 min to 3 hr, depending upon the particular experiment.

Analyses

Tissues were either extracted with $CHCl_3$/MeOH (2:1, v/v) after incubation and the cholesterol fraction isolated by thin layer chromatography (TLC) (10) or the incubations were terminated by the addition of KOH and ethyl alcohol to give a final concentration of 11% and 82%, respectively. In the latter case, the samples were heated to 65 C for 2 hr to

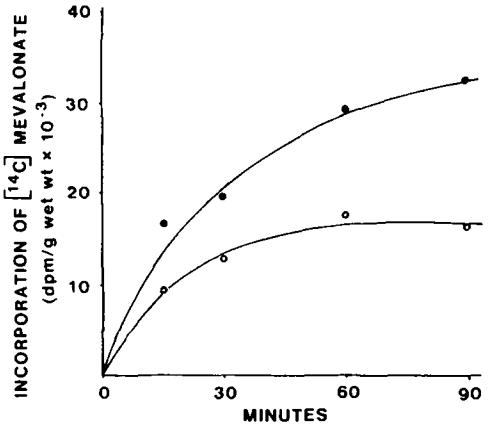

FIG. 1. Time course of the incorporation of [^{14}C] mevalonate into cholesterol in rat liver minces. Four prices of liver (500 mg each) from each of 4 rats were used to prepare liver minces for incubation with [^{14}C] mevalonate for 15, 30, 60 or 90 min. Tissue from 2 of the rats was incubated in the presence of 0.5 mM CPZ. After incubation, the tissues were extracted with $CHCl_3$/MeOH (2:1, v/v) and the cholesterol isolated by TLC as indicated under Materials and Methods. Data points are means of 2 observations (control, -●-; CPZ, -○-).

hydrolyze the tissue and to saponify the lipids (15). The nonsaponifiable lipid fraction (NSF) was extracted with *n*-hexane (15). Cholesterol, lanosterol and squalene were isolated from portions of the NSF by TLC as previously described (15). In all experiments, the lipids of interest were scraped directly into vials containing 15 ml of counting fluid (Liquifluor, New England Nuclear Corp., Boston, MA) for radioactive assay by liquid scintillation counting (16).

Digitonin-precipitable sterols were obtained by adding digitonin to portions of the NSF that were evaporated to dryness under N_2 and then redissolved in acetone/ethanol (1:1, v/v) (17). The digitonides were dissolved in 0.2 ml pyridine (18) and then assayed for radioactivity as above. All radioactivity data were corrected for quenching by the external standardization method.

Statistical analysis of paired samples was performed using Student's t-test for paired variates.

RESULTS

An inhibitory effect of chlorpromazine (CPZ) on sterol biosynthesis from mevalonic acid was demonstrated in rat liver minces by measuring the incorporation of [^{14}C] mevalonate into digitonin-precipitable sterols (sterols possessing a 3 β-OH group)over the concentration range 0.15-1.0 mM CPZ (Table 1). In a separate study, a timecourse for the incorporation of [^{14}C] mevalonate into cholesterol in the presence of 0.5 mM CPZ was performed at the intervals 15, 30, 60 and 90 min. The inhibitory effect of CPZ on cholesterogenesis was evident throughout the entire 90-min period. In subsequent studies, the effect of CPZ on the formation of cholesterol and cholesterol precursors (lanosterol and squalene) was examined (Table 2). Chlorpromazine (0.5mM) significantly reduced the incorporation of [^{14}C] mevalonate into cholesterol by ca. 65% ($p < 0.001$) in rat liver minces (Table 2). This reduction was paralleled by significant reductions in the incorporation of [^{14}C] mevalonate into the cholesterol precursors lanosterol ($p < 0.02$) and squalene ($p < 0.01$). The reduction in sterol formation in the presence of CPZ was further confirmed by the reduction (70%, $p < 0.001$) in [^{14}C] mevalonate incorporation into digitonin-precipitable sterols (Table 2). In addition, the ratio of [^{14}C] label incorporated into squalene vs cholesterol, which was 1.71± 0.14 in the control tissues, was significantly higher in tissues incubated with CPZ (3.57± 0.27, $p < 0.001$). The labeled lanosterol: cholesterol ratio was also higher in the CPZtreated tissue ($p < 0.001$).

Experiments similar to those using [^{14}C] mevalonate were conducted with [^{14}C] acetate as a sterol precursor (Table 3) and yielded results that were qualitatively similar to the

TABLE 1

Effect of Various Concentrations of Chlorpromazine on the Incorporation of [^{14}C] Mevalonate into Digitonin-precipitable Sterols in Rat Liver Minces

Chlorpromazine (mM)	N	dpm/g wet wt X 10^{-3}	% Control
0	10	75.0 ± 5.4	100
0.15	2	59.8 ± 11.6	80
0.25	5	49.0 ± 3.2	65
0.50	3	27.2 ± 2.4	36
1.00	2	13.2 ± 4.3	18

Liver minces from each of 22 mal rats (225-250 g) were incubated for 3 hr at 37 C in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 0.57 μCi DL-[2-^{14}C] mevalonic acid, dibenzylethylenediamine salt/ml and various concentrations of chlorpromazine·HCl. Digitonin-precipitable sterols were isolated from the nonsaponifiable lipid fraction after treatment of the tissue with alcoholic KOH as described under Materials and Methods. Values are means ± SEM when 3 or more rats (N) were used and means ± range when 2 rats were used.

TABLE 2

Effect of Chlorpromazine on the Incorporation of [^{14}C] Mevalonate into Squalene and Sterols in Rat Liver Minces

	Cholesterol	Lanosterol	Squalene	Digitonin-precipitable sterols	Ratio: Squalene/ Cholesterol	Ratio: Lanosterol/ Cholesterol
	(dpm/g wet wt)					
Control	20765 ±1135	10665 ±660	34600 ±1765	20785 ±1630	1.71 ±0.14	0.53 ±0.05
CPZ	6965 ±400	8930 ±685	24675 ±1720	6150 ±590	3.57 ±0.27	1.29 ±0.08
	p<0.001	p<0.02	p<0.01	p<0.001	p<0.001	p<0.001

Liver minces were prepared in duplicate from each of 7 male rats (225-250 g) and incubated for 90 min at 37 C in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 0.57 μCi DL-[2-^{14}C] mevalonic acid, dibenzylethylenediamine salt/ml in the presence and absence of 0.5 mM chlorpromazine·HCl (CPZ). Values are means ± SEM of data from 7 rats. Statistical analyses were performed using Student's t-test for paired variates.

TABLE 3

Effect of Chlorpromazine on the Incorporation of [1-^{14}C] Acetate into Squalene and Sterols in Rat Liver Minces

	Cholesterol	Lanosterol	Squalene	Digitonin-precipitable sterols	Ratio: Squalene/ Cholesterol	Ratio: Lanosterol/ Cholesterol
	(dpm/g wet wt)					
Control	1650 ±190	1150 ±385	1195 ±185	1665 ±190	0.75 ±0.10	0.73 ±0.22
CPZ	420 ±45	525 ±150	770 ±155	610 ± 75	1.83 ±0.31	1.14 ±0.33
	p<0.001	NS	p<0.01	p<0.001	p<0.01	NS

Liver minces were prepared in duplicate from each of 8 male rats (225-250 g) and incubated for 90 min at 37 C in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 0.66 μCi [1-^{14}C] Acetic acid, sodium salt/ml in the presence and absence of 0.5 mM chlorpromazine (CPZ). Values are means ± SEM of data from 8 rats. Statistical analyses were performed using Student's t-test for paired variates.

[^{14}C] mevalonate studies in that [^{14}C] acetate incorporation into cholesterol, squalene and digitonin-precipitable sterols was statistically significantly reduced by 0.5 mM CPZ; mean incorporation into lanosterol was also lowered (1150±385 vs 525±150 dpm/g wet wt liver) but did not reach a statistical level of significance ($p > 0.05$) because of a high level of variability introduced by 2 of the animals. As with [^{14}C] mevalonate, the ratio of [^{14}C] label incorporated into squalene vs cholesterol from [^{14}C] acetate was also significantly higher in the CPZ-treated tissue (0.75±0.10 vs 1.83±0.31, $p < 0.01$). The labeled lanosterol: cholesterol ratio was also elevated (1.5-fold) but not significantly, for reasons outlined above.

DISCUSSION

The mechanism of CPZ inhibition of hepatic cholesterogenesis has been investigated in rat liver, in vitro, using labeled acetate and mevalonate as cholesterol percursors. Since these precursors enter the cholesterol biosynthetic pathway prior to (i.e., acetate) and beyond (i.e., mevalonate) the rate-limiting enzyme HMGCoA reductase (β-hydroxy-β-methylglutaryl-CoA reductase, mevalonate: NADP oxidoreductase, EC 1.1.1.34) (19), the relative extent of labeling of cholesterol and post-mevalonate intermediates such as squalene and lanosterol can be used to provide information on the sites(s) of action of an inhibitor.

In these studies which confirm that CPZ inhibits cholesterogenesis (Fig. 1, Tables 1-3), an inhibition of incorporation of radiolabeled acetate and mevalonate into squalene also occurred. The fact that the decrease was similar in magnitude (36% with [^{14}C] acetate and 29% with [^{14}C] mevalonate) suggests that a CPZ blockade occurs at a site beyond HMGCoA reductase but prior to the formation of squalene. Additionally, the ratio of labeled squalene to labeled cholesterol formed from [^{14}C] acetate and [^{14}C] mevalonate (Tables 2 and 3) was statistically significantly higher in the CPZ-treated tissue than in the paired untreated control tissue; this observation indicates that CPZ also blocks the sterol pathway between squalene and cholesterol. The data further indicate that such a blockade occurs at multiple sites. For example, a reduction in the conversion of squalene into lanosterol is indicated by both the [^{14}C] acetate and [^{14}C] mevalonate data (Tables 2 and 3) and in addition there is evidence that the conversion of lanosterol to cholesterol is also somewhat impaired, i.e., the ratio of labeled lanosterol to labeled cholesterol in control tissue in Tables 2 and 3 is 0.53 and 0.73, respectively, whereas the ratio in paired CPZ-treated tissue is 1.29 and 1.14, respectively, thus indicating an accumulation of labeled lanosterol relative to cholesterol in the CPZ-treated tissues.

In summary, the data presented here suggest that CPZ exerts inhibitory effects at multiple sites in the sterol biosynthesis pathway beyond HMGCoA reductase. Overall inhibition of cholesterol synthesis in these studies reflects an inhibition of squalene synthesis, impaired conversion of squalene to lanosterol, and impaired conversion of lanosterol to cholesterol. Precise identification of the enzymes affected will require a more detailed investigation since the formation of squalene from mevalonate, of lanosterol from squalene, and of cholesterol from lanosterol all involve the participation of multiple enzymes (20).

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Reduced Plasma Lecithin Cholesterol Acyl Transferase Activity in Rats Fed Iron-Deficient Diets

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ABSTRACT

An iron-deficient diet containing no fat (FF-Fe) or containing either 14% hydrogenated coconut oil (HCNO-Fe) or 14% corn oil (CO-Fe) was fed to separate groups of rats for 10 weeks. In the control group, the corresponding iron-supplemented diets were fed FF+Fe, HCNO+Fe, CO+Fe. When rats were fed iron-deficient diets, their plasma lecithin cholesterol acyl transferase (LCAT) activity was significantly reduced as compared to controls. Their plasma also contained relatively more cholesteryl esters (CE) than free cholesterol (CH). In rats fed FF+Fe and CO+Fe diets, plasma contained similar levels of CE and CH. In those fed HCNO+Fe diet, plasma had 40% less CE than CH. Red cell CH content was significantly greater in the CO-Fe group. Iron deficiency, as indicated by low blood hemoglobin (Hb) and hematocrit (Hct) values, was also observed only in this group. The triglyceride and phospholipid contents of plasma in rats fed iron-deficient diets were significantly lower than of those in the control groups. Thus, changes in LCAT activity and CE/CH ratio in plasma showed the effect of iron-deficient diet consumption even before the blood Hb and Hct levels were reduced.
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INTRODUCTION

Numerous studies have demonstrated that dietary restriction of iron causes many changes in the lipid metabolism of animals (1-6). The type of fat consumed along with the iron-deficient diets has a role in the onset of iron deficiency as monitored by the levels of hemoglobin (Hb) and hematocrit (Hct) in blood. Consumption of low iron diets containing polyunsaturated fat promotes iron deficiency, whereas those which are fat-free or contain saturated fat delay the onset of iron deficiency (7,8). We have found that when low iron diets are fed to rats, the specific activity of liver microsomal stearoyl-CoA desaturase, an iron-containing enzyme complex, is reduced regardless of whether the blood Hb and Hct levels are altered (8).

Studies on the etiology of hyperlipidemia associated with iron deficiency have led Jain et al. (9) to suggest that plasma lecithin: cholesterol acyl transferase (LCAT) activity is significantly diminished in severely iron-deficient but not in moderately iron-deficient rats. Since the LCAT assays by Jain et al. measured only the radioactivity (cpm) in the cholesteryl ester (CE) fraction and not the amount of CE produced, it was not certain whether the reported result reflected actual changes in enzyme activity. In the present study, LCAT was quantitated as nmol cholesterol esterified/min/l plasma. We also determined whether, as in the case of Δ9 desaturase, LCAT activity was altered in rats which did not become anemic even though they consumed iron-deficient diets.

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MATERIALS AND METHODS

Plasma was obtained from the blood of the same groups of Sprague-Dawley male rats which were used to investigate the effect of feeding iron-deficient diets on the hepatic microsomal stearoyl-CoA desaturase activity (8). Thirty-six male Sprague-Dawley rats weighing ca. 75 g were obtained from Charles River, Wilmington, MD. They were divided into 6 groups of 6 each and housed in plastic cages with plastic ventilated covers. Rats had free access to deionized-distilled water fed through glass sipper tubes. Cages had sawdust bedding which was changed twice a week. The animal room had a 12-hr light and 12-hr dark cycle. It was maintained at 24 C with 40-50% relative humidity. Each group was fed ad libitum one of the iron-deficient diets (fat-free, FF-Fe; 14% hydrogenated coconut oil, HCNO-Fe; 14% corn oil, CO-Fe) or iron-supplemented (FF+Fe, HCNO+Fe, CO+Fe) diets for 10 weeks. Diets were custom-made by ICN Nutritional Biochemicals, Cleveland, OH. The composition of the FF-Fe diet was the same as that of the fat-free diet described earlier (8), except that ferrous ammonium citrate was excluded from the USP salt mixture XIV. The compositions of the CO-Fe and HCNO-Fe diets were same as the low iron diet supplied by ICN (8) except that the vegetable oil was replaced with corn oil and hydrogenated coconut oil, respectively. Low iron diets contained 13 μg Fe/g diet. The supplemented diets contained 80 μg Fe/g diet.

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/ml/300 g rat) and exsanguinated using heparin-

levels of eicosatrienoic acid, a sign of essential fatty acid deficiency, although they were less depleted of 18:2 and 20:4 as compared to the iron-supplemented controls (7).

As reported previously (7), when rats were fed the FF-Fe diet, their growth was not reduced markedly as compared to those fed the FF+Fe diet. The final body weights of rats on the FF-Fe and FF+Fe diets were 300 ± 28 g and 326 ± 36 g, respectively. A marked effect on growth by dietary deprivation of iron was also not observed in rats maintained on the HCNO diet. On the contrary, in the case of rats fed the CO diet, omission of iron caused a significant reduction in growth (7). The weights of rats fed the CO-Fe and CO+Fe diets were 379 ± 24 g and 484 ± 11 g, respectively.

Plasma LCAT Activity

In previous studies which reported that the LCAT activity was reduced in severely but not moderately iron-deficient rats (9), for the enzyme assays, labeled CH was not preincubated with plasma to allow for its equilibration with endogenous cholesterol. Such preincubations are vital to obtain optimal LCAT activity (15). Furthermore, the evaluation of LCAT was based only on the radioactivity of CE and not on the amount of CE produced (9). In the present study, the amount of CE produced was measured after the substrate CH and plasma CH were allowed to equilibrate. Our results show that when rats consumed low iron diets, their plasma had reduced LCAT activity regardless of whether they were iron-deficient or not (Table 1). It is possible that in earlier experiments, plasma LCAT would have been found to be decreased in severe as well as moderate iron-deficient rats if the enzyme activity was measured based on the amount of CE produced. Significant differences in the specific activity of labeled CH can occur due to the variations in the cholesterol content of plasma in rats fed the various diets (Table 2). These observations also suggest that the reduction of LCAT found in severely anemic rats would have been further amplified if the dilution effect of CH was taken into consideration (9).

Plasma CH and CE Levels

It is not known whether the CE/CH ratio in plasma is altered as would be expected by the reduction of LCAT activity since these parameters have not been examined previously in the same iron-deficient rat. It would be logical to expect that, when LCAT activity is reduced in animals fed iron-deficient diets (Table 1), the relative level of CE to CH in plasma would also be reduced as compared to the levels in iron-supplemented controls. However, our washed syringes and needles. Whole blood was centrifuged at 1500 rpm at room temperature in a Dynac centrifuge for 7 min, and plasma was removed. Red cells were washed 3 times with resuspension in standard incubation medium (10) and centrifugation at 1500 rpm for 7 min.

Lipids were extracted from 1-ml aliquots of plasma and red cells as described by Folch et al. (11) and by Rose and Oklander (12), respectively. Free cholesterol (CH) and CE contents were analyzed by the O-phthalaldehyde method (13). Plasma triglyceride and phospholipid contents were determined by analyzing their fatty acid composition by gas liquid chromatography and using methyl pentadecanoate as an internal standard (14).

Assay of LCAT was carried out according to the procedure by Stokke and Norum (15). Plasma (100 µl) was incubated at 37 C with albumin-[4-^{14}C] cholesterol emulsion (100 µl) and Ellman reagent (1.4 mM in 0.2 M phosphate buffer, pH 7.1, 20 µl) for 2 hr to allow the equilibration of added labeled CH with the plasma lipoprotein cholesterol. Mercaptoethanol (0.1 M, 20 µl) was then added and incubation was continued for an additional 2-hr period. The reaction was stopped by the addition of 5 ml chloroform/methanol (2:1, v/v), lipids were extracted, separated by thin layer chrom-

TABLE 1

Lecithin Cholesterol Acyl Transferase Activity in Plasma of Rats Fed Iron-Deficient or Iron-Supplemented Diets

Diet[a]	LCAT Activity[b]
FF-Fe	560 ± 79[c]
FF+Fe	1047 ± 151
HCNO-Fe	668 ± 43[c]
HCNO+Fe	1041 ± 72
CO-Fe	689 ± 97[d]
CO+Fe	1096 ± 116

[a]Diet abbreviations are: FF, fat-free; HCNO, 14% hydrogenated coconut oil diet; CO, 14% corn oil diet. Iron-deficient and iron-supplemented diets are given as -Fe and +Fe, respectively.

[b]LCAT activity is given as nmol cholesterol esterified/min/l plasma. Values are mean ± SE from duplicate determinations with plasma sample from each of the 6 rats in the diet groups.

[c]Significantly different with $p<0.01$ using 2-tailed t-test as compared to the value in the corresponding +Fe group.

[d]Significantly different with $p<0.02$ as compared to the value in the CO+Fe group.

TABLE 2

Cholesterol and Cholesteryl Ester Contents of Plasma from Rats Fed Iron-Deficient or Iron-Supplemented Diets[a]

Diet[b]	Cholesterol	Cholesteryl ester	Cholesteryl ester / Cholesterol
FF-Fe	17.6 ± 1.3	30.4 ± 1.8	1.76 ± 0.1[c]
FF+Fe	23.4 ± 1.6	21.4 ± 4.4	0.97 ± 0.23
HCNO-Fe	18.6 ± 0.4	23.9 ± 2.5	1.40 ± 0.11[c]
HCNO+Fe	26.3 ± 3.6	15.7 ± 4.9	0.72 ± 0.24
CO-Fe	19.0 ± 2.7	31.5 ± 4.0	1.64 ± 0.26[c]
CO+Fe	27.8 ± 6.4	29.8 ± 5.2	0.82 ± 0.10

[a]Plasma cholesterol and cholesteryl ester contents are given as mg %. these are mean ± SE obtained from duplicate analysis with plasma sample from each of the 6 rats in the diet groups.

[b]See Table 1 for diet designation.

[c]Significantly different from the corresponding value in the +Fe group with $p<0.001$ using the 2-tailed t-test.

atography and the (^{14}C) activities in the CH and CE fractions were quantitated. [4-^{14}C] Cholesterol (52.5 mCi/mmol, 0.01 mCi) was purchased from New England Nuclear, Boston, MA.

RESULTS AND DISCUSSION

In the present study, although rats were fed different iron-deficient diets, only those in the CO-Fe group became moderately iron-deficient as indicated by their reduced blood Hb and Hct levels (ca. 8.4 g % and 34%, respectively, as compared to ca. 14 g % and 44% in other ± Fe groups) (8). In humans, the CH content of red blood cells (RBC) has been reported to be increased markedly due to anemia (16,17). We found that the CH content of RBC was significantly ($p<0.01$) increased only in the CO-Fe group (1.41 ± 0.13 mg/ml as compared to 1.10 ± 0.18 mg/ml in the CO+Fe group). The cholesterol content of RBC of rats in the other groups was not altered significantly (FF+Fe, 1.0 ± 0.18; FF-Fe, 1.13 ± 0.09; HCNO + Fe, 1.13 ± 0.16; HCNO-Fe, 1.0 ± 0.22).

Rats maintained on the FF-Fe or HCNO-Fe diets were deprived of essential fatty acids and iron. On the other hand, those fed the CO-Fe diet were exposed only to a low intake of dietary iron. In rats on the FF-Fe and HCNO-Fe diets, the plasma lipids contained appreciable results showed that CE/CH ratio was increased significantly rather than decreased when compared to the controls (Table 2). It would appear that the LCAT activity is low under conditions when the relative level of CE to CH in the plasma is already high. On the other hand, enzyme activity is high in iron-supplemented animals when CE/CH ratio is reduced. An evaluation of the data from earlier studies also suggests that it is difficult to correlate plasma LCAT and CE/CH levels in iron-deficient animals. The relative level of CE to CH in plasma lipids had been found to be either decreased (2, 18, 20) or unchanged (3, 19) in iron-deficient rats. Furthermore, the values for CE/CH decreased mostly due to increased levels of CH and not due to decreased levels of CE (2, 18, 20). In some experiments, even though the blood Hb levels were decreased to 4.1 g % (vs 15.9 g % in controls), plasma cholesterol contents have remained unchanged (6). Hence, further experiments are needed to understand the relationship between iron deficiency, plasma CE/CH levels and LCAT activity.

Hyperlipidemia

Another reported effect of iron deficiency is the production of hyperlipidemia. However, iron deficiency during both pregnancy and lactation causes hyperlipidemia in 18-day-old offspring but not in maternal rats (1). When male weanling rats were fed iron-deficient diets for 5 weeks, their blood triglyceride levels were 5-fold greater than in those in animals fed an iron-supplemented diet (6). Marked lipemia was also observed in weanling male and female rats and chicks when they were fed iron-deficient diets (4). However, hyperlipidemia appears to be dependent on the amount and type of dietary fat fed and also on the strain of rat used (5). In some experiments, hyperlipidemia was not produced and instead the serum triglyceride levels were depressed markedly (60-75%) by iron deficiency (3,5). The present study supports this finding since the analysis of the plasma lipid contents showed that the triglyceride levels were significantly reduced in rats fed iron-deficient diets as com-

pared to the controls (Table 3). The plasma levels of phospholipids in iron-deficient rats have been reported to be increased (3), but they also have been found to be unchanged (20). We found that the plasma phospholipid content was significantly reduced in rats fed low iron diets as compared to the controls (Table 3). Our results as well as those of others (3,5) on plasma triglyceride levels demonstrate that hyperlipidemia does not always occur during iron deficiency.

TABLE 3

Triglyceride and Phospholipid Content of Plasma from Rats Fed Iron-Deficient or Iron-Supplemented Diets[a]

Diet[b]	Triglyceride	Phospholipid
FF-Fe	32 ± 8[c]	35 ± 4[c]
FF+Fe	62 ± 8	61 ± 7
HCNO-Fe	38 ± 6[d]	60 ± 4[e]
HCNO+Fe	81 ± 7	84 ± 11
CO-Fe	46 ± 8[d]	57 ± 7[e]
CO+Fe	131 ± 16	86 ± 10

[a]Values given are mg/dl and are mean ± SE from duplicate determinations with 4 different plasma samples from each group. Triglyceride and phospholipid contents are given as the amount of fatty acid methyl esters obtained from them.

[b]See Table 1 for diet abbreviations.

[c]Significantly different with $p<0.01$ using the 2-tailed t-test as compared to the value in the corresponding +Fe group.

[d]Significantly different with $p<0.001$ as compared to the value in the corresponding +Fe group.

[e]Significantly different with $p<0.02$ as compared to the value in the corresponding +Fe group.

The compositions of the diets used in the present study were the same as those of the diets in our earlier experiments (7,8). There were several differences in the contents of the FF and fat-containing diets. The FF diet contained sucrose and USP XIV salt mixture, and the CO and HCNO diets contained corn starch and the Hubbell, Mendel and Wakeman (HMW) salt mixture. Alphacel was present in the FF diet but not in the fat-containing diets. Furthermore, the USP XIV and HMW salt mixtures were devoid of zinc and did not contain adequate levels of copper and manganese. In spite of these differences and deficiencies between the 3 diets, the composition of each -Fe diet was the same as that of the corresponding +Fe diet except that iron content was low. Hence, it is likely that the effects observed due to feeding the -Fe diet in this and earlier studies (7,8) are due to the low iron content in the diet. However, whether other differences in the diet composition discussed above also play a contributory role has not yet been determined.

The present study supports and extends the conclusions of Jain et al. (9). Plasma LCAT activity is reduced not only in severe but also in moderate iron deficiency. Moreover, even in the absence of an overt iron deficiency as indicated by blood Hb and Hct levels and, in the absence of lipemia, consumption of low iron diets causes a decrease in the plasma LCAT activity. Although iron deficiency produces significant changes in lipid metabolism, it is difficult to make generalizations since many of the effects are influenced by factors such as age and strain of the experimental animal model and the dietary regimen. For this reason, the interrelationships between reduced LCAT activity, CE/CH ratio and hyperlipidemia and hematological values in iron-deficient animals remain poorly understood.

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Alterations of Phospholipids in Ischemic Canine Myocardium During Acute Arrhythmia

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ABSTRACT

Myocardial ischemia was produced in the left ventricle of the canine heart by a Harris two-stage occlusion of the left anterior descending coronary artery. The lipid content in the ischemic myocardium was analyzed and compared with the control tissue. No significant change in total phospholipid and cholesterol was detected. A 2-fold elevation in the levels of the major lysophospholipids was observed during acute ventricular arrhythmias at 24 hr after the onset of ischemia. Such increases were not caused by preferential hydrolysis of phospholipid plasmalogens from the parent phospholipids.
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The development of cardiac arrhythmias following the onset of cardiac ischemia has been well established (1). However, the exact biochemical factors involved in the production of cardiac arrhythmias in vivo remain obscure. Recently, lysophosphatidylcholine has been implicated as one of the biochemical factors that may produce membrane alterations conducive to the generation of arrhythmia. The direct action of lysophosphatidylcholine and other lysophospholipids on cardiac arrhythmias has been observed in isolated perfused hearts (2). During short intervals of experimentally produced ischemia, elevated levels of lysophosphatidylcholine in the ischemic myocardium have been reported (3,4). Exogenous addition of lysophosphatidylcholine to the superfusate of cardiac tissue preparations appears to produce electrophysiological changes similar to those seen in ischemic tissues (5,6). Although a direct relationship between lysophospholipids and cardiac arrhythmia in vivo has not been established, results obtained from the preceding studies clearly support the involvement of lysolipids in the genesis of malignant arrhythmia induced by ischemia (7,8).

To correlate the involvement of endogenous lysophospholipids and other lipids in ischemic myocardium with cardiac arrhythmia, we used an ischemic model of the canine heart which developed acute arrhythmia within 24 hr after occlusion of the left anterior descending coronary artery. The phospholipid profile in the ischemic region of the heart was then compared with tissue obtained from the control region. We report that the levels of lysophosphatidylcholine and lysophosphatidylethanolamine were elevated over 2-fold in the ischemic tissue, and the increased levels of these lysophospholipids were not due to preferential hydrolysis of plasmalogens in the ischemic tissue.

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MATERIALS AND METHODS

Phospholipid standards were obtained from Serdary Research Laboratories. [1-^{14}C-Palmitoyl] lysophosphatidylcholine and [1-^{14}C-palmitoyl] dipalmitoylphosphatidylcholine were obtained from New England Nuclear. Palmitaldehyde sodium bisulfite was purchased from K and K Laboratories, Plainville, NY. Thin layer chromatographic plates (Sil-G25) were the product of Brinkmann. Other chemicals and solvents were of reagent grade and were purchased from Fisher Scientific Company. Cholesterol oxidase, cholesterol standards and phenol/4-aminoantipyrine chromogenic agent were purchased from Sigma.

Mongrel dogs of either sex, weighing 8-15 kg, were used throughout this study. The dogs were anesthetized by intravenous injection of sodium pentobarbital (30 mg/kg body weight). Subsequent to the exposure of the heart, the left anterior descending coronary artery was isolated and occluded by the Harris two-stage technique (9). The chest cavity was closed after the occlusion of the artery and the animal was allowed to recover. Appropriate dosages of morphine sulfate and diazepam were given as analgesic and sedative. The cardiac rhythm of these animals after surgery was monitored by electrocardiac recording (lead 2). At 24 hr after the occlusion of the artery, the heart was removed from the animal and tissues from the nonischemic and ischemic areas of the left ventricle were taken. In pilot experiments, tissue samples were (a) frozen in liquid nitrogen by the Wollenberger technique (10), and (b) stored in ice and homogenized within 10 min of excision. No difference in lipid content and composition was found between these 2 modes of preparation. Hence, the latter mode was used for all subsequent experiments.

The dry weight of the tissue was determined by complete dehydration in an oven at 100 C

under reduced pressure. For histological examination, the tissue was fixed in 10% buffered formaldehyde solution, and subsequently embedded in paraffin. The tissue sections were stained with eosin and hemotoxylin. In the determination of total protein, the tissue was homogenized in 20 vol of saline, and an aliquot was used for protein determination by the method of Lowry et al. (11).

Lipids were extracted from the tissue by the method of Connor et al. (12). The tissue was homogenized in 10 vol (w/v) of chloroform/methanol (1:2, v/v) and extracted twice with the same solvent. The extracts were pooled and a biphasic mixture was obtained by the addition of 0.1 M KCl to provide a solvent mixture of $CH_3OH/CHCl_3/H_2O$ (2:1:0.8, v/v/v). The lower phase was removed and evaporated under reduced pressure. The lipid extract was reconstituted in a small volume of chloroform/methanol (2:1) and was used immediately for lipid analysis. Complete extraction of phosphatidylcholine and lysophosphatidylcholine was achieved by this mode of extraction in pilot studies. Hydrolysis of phosphatidylcholine and lysophosphatidylcholine was monitored by the addition of labeled lipids to the sample in chloroform/methanol prior to homogenization. No hydrolysis of the labeled lipids was detected.

Quantitation of total cholesterol was performed by enzymatic assay with cholesterol oxidase (13). The phospholipids in the lipid extract were separated by thin layer chromatography (TLC) in a solvent containing chloroform/methanol/water/acetic acid (70:30:4:2) (14). In some experiments, the phospholipids were separated by another solvent containing chloroform/methanol/water/ammonium hydroxide (70:30:4:1) (15). The phospholipids on the thin layer chromatographic plates were visualized by iodine vapor and identified by comparison with phospholipid standard. Quantitation of lipid phosphorus was performed by the method of Bartlett (16). To account for loss during chromatography, defined amounts of phospholipid standards were also subjected to TLC prior to the establishment of a standard curve for lipid phosphorus determination.

The plasmalogen content of the phospholipids was determined as follows. Subsequent to the separation of the phospholipids by TLC, the lipids were eluted from the silica gel by chloroform/methanol (1:2). An aliquot was used for lipid phosphorus determination and the remaining volume was used for plasmalogen determination by the procedure of Wittenberg et al. (17). Palmitaldehyde was used as a standard for this determination. The percentage of plasmalogen in each phospholipid was calculated by the molar ratio of plasmalogen to total lipid phosphorus.

RESULTS

Histological and Morphological Changes in the Ischemic Tissue

Periodic ventricular arrhythmias developed spontaneously after the occlusion of the left anterior descending coronary artery. A quiescence period was observed between 4 and 8 hr post-surgery, which was followed by another onset of ventricular arrhythmias. Ventricular arrhythmias were recorded at 24 hr post-surgery which persisted for several days. Morphological examination of the ischemic area in the left ventricle revealed that the texture and color of the ischemic tissue were quite distinct from the other tissues. The ischemic area covered ca. ¼ the size of the left ventricle and extended from the upper one-third of the left anterior papillary muscle down to the apical region. The tissue from the posterior side of the left ventricle from the same heart appeared to be similar to normal cardiac tissue under morphological and histological examination, and was used as control tissue for this study. Histological comparison betweeen the control and ischemic tissues (Fig. 1) showed that most fibers in the ischemic area underwent coagulative necrosis. The nucleii of the cell had dis-

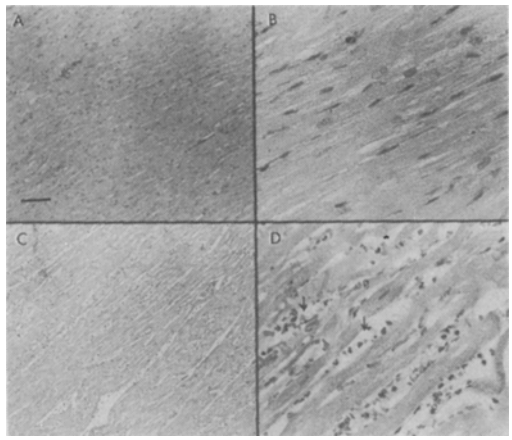

FIG. 1. Histological study of normal and ischemic tissues. Panel A (low magnification) and panel B (high magnification) depict sections obtained from the normal areas of the heart. Muscle fibers are well organized and the nuclei are clearly visible. Panel C and D show sections obtained from the ischemic areas. Most fibers have undergone coagulative necrosis and nuclei have disappeared in some cells. Interstitial inflammation with edema and neutrophil infiltration are indicated by arrows (panel D). Calibration bar is 100 μm for panels A and C; 25 μm for panels B and D.

appeared in some areas and interstitial inflammatory reaction with edema was very apparent. Neutrophil infiltration of the ischemic tissue is shown by the arrows in Figure 1. In other experiments where the left anterior descending coronary artery was not occluded, no ventricular arrhythmias were observed. Concurrently, no ischemic area in the heart was identified. This clearly established that arrhythmia resulted from the development of ischemia in the heart, and was not caused by the surgical procedures.

Lipid Content and Composition in Ischemic Tissues

Since alterations in lipid content and composition have been implicated as one of the biochemical causes for the production of cardiac arrhythmias, the lipid composition in control and ischemic tissues was compared. As depicted in Table 1, a decrease in total cholesterol and phospholipids was observed in the ischemic tissue based on tissue wet weight. Such a decrease, however, might originate from the change in tissue water content since local edema was identified in ischemic tissues in morphological studies. Therefore, the water content and total protein content (Table 2) in ischemic tissues were determined and compared with control tissues. A 14% increase in water content was observed in the ischemic tissue with a 12% decrease in total protein. When total cholesterol and phospholipids were expressed in dry weight of the tissues or by protein content, no significant difference was detected between the control and ischemic tissues. The results clearly indicate that the decrease in lipid content in ischemic tissue by wet weight determination reflects the increase in tissue water content during ischemia.

Analysis of Phospholipid Classes in Control and Ischemic Tissues

The complete analysis of phospholipid classes in control and ischemic tissues with respect to tissue protein content is depicted in Table 3. A significant increase (2-fold) in both lysophosphatidylcholine and lysophosphatidylethanolamine was seen in the ischemic tissue, with a corresponding decrease in phosphatidylcholine

TABLE 1

Total Cholesterol and Phospholipid Content in Control and Ischemic Canine Heart

	Control	Ischemic
Cholesterol		
(mg/g wet wt)	2.21[a] ± 0.24[b] (15)[c]	1.91 ± 0.27 (8)[d]
(mg/g dry wt)	10.38 ± 1.13 (15)	11.13 ± 1.53 (8)
(mg/g protein)	15.67 ± 1.70 (15)	15.40 ± 2.17 (8)
Phospholipids		
(μmol Pi/g wet wt)	24.85 ± 2.00 (7)	21.42 ± 1.54 (7)[d]
(μmol Pi/g dry wt)	116.67 ± 9.39 (7)	121.02 ± 8.70 (7)
(μmol Pi/g protein)	176.24 ± 14.18 (7)	172.74 ± 12.42 (7)

[a]Mean.
[b]Standard deviation.
[c]Number of experiments.
[d]$p < 0.05$.

TABLE 2

Tissue Water and Protein Contents in Control and Ischemic Canine Hearts

	Control	Ischemic
g H_2^2O/g wet weight	0.787[a] ± 0.021[b] (6)[c]	0.823 ± 0.11 (6)
g H_2O/g dry weight	3.880 ± 0.411 (6)	4.671 ± 0.360 (6)
dry weight/wet weight	0.207 ± 0.019 (15)	0.177 ± 0.011 (6)[d]
g protein/g wet weight	0.141 ± 0.019 (15)	0.124 ± 0.010 (15)[d]

[a]Mean.
[b]Standard deviation.
[c]Number of experiments.
[d]$p < 0.05$.

TABLE 3

Analysis of Phospholipid Classes in Control and Ischemic Canine Hearts

Phospholipids	Control	Ischemic
	(μmol/g protein)	
Lysophosphatidylcholine	1.00[a]±0.21[b](11)[c]	2.07± 0.61(8)[d]
Sphingomyelin	16.74 ±4.68 (11)	16.46± 3.61(11)
Phosphatidylcholine	88.93 ±8.80 (6)	76.07± 9.29(6)[d]
Phosphatidylserine and phosphatidylinositol	13.05 ±2.20 (8)	15.84± 3.61(8)
Lysophosphatidylethanolamine	0.51 ±0.07 (5)	1.23± 0.46(5)[d]
Phosphatidylethanolamine	46.03 ±3.26 (8)	39.23± 2.84(8)[d]
Phosphatidylglycerol and cardiolipin	28.37 ±7.94 (11)	37.30±10.76(11)[d]
Others	4.82 ±1.42 (6)	4.23± 1.23(6)

[a]Mean.
[b]Standard deviation.
[c]Number of experiments.
[d]$p < 0.05$.

TABLE 4

Plasmalogen Content of Phospholipids in Control and Ischemic Canine Hearts

Phospholipids	Control	Ischemic
	(% plasmalogen)	
Lysophosphatidylcholine	28.5[a] ± 10.6[b] (4)[c]	27.3 ± 8.6 (4)
Lysophosphatidylethanolamine	17.9 ± 11.5 (4)	18.5 ± 8.5 (4)
Phosphatidylcholine	43.6 ± 10.4 (4)	37.8 ± 8.1 (3)
Phosphatidylethanolamine	31.6 ± 5.5 (4)	36.4 ± 5.7 (4)

[a]Mean.
[b]Standard deviation.
[c]Number of experiments.

(15%) and phosphatidylethanolamine (15%). The levels of sphingomyelin, phosphatidylserine-phosphatidylinositol, and other minor phospholipids did not change significantly, but an increase in phosphatidylglycerol and cardiolipin was observed. The sums of the phospholipids in this analysis is in agreement with the values obtained from the analysis of the total phospholipid content (Table 1).

Analysis of Phospholipid Plasmalogens

Since a significant amount of phospholipid in the heart is present in plasmalogen form, the plasmalogen content in each phospholipid class between control and ischemic tissues was determined. The plasmalogen content in the lysophospholipids between control and ischemic tissues did not seem to change (Table 4), but were significantly decreased when compared with the parent phospholipid. The high standard deviations obtained in this study are probably due to the variation of plasmalogen content in these phospholipids between experimental animals.

DISCUSSION

In previous studies, the effect of short-term ischemia on tissue lysophospholipids has been reported (3,4,18). The arrhythmogenic nature of exogenous lysophospholipids in the isolated heart has been identified (2,19). In the present study, we have clearly established a correlation between the elevated levels of lysophospholipids in ischemic tissues and acute arrhythmias. Although changes in other phospholipids were also observed in this ischemic model, none of these phospholipids were found to be arrhythmogenic (19). It was shown that lysophosphatidylserine and lysophosphatidylglycerol were able to cause cardiac arrhythmias at the same concentrations as lysophosphatidylcholine or lysophosphatidylethanolamine (19). However, the levels of these minor lysolipids were low in both the control and ischemic tissues (less than 0.1 μmol/g protein) and hence, may not play a significant role in the production of cardiac arrhythmia.

Although a 2-fold increase in the levels of lysophosphatidylcholine and lysophosphatidyl-

ethanolamine was observed in ischemic tissues, the percentages of plasmalogen in these lysolipids were similar to those found in the control tissues. Since the percentage of plasmalogen form the parent phospholipids was also similar in both the control and ischemic tissues, it can be concluded that the increase in lysophospholipid levels during ischemia was not caused by preferential hydrolysis of the diacylphospholipids or the phospholipid plasmalogens.

One intriguing aspect pertaining to this study is the decrease of phosphatidylcholine and phosphatidylethanolamine levels in ischemic tissues. The quantitative increases in the corresponding lysophospholipids did not account for the amount of decrease from the parent phospholipids. Although it could be argued that the diminished levels of high energy nucleotides during ischemia (20-22) caused a reduction in the biosynthesis of these phospholipids (23, 24), this appears to be unlikely since the levels of other phospholipids either remained the same or elevated in ischemic tissues (Table 3). Another explanation is that the choline and ethanolamine containing phospholipids were subjected to selective hydrolysis during ischemia, which might result in the increase in formation of the respective lysophospholipids. Due to the cytolytic nature of the lysophospholipids (25), the majority of the lysophospholipids formed during ischemia was removed, either by degradation or extracellular dispersion. We postulate that the 2-fold increase in the major lysophospholipids during ischemia only reflects a fraction of the total increase in lysophospholipid production during prolonged ischemia.

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Urinary Prostaglandin E_2 and Vasopressin Excretion in Essential Fatty Acid-Deficient Rats: Effect of Linolenic Acid Supplementation[1]

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ABSTRACT

Three groups of weanling male rats were fed on a fat-free diet for 13 weeks. One group received only the fat-free diet (FF rats), the other 2 groups received the fat-free diet and a daily supplement of 2 energy% ethyl linoleate ([n-6] rats), or 2 energy% ethyl linolenate ([n-3] rats). Urinary excretion of prostaglandin E_2 (PGE_2), immunoreactive arginine vasopressin (iAVP), and kallikrein were determined. PGE_2 was quantitated with a radioimmunoassay having 4.9% cross-reactivity with prostaglandin E_3 (PGE_3). After 4 weeks on the diet, water consumption and urinary iAVP excretion increased significantly in the FF rats and the (n-3) rats compared with the (n-6) rats. Urinary PGE_2 excretion was the same for all 3 groups during the first 10 weeks; thereafter it decreased in FF rats and (n-3) rats compared with the (n-6) rats. There was no difference in urinary PGE_2 excretion between the FF rats and the (n-3) rats, even though large differences were found in the percentage of arachidonic acid (20:4[n-6]), icosapentaenoic acid (20:5[n-3]), and icosatrienoic acid (20:3[n-9]) of total kidney fatty acids as well as of kidney phosphatidylinositol fatty acids. Fractionation of urine extracts on high performance liquid chromatography with radioimmunoassay detection indicated that (n-3) rats excreted very little PGE_3, if any. Urine output followed the same pattern, as did urinary PGE_2 excretion. Urinary kallikrein was estimated at week 12 only. It was found to be significantly lower in FF rats and (n-3) rats. Increased water consumption and increased urinary iAVP excretion seem to be early symptoms (after 4 weeks) of EFA deficiency, whereas decreased urine output and decreased urinary PGE_2 excretion occur much later (after 10 weeks). Two energy% linolenate supplementation to a fat-free diet did not change the appearance of any of the measured EFA-deficiency symptoms except for a slightly improved growth rate. There was no evidence of a significant urinary PGE_3 excretion in spite of an extreme enrichment of kidney lipids with 20:5(n-3). It is suggested that urinary PGE_2 is derived from precursors delivered from an arachidonic acid pool, which is rather resistant to restriction in dietary linoleate.

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In previous experiments, we have found that EFA deficiency in rats induces decreased urinary PGE_2 excretion (1) and increased urinary iAVP excretion (2). PGE_2 and iAVP determinations were carried out after 13 and 22 weeks, respectively, on the experimental diet (1,2). Vasopressin is the principal hormone for regulation of renal water excretion, and there is evidence that PGE_2 formed in the kidney functions as a modulator of vasopressin-induced reabsorption of water in the kidney (3,4). Urinary PGE_2 excretion is considered to reflect renal prostaglandin production (5).

As increased water consumption and, occasionally, reduced urine output are well known EFA-deficiency symptoms (6,7), we found it of interest to investigate the time course of these 4 parameters, i.e., urinary excretion of PGE_2 and iAVP, urine output and water consumption in young rats reared on fat-free diets. In addition, it would be of value to examine the possible influence of dietary (n-3) fatty acids on the prostaglandin production in a linoleate deficiency.

Icosapentaenoic acid (20:5[n-3]) is the precursor for the trienoic prostaglandins. However, this fatty acid is a poor substrate for the cyclooxygenase in vitro (8,9) as well as a competitive inhibitor of the cyclooxygenase-catalyzed arachidonate conversion (10). ten Hoor et al. (11) have reported that feeding rats fish oil high in 20:5(n-3) results in reduced prostaglandin formation in vitro, a reduction to the same extent as that seen for EFA-deficient rats. However, the arachidonic acid pool, which delivers the precursor for in vivo prostaglandin formation, may not be the same as the one seen in vitro (12). Thus, it is important that attempts to correlate dietary EFA intake to prostaglandin production involve quantitation of in vivo prostaglandin production.

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Abbreviations: EFA = essential fatty acid; PGE_2, PGE_3 = prostaglandin E_2, prostaglandin E_3, etc.; iAVP = immunoreactive arginine-vasopressin; HPLC = high performance liquid chromatography; TLC = thin layer chromatography; PI = phosphatidylinositol.

The purpose of the present experiment was to establish when the EFA-deficiency symptoms discussed above become apparent; whether an extreme enrichment of the tissues with icosapentaenoic acid (20:5[n-3]) further depresses urinary PGE_2 excretion relative to that of EFA-deficient rats; and whether a high tissue content of 20:5(n-3) results in the formation of PGE_3 in vivo.

MATERIALS AND METHODS

Animals and Procedures

Sixteen 21-day-old male Mol-Wistar rats (K. Møllegaard-Hansens Avlslaboratorium A/S, Ll. Skensved, Denmark) were reared on a semi-synthetic fat-free diet which contained by weight: vitamin-free casein (Nutritional Biochemicals Corp., ICN, Cleveland, OH), 20%; sucrose, 74%; choline chloride, 0.5%; vitamin mixture, 0.5%; salt mixture, 5%. The compositions of the vitamin and salt mixtures were largely as described by Aaes-Jørgensen and Hølmer (13) and by Jensen (14), respectively. The compositions of the vitamin and salt mixtures ensured that the recommendations given by the Laboratory Animal Science Association, London (15) were fulfilled or exceeded.

The rats were divided into 3 groups with 5 or 6 animals per group. One group received only the fat-free diet (FF rats), the other groups received the fat-free diet and a daily dose of either ethyl linoleate (all *cis*-9,12-octadecadienoic acid ethyl ester) ([n-6] rats) or ethyl linolenate (all *cis*-9,12,15-octadecatrienoic acid ethyl ester) ([n-3] rats) (99% pure; NuChek-Prep, Inc., Elysian, MN). These supplements were given per os, with a plastic-tip pipette. The daily amounts of the 2 fatty acid esters were adjusted in proportion to the consumption of the basal diet to ensure a dosage corresponding to ca. 2% of the daily energy intake. The same animals have been used for studies of in vitro lung prostaglandin production (16).

The linoleic acid content of the fat-free diet was less than 0.001 energy%. The fat-free diet and water were supplied ad libitum throughout the experiment in a 12 hr/12 hr light-dark period. The rats were housed in wire mesh cages, 2 animals/cage. Once a week the rats were housed individually in metabolic cages for 24 hr with free access to food and water. The 24 hr urine was collected in a polyethylene tube packed in dry ice, which ensured immediate freezing of the urine after voiding. The collected urine samples were stored at −20 C until PGE_2 and iAVP determinations were performed. The experimental feeding period started in February and ended in May. The relative humidity was not controlled, and increased from ca. 20% to ca. 50% within this period. The room temperature was 24 ± 1 C.

After 13 weeks of experiment, the rats were decapitated, the various organs were excised, weighed, wrapped in aluminium foil, frozen in liquid nitrogen, and stored at −80 C until analyzed.

PGE_2-Radioimmunoassay

[^{3}H]PGE_2, 160 Ci/mmol, was obtained from the Radiochemical Centre, Amersham, England, and unlabeled prostaglandins were kindly provided by Dr. J. Pike, The Upjohn Co., Kalamazoo, MI. ^{3}H-PGE_2 was routinely purified on Sephadex LH 20 columns before use (17). The PGE_2 antiserum was kindly provided by Dr. P. Christensen, Institute of Experimental Medicine, Copenhagen, Denmark (17). The radioimmunoassay was performed as described previously (1), with the modification, however, that the standards were extracted in the same way as the samples, making the use of ^{3}H-PGE_2 as internal standard unnecessary. Storage of urine samples at −20 C resulted in a slow, apparently monoexponential decay of immunoreactive PGE_2 with time. Ca. 20% had disappeared after 200 days of storage. Urinary PGE_2 determinations have been corrected for storage decay according to this observation.

AVP-Radioimmunoassay

^{125}I-AVP, ca 1215 μCi/μg, was obtained from New England Nuclear, Boston, MA, and unlabeled AVP, 345 U/mg, was from Ferring AB, Malmø, Sweden. The AVP antiserum was kindly provided by Dr. J. D. Christensen, Biological Laboratory, Royal Danish School of Pharmacy, Denmark (18). The radioimmunoassay was performed as described earlier (2).

Fractionation of Urinary PGE on HPLC

To test for PGE_3 formation in the (n-3) rats, an HPLC system was developed for separation of diene and triene prostaglandins. HPLC was carried out using a P-45 pump (Waters Associates, Milford, MA) equipped with a 20-μl sample injector (Rheodyne Inc., Cotati, CA) and a Knauer column (Wissenschaftliche Gerätebau, Dr. Ing. H. Knauer, Gmbh, Berlin, G.F.R.) (125 × 4.6 mm ID) packed with Nucleosil 5 C_{18} (Mackerey-Nagel, Düren, G.F.R.). The solvent system was water/acetonitrile/tetrahydrofuran/acetic acid (72:28:2:2). Flow rate was 1 ml/min and 1-ml fractions were collected on an Ultrarac fraction collector (LKB, Bromma, Sweden). Elution of standard PGE_3 and

PGE_2 was detected at 278 nm after alkaline treatment as described by Bygdeman and Samuelsson (19). PGB_2 and PGB_3 thus formed have the same molar absorption. To 2 ml urine was added a known amount of [^{14}C]PGE_1 (55 Ci/mol from New England Nuclear, Boston, MA) as internal standard. The urine was acidified to pH 3 with acetic acid, applied to a Sep Pak C_{18} cartridge (Waters Associates, Milford, MA), and prostaglandins were eluted as described by Powell (20). In short, the cartridge was washed successively with water, 15% ethanol and petroleum ether, and the prostaglandins were eluted with diethyl ether. The diethyl ether was evaporated under a stream of nitrogen, and the residue was dissolved in a small volume of ethanol for injection into the HPLC column. Urine content of PGE_2 and PGE_3 was detected after HPLC separation with the PGE_2 radioimmunoassay. PGE_3 cross-reacted 4.9% at the 50% displacement level, showing a displacement curve parallel to the PGE_2 standard curve.

Kallikrein Determinations

Urine kallikrein was determined by the kininogenase method with heated (59 C for 3 hr) dog citrate plasma as substrate followed by bioassay of the liberated kinins on isolated rat uterus (21). One unit of kallikrein is defined as the amount of enzyme which forms 1 μg of bradykinin equivalents after 2 min of incubation at pH 7.6 and room temperature (21 C).

Urine osmolality was determined on a freezing-point osmometer (Knaver Halbmicro-Osmometer, Eppelheim bei Heidelberg, G.F.R.).

Lipid Analysis

One kidney from each rat was cut into smaller pieces and homogenized in 20 vol chloroform/methanol (2:1, v/v) using an Ultra-Turrax homogenizer with a No. 10 shaft. The tube was kept in an ice bath and flushed with nitrogen during the 60-sec homogenization. The extraction mixture was centrifuged for 10 min, and the residue was reextracted with 10 vol chloroform/methanol (1:2, v/v) using the Ultra-Turrax. The combined extracts were evaporated under reduced pressure and at a maximum temperature of 30 C. The crude lipids were redissolved in chloroform to a known volume. Methyl esters of fatty acids were prepared by transesterification with 10% (w/w) hydrogen chloride in methanol by heating to 100 C for 1.5 hr. After methylation, 1 ml water was added to the vials, and the esters were extracted with petroleum ether.

The methyl ester extract was gas chromatographed twice on a packed glass column (10% SP 2330 on 100/120 mesh Chromosorb W/AW (Supelco, Bellefonte, PA) in a 6 mm x 1.8 m column, temperature-programmed from 160 C to 210 C (Perkin-Elmer 3920 gas chromatograph with flame ionization detector). The peak areas were quantitated using a Hewlett-Packard 3390A recording integrator. The retention times of the prepared methyl esters were compared with those of standards for tentative identification.

Phosphatidylinositol Analysis

PI fatty acids of the kidney lipid extracts were analyzed after preparative TLC. Ca. 1 mg crude lipid was applied in a band to the concentrating zone of a 10 X 20 cm silica gel plate (Merck Art. 11844, Darmstadt, G.F.R.), which was developed twice in chloroform/2-propanol/ethyl acetate/methanol/0.25% aqueous potassium chloride (30:25:18:9:6) as described by Hedegaard and Jensen (22). The plate was dried in a nitrogen atmosphere. Butylated hydroxytoluene (0.01% final concentration) was added to the solvent and to the spray reagent. The PI band was localized by comparison with phospholipid standards (Serdary, London, Ontario, Canada) after spraying with 0.1% 2′,7′-dichlorofluorescein. The PI band was scraped into a minivial, transesterified, and fatty acids were analyzed as above. Unless otherwise specified, all organic solvents and reagents used were analytical grade (E. Merck, Darmstadt, G.F.R.).

Statistical analyses were done using Student's t-test.

RESULTS

The urinary PGE_2 excretion was of the same magnitude in the 3 groups of rats for the first 10 weeks of experiment. Only at weeks 12 and 13 did separate t-test reveal any significant differences: the urinary PGE_2 excretion was significantly lower in the FF rats and (n-3) rats as compared with the (n-6) rats (Fig. 1). At week 8, 2 of the 5 (n-6) rats had a very high urinary PGE_2 excretion, i.e., 122 and 144 ng/24 hr, which was about twice their previous and subsequent excretion rates. Therefore, in Figure 1, these 2 values are not included in the mean value for week 8. Thus, the mean value was 36 ± 9 ng/24 hr (n=3). If the 2 excluded values were included, the obtained mean value, 69 ± 45 ng/24 hr (n=5), would still not be significantly different from the mean values for the 2 other groups at week 8. There was no significant difference in the urinary PGE_2 excretion between the FF rats and the (n-3) rats. Analysis on HPLC of the urine samples collected at week

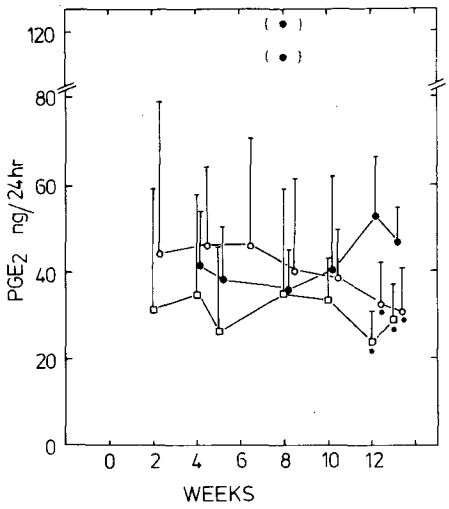

FIG. 1. Urinary PGE_2 excretion of male rats reared on fat-free diet supplemented with 2 energy% ethyl linolenate (n-3) □-□, with 2 energy% ethyl linoleate (n-6) ●-●, or with no supplementation (FF) ○-○. The fatty acid esters were given per os daily to the rats. The rats were 21 days old when they were started on the experimental diet. Once a week the rats were housed individually in metabolic cages, and the 24-hr urine was collected in a tube packed in dry ice. Urinary PGE_2 was estimated by radioimmunoassay. The bars indicate one SD for n = 5 or 6, except for (n-6) group at week 8, where n = 3; for details see Results. *Statistically significant difference ($p < 0.05$) compared with (n-6) group.

13 revealed a small peak (fractions 13-16) of immunoreactive PGE_2 material (less than 2% of the PGE_2 peak at fractions 21-24) which migrated as PGE_3 (Figs. 2 and 3).

However, this peak was observed in both the (n-3) rats (Fig. 2A) and in the (n-6) rats (Fig. 2B). Since there was extremely little, if any, 20:5(n-3) in the kidneys of the (n-6) rats (Tables 1 and 2), this peak probably does not represent PGE_3.

Fatty acid analysis of total kidney lipids showed marked differences in 20:3(n-9), 20:4 (n-6), and 20:5(n-3) (Table 1). In the (n-3) rats, the 20:4(n-6) was only half of that seen in the FF rats, whereas their urinary PGE_2 excretion was the same (Fig. 1).

Fatty acid analysis of kidney PI showed that 20:4(n-6) was reduced to the same extent in both FF rats and (n-3) rats (Table 2). The ratio between 20:5(n-3)/20:4(n-6) was 5.1 in total kidney lipids and 2.1 in kidney PI of the (n-3) rats. The content of linolenic acid was rather low, indicating desaturation and elongation of dietary 18:3(n-3) to 20:5(n-3). The triene/tetraene ratio in the FF rats was 1.9 in total kidney lipids and 6.7 in kidney PI.

Urinary iAVP excretion was the same in the 3 groups of rats at week 2; thereafter, it increased in both the FF rats and the (n-3) rats (Fig. 4). Using separate t-tests, significant differences from the (n-6) rats ($p < 0.05$) were found for the FF rats from week 4, and for the (n-3) rats from week 8. There was no significant difference between the 3 groups either in urine osmolality or in osmotic load (data not shown). At week 2, the urine osmolality was 2300-2600 mOsmol/kg, gradually falling to 1500-2300 mOsmol/kg at week 13, being highest in the FF rats and lowest in (n-6) rats. At week 2, the osmotic load was 11-15 mOsmol/24 hr, slowly increasing to 18-21 mOsmol/24 hr at week 13, generally being highest in the (n-6) rats and lowest in the (n-3) rats.

Urine output was the same in the 3 groups of rats for the first 10 weeks of experiment (Fig. 5). At week 12, the urine output was significantly ($p < 0.05$) lower, both in the FF rats and the (n-3) rats, compared with the (n-6) rats. The following week, the difference in mean urine output was increased between the (n-6) rats and the 2 other groups. However, the difference was not significant ($0.1 > p > 0.05$).

Urinary kallikrein excretion was measured by the kininogenase method at week 12 only, giving values of 295 ± 36 U/24 hr (mean ± SD, n = 5), 317 ± 14 U/24 hr (n = 6), and 417 ± 65 U/24 hr (n = 5), for the (n-3) rats, the FF rats, and the (n-6) rats, respectively. The urinary kallikrein excretion in the first 2 groups was significantly ($p < 0.05$) lower than that in the (n-6) rats. There was a positive correlation between urinary kallikrein and urine output (Fig. 6).

The water consumption of the FF rats and the (n-3) rats increased in a parallel manner (Fig. 7) over that of the (n-6) rats. This effect was significant already at week 2 for the (n-3) rats. The FF rats and the (n-3) rats showed the well known EFA-deficiency symptoms: reduced growth rate, increase of relative kidney weights, and decrease of testis weight (Fig. 8 and Table 3), as well as deficiency of pigmentation of the back and scaliness of the skin (data not shown).

DISCUSSION

The data of this report (Figs. 1, 4, 5 and 7) are presented per animal instead of per unit body weight. This is consistent with the presentation in our earlier reports (1,2). The arguments for our per animal presentation are, first, that the rats have the same absolute kidney weights (Table 3) and, secondly, that the disturbed water balance in the rats probably is of

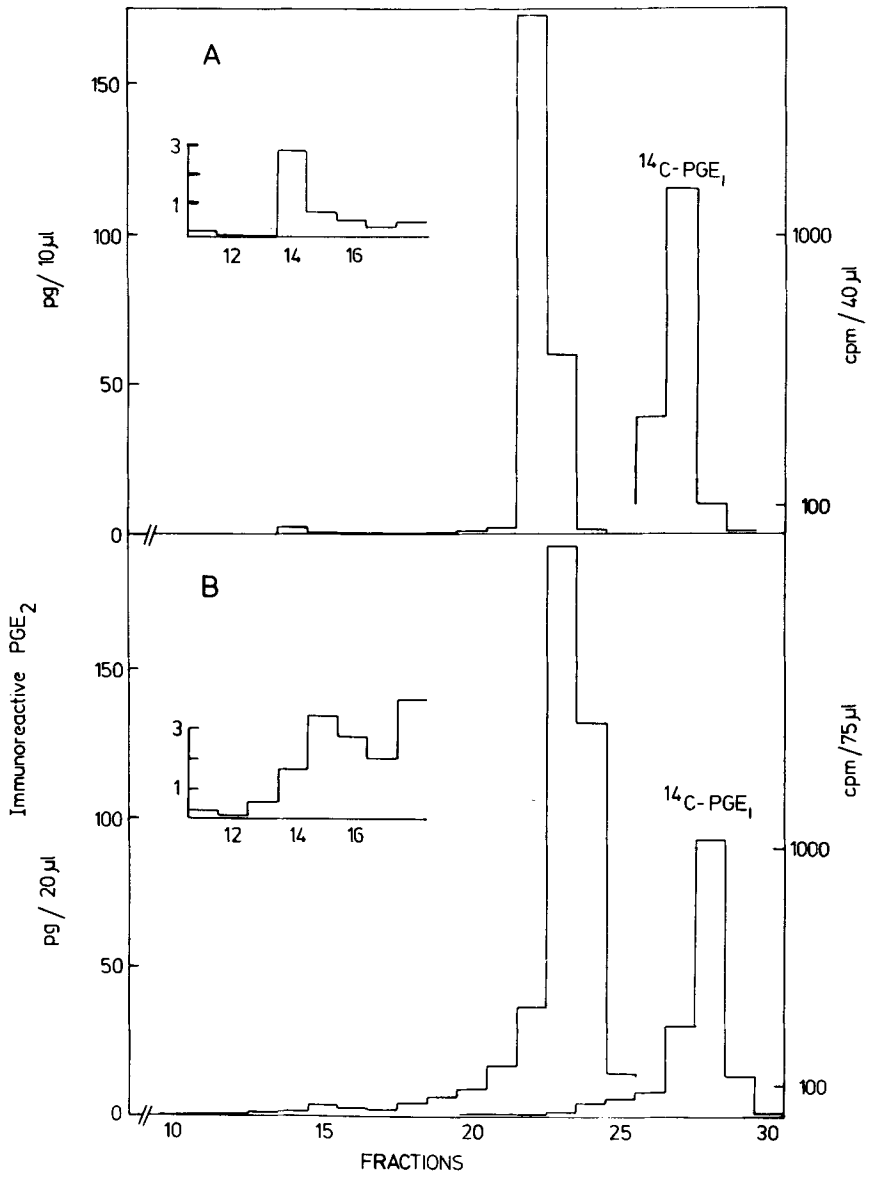

FIG. 2. Separation of urinary immunoreactive PGE_2 material on a reversed-phase HPLC column. Two ml of urine from a linolenate (n-3)-supplemented rat (A) and from a linoleate (n-6)-supplemented rat (B) were fractionated on Sep Pak C_{18} and the prostaglandin fraction was then applied to the HPLC column (125 × 4.6 mm, Nucleosil C_{18}). Flow rate was 1 ml/min and fractions of 1 ml were collected. A 10-fold enlargement of immunoreactive PGE_2 detection in fractions 11-18 is shown in the upper left corners.

greater significance for the urine volume and the iAVP excretion than is the body weight. Since the high water consumption probably was caused by a change in the water permeability of the skin (6), it could be relevant to express these data per surface area of the animal. This would only tend to increase the observed difference between the (n-6) rats and the other two groups.

It is remarkable that the urinary PGE_2 excretion seemed to be unaffected by the dietary intake of EFA for such a long time (Fig. 1) and that enrichment of the tissue lipids with icosapentaenoic acid did not result in a further depression of renal PGE_2 production as compared with the EFA-deficient group (FF rats).

TABLE 1

Fatty Acid Composition of Total Kidney Lipids after 13 Weeks of Experiment (peak area %, mean ± SD)

Group	(n-3) rats n = 6	FF rats n = 6	(n-6) rats n = 5
16:0	21.1 ± 0.5[c]	19.5 ± 1.5[c]	21.1 ± 1.4
16:1	4.3 ± 0.5[d]	5.4 ± 0.7[d,e]	3.5 ± 1.1[e]
18:0	14.0 ± 0.7	13.5 ± 1.0	14.8 ± 1.3
18:1	24.4 ± 2.0[f]	26.6 ± 1.7[g]	19.1 ± 1.8[f,g]
18:2	0.8 ± 0.4[h]	0.8 ± 0.1[i]	4.0 ± 0.5[h,i]
18:3	0.6 ± 0.1	tr	nd
20:3 (n-9)	1.4 ± 0.3[j,l]	14.4 ± 1.3[k,l]	0.7 ± 0.2[j,k]
20:3 (n-6)	tr[a]	nd[b]	0.7 ± 0.2
20:4 (n-6)	3.2 ± 0.5[m,o]	7.5 ± 0.7[n,o]	25.4 ± 2.5[m,n]
20:5 (n-3)	16.4 ± 1.9	nd	nd
22:4	2.9 ± 0.6	2.8 ± 0.7	2.4 ± 0.6
22:5	1.0 ± 0.6[p,q]	1.9 ± 0.3[p]	2.2 ± 0.5[q]
22:6	3.1 ± 0.1[r,s]	0.4 ± 0.1[r,t]	1.7 ± 1.0[s,t]

[a]tr = trace.

[b]nd = not detected.

[c-t]Values with same superscript are significantly different ($p < 0.05$; Student's t-test).

TABLE 2

Fatty Acid Composition of Kidney PI after 13 Weeks of Experiment (peak area %, mean ± SD)

Group	(n-3) rats n = 6	FF rats n = 5	(n-6) rats n = 5
16:0	9.9 ± 2.0[c,d]	5.3 ± 0.9[c]	5.8 ± 1.2[d]
16:1	1.5 ± 1.1	1.2 ± 1.3	0.3 ± 0.7
18:0	29.8 ± 5.7[e,f]	38.3 ± 6.1[e]	36.8 ± 3.0[f]
18:1	12.7 ± 4.4[g,i]	6.9 ± 0.8[h,i]	4.2 ± 1.3[g,h]
18:2	0.5 ± 0.2	1.2 ± 1.1	1.1 ± 1.0
18:3	1.3 ± 0.6[j]	0.4 ± 0.3[j]	1.3 ± 1.5
20:3 (n-9)	8.1 ± 3.2[k,m]	35.0 ± 4.5[l,m]	2.8 ± 0.9[k,l]
20:3 (n-6)	nd[a]	nd	tr[b]
20:4 (n-6)	6.3 ± 2.5[n]	5.1 ± 2.2[o]	38.4 ± 4.3[n,o]
20:5 (n-3)	13.6 ± 2.7[p]	0.7 ± 0.5[p]	tr

[a]nd = not detected.

[b]tr = trace.

[c-p]Values with same superscript are significantly different ($p < 0.05$; Student's t-test).

Galli et al. (23) have reported that a linolenic acid-rich diet suppressed drug-induced $PGF_{2\alpha}$ production in rat brain to a greater extent than that seen in EFA-deficient rats. These rats were sacrificed by microwave radiation, which should minimize post-mortem production of prostaglandins (12,24).

However, the use of a drug to induce prostaglandin synthesis in the brain could conceivably involve mobilization of arachidonic acid from another storage pool than that seen in the kidney of nontreated rats in vivo. We have found that PGE_2 release in vitro from the lungs of the same rats which are described in this report was reduced to a greater extent in the linolenate-supplemented group than in the EFA-deficient group (16). The fatty acid composition of total kidney lipids and kidney PI both showed a marked reduction of the 20:4 (n-6) content (Tables 1 and 2). The 20:4(n-6) percentage in kidney PI of the (n-3) rats was not different from that of the FF rats, and there was no difference between the two groups in their urinary PGE_2 excretion. This could support the hypothesis that PI could be a storage pool of arachidonic acid available for prostaglandin production in vivo (25).

We tried to detect PGE_3 in the urine from

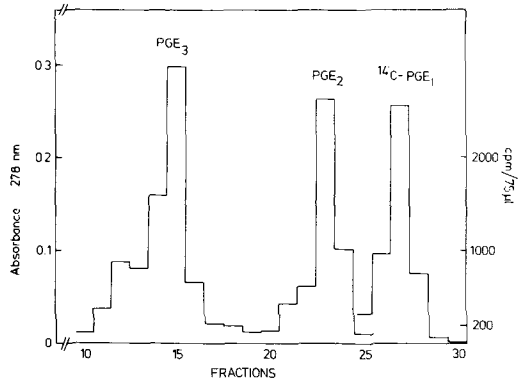

FIG. 3. Separation of standards: PGE_3, PGE_2 and [^{14}C]PGE_1 on reversed-phase HPLC column (125 × 4.6 mm, Nucleosil C_{18}). 35 µg PGE_3, 35 µg PGE_2 and 360,000 cpm [^{14}C]PGE_1 in 20 µl ethanol were injected on the column. Flow rate 1 ml/min and fractions of 1 ml. PGE_3 and PGE_2 in the fractions were determined after alkaline treatment as described under Methods.

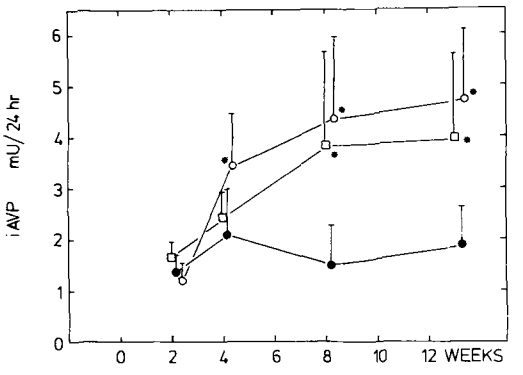

FIG. 4. Urinary iAVP excretion of male rats reared on fat-free diet supplemented with linolenate (n-3) □-□ with linoleate (n-6) ●-●, or with no supplementation (FF) ○-○. For details, see legend to Figure 1. *Statistically significant difference ($p < 0.05$) when compared with (n-6) group.

the (n-3) rats using HPLC and PGE_2 radioimmunoassay. Although we did obtain a peak of immunoreactive material with an elution volume corresponding to that of PGE_3 standard (Figs. 2A and 3), a similar peak was seen in the urine extracts from (n-6) rats also (Fig. 2B). Since there was extremely little, if any, icosapentaenoic acid in the kidneys of (n-6) rats (Tables 1 and 2), it appears likely that the observed peak (fractions 13-16 in Figs. 2A and 2B) was due to cross-reactivity to one of the many PGE metabolites found in rat urine (26).

Ferretti et al. (27) also could find no evidence for urinary PGE_3 excretion in fish oil-fed rats; however, the 20:5(n-3)/20:4(n-6) ratio

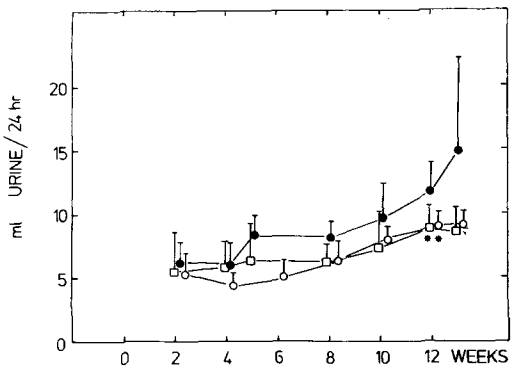

FIG. 5. Urine output from male rats reared on fat-free diet supplemented with linolenate (n-3) □-□, with linoleate (n-6) ●-●, or with no supplementation (FF) ○-○. For details, see legend to Figure 1. *Statistically significant difference ($p < 0.05$) when compared with (n-6) group.

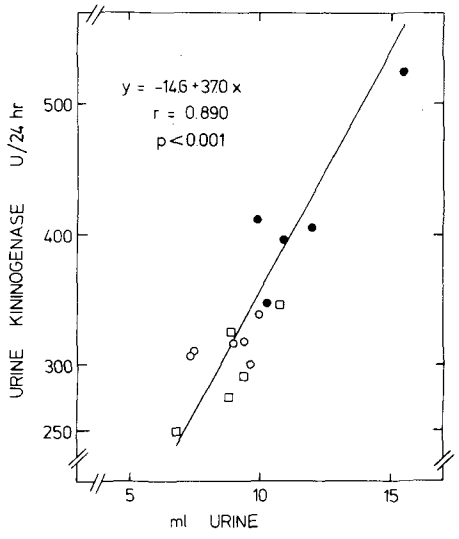

FIG. 6. Urinary kallikrein excretion of male rats reared on fat-free diet supplemented with linolenate (n-3) □-□, with linoleate (n-6) ●-●, or with no supplementation (FF) ○-○. The measurements are performed on urine collected at week 12, when there were statistically significant differences between the urine output of the (n-6) group and the 2 other groups.

was considerably smaller in the kidney lipids of their rats (28). The urinary iAVP excretion increased in a parallel manner both in the (n-3) rats and FF rats (Fig. 4). This confirms our recent findings of increased iAVP in EFA-deficient rats (2). However, we found no significant differences between the groups in urine osmolality (data not shown), and the urine output in both the FF rats and the (n-3) rats was not decreased compared with the (n-6) rats until after the 10th week of experiment (Fig. 5). Whereas the

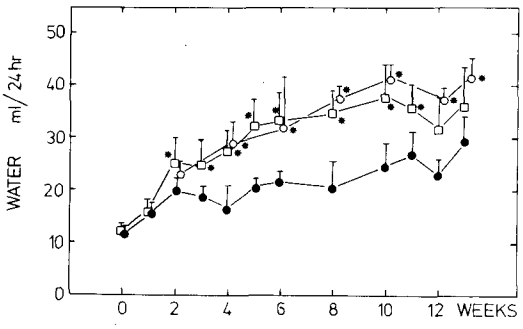

FIG. 7. Water consumption of male rats reared on fat-free diet supplemented with linolenate (n-3) □-□, with linoleate (n-6) ●-●, or with no supplementation (FF) ○-○. For details, see legend to Figure 1. *Statistically significant difference ($p < 0.05$) when compared with (n-6) group.

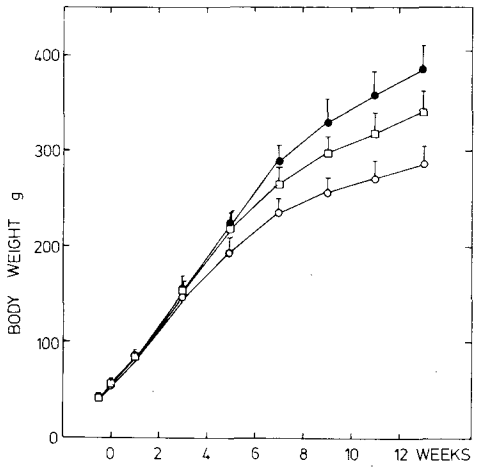

FIG. 8. Growth rate of male rats reared on fat-free diet supplemented with linolenate (n-3) □-□, with linoleate (n-6) ●-●, or with no supplementation (FF) ○-○. For details, see legend to Figure 1.

urine output did not parallel the urinary iAVP excretion, a parallelism was found in all 3 groups for water consumption (Fig. 7) and iAVP excretion (Fig. 4). Since it may be assumed that urinary vasopressin excretion is an index of vasopressin secretion from the neurohypophysis, both the increased water consumption and the increased urinary iAVP excretion indicate that the FF rats and the (n-3) rats after a few weeks of experiment were in a state of dehydration. Al-Nagdy et al. (29) have previously obtained results indicating that EFA deficiency results in dehydration of the rat. It is remarkable that urinary iAVP excretion increases in the FF rats and the (n-3) rats early in the experiment, whereas the urine output is unaffected for the first 10 weeks of experiment. Maybe the responsiveness of the kidney to vasopressin is decreased in the 2 groups of rats due to the dietary regimen, so that an effect of increased vasopressin secretion on kidney function was expressed only late in the experiment. Sakr and Dunham (30), who have induced an EFA deficiency in grown rats through starvation and refeeding, have reported that the renal vascular bed of presumably EFA-deficient rats showed increased responsiveness to angiotensin II and norephinephrine.

The renal kallikrein-kinin system has been suggested to be involved, together with the prostaglandins, in the regulation of renal functions (31). We have looked at urinary kallikrein excretion in our rats, however, only at week 12. We found a significantly lowered urinary kallikrein excretion in both the FF rats and in the (n-3) rats. However, since there was a positive correlation between urinary kallikrein excretion and urine volume (Fig. 6), and since, at week 12, there was a decrease in urine output (Fig. 5) corresponding to that seen for kallikrein excretion, it could be hypothesized that urinary kallikrein excretion was more related to renal water excretion than to the state of EFA defi-

TABLE 3

Body and Organ Weight after 13 Weeks of Experiment (mean ± SD, n = 5 or 6)

Group	(n-3) rats	FF rats	(n-6) rats
Body weight (g)	328 ± 20[a]	283 ± 17[a]	357 ± 27
Kidney weight (g/pair)	2.34 ± 0.15	2.32 ± 0.23	2.29 ± 0.14
Testis weight (g/pair)	2.65 ± 0.79[a]	2.65 ± 0.74[a]	3.89 ± 0.20

[a]Statistically significant difference ($p < 0.05$) from the (n-6) rats.

ciency of the animal. The 2 supplemented fatty acids were given in an amount of 2 energy%, which—according to Mohrhauer and Holman (32)—secured optimal growth of the rats. Except for the growth rate, the linolenate-supplemented rats ([n-3] rats) resembled the EFA-deficient rats (FF rats) in all the investigated parameters, i.e., urinary PGE_2 excretion, urinary iAVP excretion, water consumption, urine output, urinary kallikrein excretion, and kidney and testis weight. Thomasson (33) tried to quantitate the essentiality of different polyunsaturated fatty acids using a growth test which involved restriction of the water intake. In this test, linolenic acid was a very poor substitute for linoleic acid (33,34).

In summary, we have found that increased water consumption and increased urinary iAVP excretion are early symptoms of EFA deficiency. Both of these symptoms are probably due to increased transepidermal water loss. Decreased urine output as well as decreased urinary PGE_2 excretion are EFA-deficiency symptoms which developed much later, i.e., after 10 weeks on the diet. Extreme enrichment of the kidney lipids with icosapentaenoic acid does not suppress urinary PGE_2 excretion to a greater extent than that seen in EFA-deficient rats. This is in contrast to the effect on PGE_2 release in vitro from the lungs obtained from the same rats (16). We could not detect any urinary PGE_3 excretion in the linolenate-supplemented rats in spite of a very high 20:5(n-3)/20:(n-6) ratio of both total kidney lipids and kidney PI.

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Extremely Decreased Release of Prostaglandin E_2-Like Activity from Chopped Lung of Ethyl Linolenate-Supplemented Rats[1]

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ABSTRACT

Three groups of weanling male rats were reared on a fat-free diet for 13 weeks. One group received only the fat-free diet (FF rats), the other 2 groups received the fat-free diet and a daily supplement of 2 energy% ethyl linoleate ([n-6] rats), or 2 energy% ethyl linolenate ([n-3] rats). The chopped lung preparation was used to illustrate an in vitro prostaglandin formation. PGE_2-like activity was quantified on rat stomach strip. The release of PGE_2-like activity expressed as ng PGE_2-equivalent per g lung tissue (mean ± SD) was 23 ± 7, < 6, and 65 ± 20 for the FF rats, the (n-3) rats, and the (n-6) rats, respectively. PGE_2 quantification by radioimmunoassay of the chopped lung effluent collected after passing over the rat stomach strip revealed the same release pattern as the bioassay. Fractionation of chopped lung effluent on HPLC with radioimmunoassay detection indicated that the lung tissue from (n-3) rats released very little PGE_3, if any, in spite of a 20:5(n-3)/20:4(n-6) ratio of 5.2 in the lipids of the lung. It is suggested that the pool of arachidonic acid for prostaglandin production in vitro is different from the one which functions in vivo, and that these pools are differently affected by dietary EFA.

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Formation of dienoic prostaglandins in vitro has been reported to be decreased in tissues of rats fed EFA-deficient diets (1, and references therein). ten Hoor et al. (2) reported that dienoic prostaglandin production was reduced in the tissues of rats fed diets containing linseed or cod liver oil to the same extent as that seen from tissues of EFA-deficient rats. Linseed oil and cod liver oil are rich in (n-3) fatty acids. Ingestion of linolenic acid can result in accumulation of icosapentaenoic acid (20:5[n-3]) in the phospholipids of the tissues at the expense of arachidonic acid (20:4[n-6]) (3).

Arachidonic and icosapentaenoic acid are precursors for the dienoic and trienoic prostaglandins, respectively (4). However, the latter fatty acid is a relatively poor substrate for the cyclooxygenase (4,5) and it also is a competitive inhibitor for arachidonic acid oxygenation by the same enzyme (6). Prostaglandin production can be stimulated by different stimuli, e.g., mechanical, neuronal, hormonal or electrical (7). It is likely that these different stimuli involve different precursor pools (8) and that these pools are influenced differently by dietary essential fatty acids (9).

The purpose of the present experiment was to see whether extreme enrichment of the tissues with icosapentaenoic acid (20:5[n-3]) further depresses PGE_2 formation in relation to that seen in EFA-deficient rats; and whether a high tissue content of 20:5(n-3) results in formation of PGE_3. To answer these questions, we have used both an example of in vivo prostaglandin formation, i.e., urinary PGE_2 excretion, as well as an example of in vitro prostaglandin formation, i.e., release of PGE_2 from chopped lung. Results of the in vivo studies are described in the previous paper (10).

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Abbreviations: EFA = essential fatty acid; PGE_2, PGE_3 = prostaglandin E_2, prostaglandin E_3, etc.; HPLC = high performance liquid chromatography; RIA = radioimmunoassay.

MATERIALS AND METHODS

Animals and Diets

Sixteen 21-day-old male Mol-Wistar rats (K. Møllegaard-Hansens Avlslaboratorium A/S, Ll. Skensved, Denmark) were reared on a semi-synthetic fat-free diet. The diet is described in our previous paper (10), which is based on the same animals.

The rats were divided into 3 groups with 5 or 6 animals per group. One group received only the fat-free diet (FF rats), the other 2 groups received the same diet and a daily dose of either ethyl linoleate (all *cis*-9,12-octadecadienoic acid ethyl ester) ([n-6] rats) or ethyl linolenate (all *cis*-9,12,15-octadecatrienoic acid ethyl ester) ([n-3] rats) (99% pure; NuChek-Prep, Inc., Elysian, MN). Details of the experimental conditions are given in our previous paper (10).

Chopped Lung Preparation and Assay of PGE_2-Like Activity

After the 13-week feeding period, the rats

were decapitated, the chest opened, and the heart and lungs removed. The chopped lung preparation was prepared as described by Fjalland (11). Accordingly, the pulmonary artery was cannulated with a polyethylene cannula which was passed through the right ventricle. The lungs were perfused free of blood with 37 C warm carbogenated Krebs solution (NaCl 118, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10, $MgCl_2$ 1.2, and $CaCl_2$ 2.5 mM), dissected free from the heart and frozen in liquid nitrogen. The lungs were stored at −80 C until use. The lung tissue was chopped with scissors into pieces of ca. 2 mm^3, washed and placed in a stainless steel wire-mesh basket. Krebs solution containing combined antagonists according to Gilmore et al. (12) was dripped through the chopped lung at a rate of 5 ml/min. The effluent from the lung tissue superfused a rat stomach strip as described by Vane (13). Release of PGE_2-like activity was induced by mechanical agitation of the lung tissue with a blunt rod for 45 sec. The contraction produced by different concentrations of PGE_2 directly applied to the rat stomach strip was compared with that obtained after mechanical agitation of the lung tissue. The contractions of the isolated stomach strips were recorded isometrically by means of force displacement transducers, Grass FTO3 (Grass, Quincy, MA) coupled to a Servogor polygraph, model 330 (BBC, Nürnberg, F.R.G.). The tissues were suspended under a resting tension of 1 g.

PGE_2 Radioimmunoassay

[^{3}H]PGE_2, 160 Ci/mmol was obtained from the Radiochemical Centre, Amersham, England, and unlabeled prostaglandins were kindly provided by Dr. J. Pike, The Upjohn Co., Kalamazoo, MI. ^{3}H-PGE_2 was routinely purified on Sephadex LH 20 columns before use (14). The PGE_3 sample was also purified on a Sephadex LH 20 column. The PGE_2 antiserum was kindly provided by Dr. P. Christensen, Institute of Experimental Medicine, Copenhagen, Denmark (14). The radioimmunoassay was performed as described previously (15) with the modification, however, that the standards were extracted in the same way as the samples, making the use of ^{3}H-PGE_2 as internal standard unnecessary.

Fractionation of Chopped Lung Released PGE on HPLC

A known amount of [^{14}C]-PGE_1 (55 mCi/mmol from New England Nuclear, Boston, MA) was added as internal standard to 8-12 ml chopped lung effluent which was collected after passing over the rat stomach strip. The prostaglandins were extracted by the use of Sep Pak C_{18} cartridges (Waters Associates, Milford, MA) using the method of Powell (16) as described in the previous paper (10). After purification, the prostaglandins were injected into a HPLC system to separate PGE_3, PGE_2 and ^{14}C-PGE_1 (10).

Lipid Analysis

Lipid analysis was carried out as described in our previous paper (10). Unless otherwise specified, all organic solvents and reagents used were of analytical grade (E. Merck, Darmstadt, G.F.R.).

Statistical analyses were done using Student's t-test.

RESULTS

The mechanically stimulated release of PGE_2-like activity from the lungs of the three groups of rats is shown in Table 1.

The PGE_2-like activity was stable for more than 12 min in buffer, thereby excluding the possibility that prostacyclin contributed to the measured activity. PGE_2 radioimmunoassay of 10- and 20-μl samples of lung effluents collected after superfusion of the rat stomach strip was estimated for two lungs from each group (Table 1). In both the bioassay and the PGE_2 radioimmunoassay, the release of PGE_2 from

TABLE 1

PGE Release from Chopped Lung Tissue

Group	Bioassay PGE_2-equiv (ng/g)	PGE_2-RIA of collected superfusion fluid 2 lungs from each group (ng/g)
(n-3) rats	< 6 (n = 5)	< 0.90 & < 0.40
FF rats	23 ± 7 (n = 6)[a]	4.65 & 12.84
(n-6) rats	65 ± 20 (n = 3)[b]	25.57 & 20.93

[a]Mean ± SD, statistically significant difference from (n-6) rats ($p < 0.01$).

[b]The lung preparation from one of the (n-6) rats showed a PGE_2-like activity release of 235 ng/g tissue, and this has been excluded from the table.

the lungs of (n-3) rats was below the detection limit of the assay. The cross-reaction of PGE_3 with the PGE_2 radioimmunoassay was 4.9% at the 50% displacement level. Thus, the radioimmunoassay and the bioassay both (Fig. 1) had low sensitivity for detection of PGE_3 release.

Extract of the lung effluent, collected after it had passed over the rat stomach strip, was analyzed by HPLC using the PGE_2 radioimmunoassay for detection of significant PGE_3 release. Ten pg immunoreactive PGE_2 was equivalent to 230 pg PGE_3. In Figure 2 are shown 2 representative HPLC chromatograms of PGE_2 immunoreactive material released from chopped lungs from an (n-3) rat (Fig. 2A) and from an (n-6) rat (Fig. 2B), respectively. The small peaks at fractions 13-16, which have an elution volume equilvalent to a PGE_3 standard as described in our previous paper (10), amount to ca. 2% of the immunoreactive material in the PGE_2 peak for both the (n-3) rats and the (n-6) rats. Since there was extremely little, if any, 20:5(n-3) in the lungs of the (n-6) rats, Table 2, these small peaks of immunoreactive material probably do not represent PGE_3.

Fatty acid analyses of lung lipids revealed marked differences in 20:3(n-9), 20:4(n-6) and 20:5(n-3) between the 3 experimental groups (Table 2). The ratio of 20:5(n-3)/20:4(n-6) was 5.2 in the lungs of the (n-3) rats.

DISCUSSION

Chopped lung preparations of (n-3) rats released very little PGE_2 as was seen from both bioassay and PGE_2 radioimmunoassay (Table 1). The release was less than half that seen in lungs from FF rats. Lower values were obtained by radioimmunoassay than by bioassay, probably because some of the released PGE_2 was absorbed or metabolized by the rat stomach strip. The radioimmunological quantification was done on lung effluents after they were passed over the stomach strip. HPLC analysis of concentrated extracts of lung effluents revealed that lungs of (n-3) rats indeed released PGE_2 and a small peak (shoulder) of immunoreactive PGE_2 material (ca. 2%) which could be PGE_3 (Fig. 2A). A similar peak, however, was also seen in extracts of lung effluent from (n-6) rats (Fig. 2B), which had negligible amounts of the triene prostaglandin precursor, icosapentaenoic acid, in lung lipids. Thus, it appears more likely that these small peaks of immunoreactive material were due to compounds other than PGE_3. Ferretti et al. (17) found a PGE_3 production from kidney medulla homogenates to be 14-20% of the PGE_2 production, although the 20:5(n-3)/20:4(n-6) ratio in the lipids of kidney medulla was only 0.29 (18) as compared with a ratio of 5.2 in the lungs of the present experiment. This difference between their result and ours is probably more likely due to different ways of stimulating prostaglandin formation, i.e., tissue homogenates vs mechanical stimulation of lung pieces, than to the difference in organs. There seem to be no differences in the kinetics of the cyclooxygenases from different tissues (19).

The arachidonic acid percentages in total kidney lipids in the (n-3) rats, the FF rats and the (n-6) rats were 3.2%, 7.5% and 25.4%, respectively (10). The ratios between these percentages are 1:2.3:7.9. In total lung lipids, the corresponding ratios are 1:1.9:5.9 (Table 2). Thus, the relative arachidonic acid percentages between the dietary groups are similar for kidney and for lung total lipids. Nonetheless, the relative PGE_2 production from the same tissues was quite different, i.e., urinary PGE_2 production was of the same magnitude in (n-3) rats and FF rats (10), whereas this was not the case for PGE_2 release from the chopped lung preparation (Table 1). This difference could be due to different availability of arachidonic acid in the kidney in vivo as compared with the lung in vitro. Although we compare 2 different organs and in vitro/in vivo conditions, we suggest that it was the in vivo vs the in vitro situation which caused the difference between the PGE_2 production of the kidney and the lungs of the (n-3) rats. From several studies, there is circumstantial evidence that in vitro preparations often release free fatty acids, resulting in an artifact of prostaglandin production which has no physiological relevance (9,20).

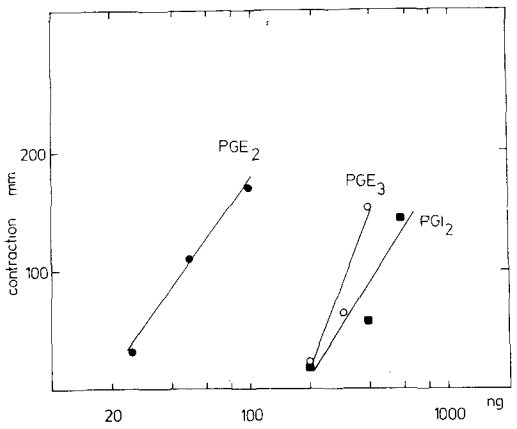

FIG. 1. Dose response curves for rat stomach strip to PGE_2, PGE_3 and PGI_2. For details of the bioassay, see Materials and Methods.

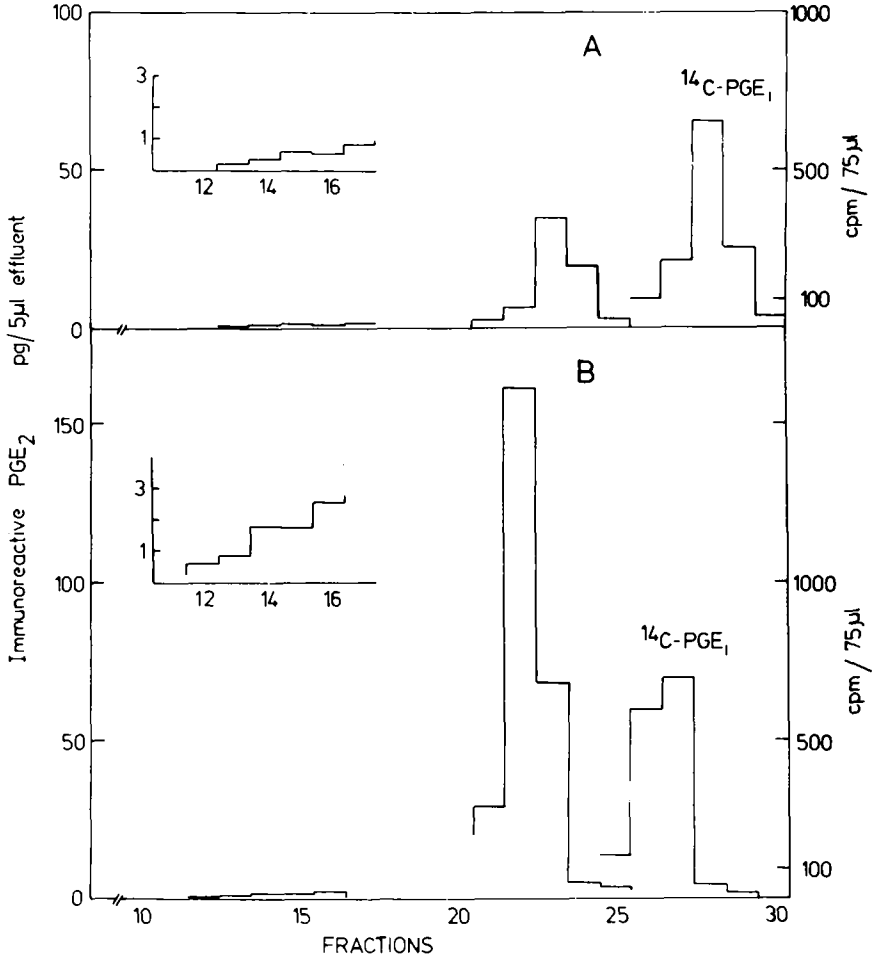

FIG. 2. Separation of chopped lung released immunoreactive PGE_2 material on a reversed-phase HPLC column. Lung effluents (8-12 ml) from one linolenate-supplemented rat (A) and from one linoleate-supplemented rat (B) were collected after passing over the rat stomach strip. The effluents were fractionated on Sep Pak C_{18} and the prostaglandin fractions were then applied to the HPLC column (125 X 4.6 mm, Nucleosil C_{18}). [^{14}C]PGE_1 was used as internal standard. Flow rate was 1 ml/min, and fractions of 1 ml were collected. A 10-fold enlargement of immunoreactive PGE_2 detection in fractions 11-17 is shown in the upper left corner.

TABLE 2

Fatty Acid Composition of Total Lung Lipids after 13 Weeks of Experiment (peak area %, mean ± SD)

Group	(n-3) rats n = 6	FF rats n = 5	(n-6) rats n = 5
16:0	27.3 ± 1.5	26.2 ± 1.5	27.8 ± 1.1
16:1	8.6 ± 1.0	9.3 ± 0.9	9.7 ± 1.5
18:0	8.9 ± 1.2[c]	8.5 ± 1.8	6.7 ± 1.5[c]
18:1	29.9 ± 4.7[d]	33.7 ± 7.8	37.0 ± 5.2[d]
18:2 (n-6)	tr[a]	0.6 ± 0.2[e]	3.1 ± 0.5[e]
18:3 (n-3)	0.7 ± 0.2[f]	1.1 ± 0.2[f,g]	0.6 ± 0.1[g]
20:3 (n-9)	0.6 ± 0.1[h]	6.7 ± 2.0[h,i]	0.5 ± 0.1[i]
20:3 (n-6)	0.9 ± 0.3[j]	0.7 ± 0.3	0.5 ± 0.2[j]
20:4 (n-6)	0.9 ± 0.2[k]	1.7 ± 1.1[l]	5.3 ± 2.0[k,l]
20:5 (n-3)	4.7 ± 0.9	tr	tr
22:3	tr	3.0 ± 0.8	tr
22:4	1.7 ± 0.5[m]	1.3 ± 0.8	0.6 ± 0.2[m]
22:5	1.8 ± 0.5	1.8 ± 0.8	1.4 ± 0.7
22:6	2.0 ± 0.3	nd[b]	tr

[a]tr = trace.

[b]nd = not detected.

[c-m]Values with same superscript are significantly different ($p < 0.05$; Student's t-test).

In summary, the PGE_2 production in the chopped lung preparation of linolenate-supplemented rats was decreased to a greater extent than that seen from lungs of EFA-deficient rats. No such difference between the 2 groups was seen for urinary PGE_2 excretion (10). It is suggested that the pool of arachidonic acid for prostaglandin production in vitro is different from that which functions in the kidney in vivo. We could not detect any PGE_3 formation in the chopped lung preparations of linolenate-supplemented rats, although the 20:5(n-3)/20:4(n-6) ratio in lung lipids was very high.

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Changes in Renal Phospholipid Fatty Acids in Diabetes Mellitus: Correlation with Changes in Adenylate Cyclase Activity

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ABSTRACT

Male Sprague-Dawley rats made diabetic with alloxan (37.5 mg/kg) or streptozotocin (65 mg/kg) were killed after 3-6 weeks of disease; renal tissues were studied for phospholipid content and for fatty acid composition of the phospholipids. No consistent change was noted in total phospholipid content nor in the proportion of various phospholipids in diabetics. However, diabetic animals showed a consistent reduction of arachidonic acid content in phosphatidylcholine (PC) and phosphatidylethanolamine in whole renal cortex, plasma membranes purified from renal cortex, and in isolated glomeruli. Associated with the fall in arachidonic acid was a rise in linoleic acid in the samples studied. Insulin therapy returned the fatty acid profiles to normal. These results are similar to patterns observed in other diabetic tissues and suggest that diabetes is associated with generalized changes in cell membranes. That these structural changes may have functional significance is suggested by demonstrated alterations in the temperature-dependence of adenylate cyclase in renal plasma membranes of diabetic animals. Adenylate cyclase is thought to be intimately associated with PC in plasma membranes, a phospholipid showing significant changes in fatty acid content in diabetes (unsaturation index 165 ± 2 for normals, 147 ± 5 for diabetics). Na^+,K^+-ATPase which is thought to be primarily associated in vivo with phosphatidylinositol (PI), shows no change in apparent energy of activation in diabetes. The fatty acid content of PI is minimally altered in diabetes, and the unsaturation index is unchanged.
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INTRODUCTION

Diabetes mellitus extensively alters lipid metabolism in several tissues. Most of the work in this area has focused on fatty acid metabolism in liver (1-3), or on prostacyclin and thromboxane ratios in vascular tissues, including platelets (4,5). Reported renal effects of diabetes mellitus on lipid metabolism include lowered prostacyclin synthesis in renal cortex (4), elevated thromboxane and lipoxygenase pathway products in glomeruli (6), and increased fatty acid synthesis (7).

Our interest in renal lipid composition and metabolism derived from biophysical studies we had performed on renal plasma membranes from alloxan- and streptozotocin-diabetic rats (8). In those studies, changes suggestive of altered lipid composition were seen in biophysical parameters measured by electron spin resonance. The present study documents the alteration of renal lipid composition in experimental models of diabetes mellitus.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (160-200 g) were injected via the tail vein with a saline solution of alloxan (35-37.5 mg/kg) or streptozotocin (65 mg/kg). Plasma glucose was monitored with a Beckman glucose analyzer. Insulin therapy was by continuous infusion of regular insulin via an osmotic minipump.

Chemicals

Reference lipids and reagents came from Supelco (Bellefonte, PA) or NuChek Prep, Inc. (Elysian, MN). Radioactive compounds were from New England Nuclear (Boston, MA).

Sample Preparation

Kidneys were removed after cervical dislocation and exsanguination of the rats. Renal cortex was homogenized for study or used as a source of plasma membranes or glomeruli according to published procedures (9,10). Lipids were extracted with chloroform/methanol (2:1, v/v) containing 1 μg/ml 2,6-ditert-butyl-4-methylphenol as antioxidant, purified by Sephadex chromatography (G-25 coarse; Pharmacia, Piscataway, NJ), and analyzed by thin layer chromatography (TLC) according to standard procedures (11). Briefly, phospholipids were resolved with 2-dimensional TLC on prewashed, heat-activated Redi-Coat 2D plates (Supelco) developed first in chloroform/methanol/ammonium hydroxide (65:25:5, v/v/v) then, after 90° rotation, in chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5, v/v/v/v/v). Phosphorus analysis was by pre-

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viously described methods (12). Phospholipids resolved and analyzed in this way were hydrolyzed and methylated with boron trifluoride (14% in methanol). The methyl esters were analyzed with a Packard Gas Chromatograph (Model 427) equipped with a flame ionization detector using 6 in. x 2 mm id glass columns packed with 10% SP-2330 on 100/120 Chromosorb W AW (Supelco). The separation was performed over a temperature range of 170-250 C and the peaks were integrated using a Hewlett Packard 3390A integrator. Methyl esters were identified by comparison of retention times to those of known standards.

Biochemical Assays

Adenylate cyclase and Na^+,K^+-ATPase were assayed by published techniques previously employed in this laboratory (13,14). Acetate incorporation studies using kidney slices (60-90 mg) incubated in Krebs-Ringer phosphate buffer followed earlier protocols (15).

Protein was measured by the biuret assay (16).

Statistics

Values reported in Tables 1, 2, 9 and 10 represent means ± SEM for measurements on individual animals. All other entries are derived from pooled samples as described in the legend to the table. Statistical significance was tested with Student's t-test.

RESULTS

The fatty acid content of phospholipids from normal and diabetic renal cortex is shown in Table 1. The largest differences between normal and diabetic tissues occur in phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Within these phospholipids, diabetic renal cortex contains higher linoleic acid (18:2) and lower arachidonic acid (20:4) than normal. Within phosphatidylinositol (PI) and phosphatidylserine (PS), the changes between normal and diabetic are much smaller; statistical significance is achieved only with the small percentage increase in linoleic acid in PI from diabetic renal cortex.

Analysis of individual phospholipids did not demonstrate any significant change in the proportion of major phospholipids present, although a small decline was observed both for PS and sphingomyelin (Table 2).

A subpopulation of renal cortical membranes enriched for plasma membranes was isolated and analyzed for fatty acid content of phospholipids (Table 3). In these studies, sphingomyelin and diphosphoglycerol were measured along with the four phospholipids measured in renal cortical homogenates. With this more homogeneous sample, linoleic acid increased and arachidonic acid decreased most prominently in PC. PE and PS isolated from diabetic renal plasma membranes showed smaller changes, similar to those observed in Table 2 for whole cortex. PI shows an anomalous low value for arachidonic acid in the normal sample.

Analysis of the proportion of phospholipids in these normal and diabetic plasma membranes showed no significant differences (Table 4).

Insulin therapy by continuous minipump

TABLE 1

Fatty Acid Content of Phospholipids from Normal and Diabetic Rat Renal Cortex

	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylinositol		Phosphatidylserine	
Fatty acid	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
16:0	32.1±0.1	31.4±0.2	11.8±0.4	11.8±0.4	11.2±1.1	11.7±0.7	5.0±0.5	5.5±0.2
16:1	1.0±0.1	0.8±0.2	0.5±0.1	0.4±0.1	0.5±0.1	0.4±0.1	0.6±0.2	0.5±0.2
18:0	11.7±0.4	11.7±0.6	21.9±0.5	22.3±0.2	37.3±1.4	36.4±0.9	41.2±1.8	41.8±1.1
18:1	9.2±0.2	9.9±0.1[a]	8.7±0.2	9.8±0.2	3.5±0.1	4.3±0.4	5.5±0.2	6.0±0.7
18:2	18.1±0.6	24.5±1.1[a]	6.5±0.2	10.1±0.5[a]	2.6±0.2	4.2±0.2[a]	3.9±0.1	5.4±0.5
20:3	1.4±0.1	1.9±0.2	0.6±0.1	0.6±0.1	3.7±0.6	4.0±0.4	1.1±0.3	1.4±0.1
20:4	22.3±0.7	14.5±1.0[a]	45.2±0.8	40.8±1.0[a]	38.1±1.7	36.3±0.7	38.8±1.1	35.3±0.5
20:5	0.9±0.1	0.7±0.1	1.5±0.1	1.5±0.2	1.3±0.1	1.0±0.3	1.8±0.1	1.8±0.2
22:4	0.3±0.1	0.4±0.2	0.8±0.1	0.6±0.1	1.0±0.1	0.8±0.2	1.3±0.2	1.2±0.4
22:5	0.4±0.1	0.4±0.1	0.5±0.1	0.5±0.1	–	–	0.5±0.1	0.6±0.1
22:6	2.3±0.1	3.3±0.8	2.1±0.4	2.4±0.6	0.9±0.2	0.9±0.2	0.6±0.2	0.9±0.2

[a] $p < 0.020$; $n = 4$.

Values are mean area % (directly related to concentration) ± SEM, as determined by integrating the peaks observed by gas chromatography. The animals used in this study were alloxan diabetics (37.5 mg/kg) killed after 3 weeks of diabetes, and age-matched controls. Blood glucose values exceeded 500 mg/dl throughout for the diabetics; normals ranged 110-120 mg/dl.

TABLE 2

Phospholipid Content of Normal and Diabetic Rat Renal Cortex

Phospholipid	μg Phospholipid/400 μg total lipid		% of total phospholipid	
	Normal	Diabetic	Normal	Diabetic
PC	98.01±5.82	102.00±4.38	35.2±2.1	36.6±1.6
PE	88.38±2.46	88.20±4.11	31.7±0.9	31.7±1.5
PS	23.70±0.48	21.15±0.87[a]	8.5±0.2	7.6±0.3
PI	19.98±1.83	22.20±2.43	7.2±0.7	8.0±0.9
Sph	26.04±0.93	21.00±1.62[a]	9.3±0.3	7.5±0.6
DPG	22.41±0.84	23.97±2.04	8.0±0.3	8.6±0.7

[a]Statistically significant reduction, $p < 0.05$.

Values are means ± SEM; n = 4. Phospholipids are analyzed after resolution on 2-dimensional TLC as described in Methods. Recovery of lipid phosphorus from the plates was 104±3%. The lipid/protein ratios for the samples analyzed are 0.298±0.016 for normals and 0.274±0.015 for diabetics. The samples analyzed were the same as those in Table 1.

infusion in previously diabetic animals returned the fatty acid profiles to normal (Table 5). This study on pooled samples reconfirms the changes in linoleic and arachidonic acid content in PC and PE. In addition, these longer-term diabetics show changes in PS and PI that were not clearly demonstrated in the shorter-term diabetics reported in Table 1. The insulin-treated diabetic samples were indistinguishable from normals for PC. Linoleic acid content was returned to normal by insulin for each of the phospholipids. Insulin therapy was without effect on the proportion of phospholipids present (results not shown).

Fatty acid profiles were also measured in glomeruli isolated from normal, diabetic, and insulin-treated diabetic rat kidneys (Table 6). In this renal structure, diabetes decreased arachidonic acid in all phospholipids tested except diphosphoglycerol. Concomitant nearly equal percentage increases in linoleic acid content were seen in PC and PE, but not in PS or PI. Also noted in these samples was a diabetes-induced increase in the longer-chain highly unsaturated fatty acid 22:6 in all phospholipids in which it could be measured. Although normal content of 22:6 is low in these tissues, the content was increased 2- to 3-fold in diabetes. This and all other observed diabetic changes were reversed by insulin therapy. No significant changes in phospholipid content was obvious in diabetic or insulin-treated diabetic glomeruli (Table 7).

The tendency to shift from arachidonic acid to linoleic acid in diabetic renal samples suggested that the disease might alter the unsaturation index of the phospholipid fatty acids. In fact, large decreases in the unsaturation index were observed in PC and PE from diabetic kidneys (Table 8). No significant changes in the unsaturation index could be demonstrated for the fatty acids acylated to PS or PI.

De novo synthesis of lipids was estimated by measuring acetate incorporation into various lipid classes (Table 9). In kidney, ^{14}[C]-acetate is roughly equally distributed in neutral lipid and phospholipid fractions, with very little being incorporated into glycolipids. Although acetate incorporation into lipids was increased in diabetic kidney, the proportion of neutral lipid to phospholipid remained relatively constant.

The two main phospholipids shown to have altered fatty acid composition in diabetes were also studied by acetate incorporation, along with cholesterol (Table 10). These studies again demonstrated a higher acetate incorporation in all 3 lipids in diabetic kidney; each of the 3 increased between 2- and 2.5-fold from normal. The ratio of PC to PE was similar in normals and diabetics, as was the ratio of cholesterol to PC.

Since our studies had suggested structural changes in the fatty acids acylated to phospholipids in renal diabetic membranes, we attempted to relate these changes to the behavior of membrane-bound enzymes. The plasma membrane marker enzymes adenylate cyclase and Na^+,K^+-ATPase were selected since these enzymes are dependent on the plasma membrane and are influenced by lipids within the membranes.

Arrhenius analyses were performed to assess transition temperatures and energies of activation for the enzymes from normal and diabetic renal cortical plasma membranes similar to those analyzed in Tables 3 and 4. With Na^+,K^+-ATPase in these membranes, no transition temperatures were observed between 40 and 15 C (Fig. 1). The energy of activation calculated at 37 C was 13.0 kcal/mol for both the enzyme from normal and the enzyme from

TABLE 3

Fatty Acid Content of Phospholipids from Normal and Diabetic Rat Partially Purified Renal Plasma Membranes

Fatty acid	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylinositol		Phosphatidylserine		Sphingomyelin		Diphosphoglycerol	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
16:0	27.8	28.5	10.3	10.4	10.1	11.6	4.8	6.2	21.4	20.8	2.8	3.5
16:1	1.4	0.9	0.2	0.5	2.5	0.7	–	0.9	4.3	1.1	0.7	1.0
18:0	12.2	13.1	24.6	24.3	41.7	37.4	43.5	39.9	17.9	16.2	1.9	2.0
18:1	9.1	9.0	7.9	9.0	4.0	2.5	3.9	4.6	10.5	3.0	10.4	9.4
18:2	16.0	22.4	5.9	8.0	1.3	3.2	2.4	3.8	1.7	1.9	71.9	73.5
20:3	1.9	2.1	0.4	0.5	1.6	3.6	–	0.8	–	–	2.2	1.6
20:4	22.9	17.9	45.5	42.6	20.7	34.9	42.3	39.1	12.5	21.2	6.6	3.9
20:5	0.7	0.7	0.8	1.3	2.0	2.0	1.3	1.6	4.4	5.9	0.5	1.6
22:4	1.0	–	0.5	–	8.1	2.3	–	0.6	–	–	–	0.5
22:5	0.4	0.4	0.2	0.2	–	–	0.2	0.2	–	–	0.4	0.3
22:6	2.7	1.9	1.9	1.4	0.7	0.4	0.5	0.3			0.9	0.5

Values are area % (directly related to concentration) as determined by integrating the peaks observed by gas chromatography. The animals used in this study were alloxan diabetics (37.5 mg/kg; n = 2), killed after 6 weeks of diabetes and age-matched controls (n = 2). Blood glucose values exceeded 500 mg/dl throughout for the diabetics, as compared to a normal range of 110-120 mg/dl. Partially purified plasma membranes were prepared as for enzyme assays (9,13).

diabetic kidneys. The diabetic membranes tended to have lower specific activity of Na^+,K^+-ATPase, as illustrated by the downward shift of the diabetic curve.

Arrhenius analyses of fluoride-stimulated adenylate cyclase showed a transition temperature of 22 C for normal and 18 C for diabetic enzyme (Fig. 2). No changes were noted in energies of activation (18.0 kcal/mol for both samples measured above the break points). Specific activity of adenylate cyclase in the diabetic sample was lower than normal.

DISCUSSION

The largest diabetes-induced changes observed in these studies occur in the fatty acids acylated to PC and PE. Linoleic acid (18:2) increases, whereas arachidonic acid (20:4) decreases significantly in diabetic kidney. These changes are observed in whole renal homogenate, partially purified plasma membranes, and in isolated glomeruli. When isolated glomeruli were analyzed, additional alterations were also revealed. In these samples, arachidonic acid was decreased and linoleic acid was increased in all phospholipids measured, not just in PC and PE. In addition, docosahexaenoic acid increased in all phospholipid fractions from diabetic glomeruli.

The changes in fatty acid composition tended to be greater in animals that had been diabetic longer (Table 5 vs Table 1), suggesting that these are progressive changes. Insulin returned the fatty acid composition to normal, suggesting that these were diabetes-specific changes. It should be noted that the animals received insulin by minipump infusion, a method of administration that maintained the animals near normal for plasma glucose. These well controlled diabetic animals may not model the human diabetic taking insulin by intermittent injection and who may suffer periods of hypoglycemia and hyperglycemia.

The changes we observe in diabetic kidney fatty acid composition in phospholipids are similar to changes previously observed in diabetic liver (2). In that tissue, the patterns were associated with a diabetes-induced fall in Δ6 and Δ9 desaturases. The Δ6 desaturase is the first enzyme in the pathway and the regulatory step in the conversion of linoleic acid to arachidonic acid. A decrease in the activity of Δ6 desaturase tends to cause an accumulation of its substrate, linoleic acid (18:2), and a relative depletion of the intermediates and final product in the pathway. These compounds in order are 18:3, 20:3 and 20:4 (arachidonic acid). In our renal samples, 18:3 was too low to be

TABLE 4

Phospholipid Content of Normal and Diabetic Rat Partially Purified Renal Plasma Membranes

Phospholipid	μg Phospholipid/400 μg total lipid		% of total phospholipid	
	Normal	Diabetic	Normal	Diabetic
PC	109.7	103.7	33.8	32.8
PE	100.4	96.3	31.0	30.4
PS	27.0	25.9	8.3	8.2
PI	20.8	16.1	6.4	5.1
Sph	34.6	35.8	10.7	11.3
DPG	31.7	38.7	9.8	12.2

Phospholipids are analyzed after resolution on 2-dimensional TLC as described in Methods. Recovery of lipid phosphorus from the plates averaged 92%. Each partially purified renal plasma membrane sample represents a pool of membranes from two animals. The lipid/protein ratios for the membranes analyzed are 0.35 for normals and 0.44 for diabetics. The samples analyzed were the same as those in Table 3.

measured, suggesting that in kidney as in liver the activity of the elongation enzyme is greater than the desaturases in the pathway. We do measure 20:3, but find it not to be significantly altered in diabetic samples, suggesting that the last enzyme in the pathway, a Δ5 desaturase, may not be greatly altered by diabetes. Therefore, our results suggest that the increased levels of linoleic acid and the decreased levels of arachidonic acid are primarily a result of a loss of Δ6 desaturase activity in the diabetic kidney.

In diabetic liver, Δ9 desaturase activity is also diminished (2). This enzyme converts saturated fatty acids to monounsaturated forms. A loss of this activity might be expected to increase the saturated fatty acids at the expense of the monounsaturated ones. We do not observe such a pattern in the diabetic renal samples.

Some studies on renal lipid metabolism exist (17,18), but no full analysis of renal lipid composition in normal and diabetic rats has appeared. Our results do compare well with previously published fatty acid profiles for normal rats (19).

The relative proportions of phospholipids remained constant between normals and diabetics for all renal samples studied (Tables 2, 4 and 7). Lipid to protein ratios were also not significantly altered. Acetate incorporation into lipids was greater in samples from diabetic kidneys than from normal kidneys (Tables 9 and 10), but the proportion of acetate incorporated into various lipid classes was similar in normals and in diabetics (Table 9). The incorporation of acetate into phospholipids reflects at least two processes: fatty acid synthesis and fatty acid acylation of phospholipids. In addition, the apparent rates of synthesis can be affected by uptake rates for acetate into cells and the ability of acetate to equilibrate with various intracellular pools. Differences in intracellular acetate pool sizes could exist between normals and diabetics. Andersen and Dietschy (20) showed that acetate freely enters cytosolic pools where fatty acids and cholesterol are synthesized. Moreover, they demonstrated that acetate also equilibrates with intramitochondrial pools. Our work also suggests that acetate enters the mitochondria and is freely and rapidly metabolized to CO_2. Acetate oxidation to CO_2 increased in our diabetic samples (unpublished observations). In work where rates of sterol synthesis in kidney and liver were compared using both tritiated water and acetate incorporation, the rate measured with acetate was 61.7% of that measured with tritiated water (20). In other studies in diabetic kidney, rates of fatty acid synthesis, measured by incorporation of tritiated water, were increased 1.6-fold by diabetes (7). In our studies, diabetes apparently increased cholesterol synthesis 2.5-fold and increased labeling of phospholipid with acetate 1.93- to 2.25-fold. Our results are expressed per wet weight of tissue. Since the kidneys greatly enlarge in diabetes and body weight goes down, increases would be magnified if expressed as total synthetic ability per kidney pair or as synthetic ability per 100 g body weight.

Our major interest in renal lipids was related to observations we had made on the biophysical properties of certain membrane-bound enzymes in diabetes. The one clear compositional change in renal membrane lipids is a shift toward more saturated, shorter-chain fatty acids in diabetes. The unsaturation index of PC and PE, phospholipids comprising 60-70% of total membrane phospholipid, is significantly reduced. The expected tendency of such a shift would be to

TABLE 5

Fatty Acid Content of Phospholipids from Renal Cortices of Normal, Diabetic, and Insulin-Treated Diabetic Rats

Fatty acid	Phosphatidylcholine			Phosphatidylethanolamine			Phosphatidylinositol			Phosphatidylserine			Diphosphoglycerol		
	Normal	Diabetic	Insulin Rx	Normal	Diabetic	Insulin Rx	Normal	Diabetic	Insulin Rx	Normal	Diabetic	Insulin Rx	Normal	Diabetic	Insulin Rx
16:0	27.4	31.0	28.9	10.3	12.4	11.8	19.6	14.9	9.9	4.5	5.2	5.3	2.7	2.1	3.8
16:1	0.2	0.7	0.8	0.5	0.1	0.5	–	–	–	0.6	0.6	1.2	0.8	0.8	0.9
18:0	13.6	14.0	15.9	20.2	27.6	25.9	36.8	43.7	43.4	42.7	45.9	47.7	1.0	1.1	1.2
18:1	9.2	10.0	10.0	8.8	9.4	9.5	–	3.6	5.2	4.8	5.9	6.8	10.8	10.9	12.0
18:2	19.5	26.6	16.9	7.8	11.9	7.0	2.1	4.2	2.4	3.3	6.7	2.7	76.3	79.7	75.4
20:3	1.8	1.9	1.4	0.6	0.6	0.6	4.4	5.2	4.0	0.9	1.9	1.4	2.4	1.9	1.8
20:4	24.5	11.7	23.0	46.7	33.3	40.5	32.6	26.7	32.4	38.8	30.5	30.5	4.1	2.2	3.2
20:5	0.6	0.4	0.5	1.0	1.1	0.9	–	0.3	1.0	1.6	1.4	0.8	0.4	0.3	0.6
22:4	0.4	0.3	0.3	1.0	0.6	0.9	3.3	0.6	0.8	1.7	0.9	1.5	0.7	0.6	0.7
22:5	0.3	0.4	0.3	0.5	0.3	0.4	–	–	–	0.5	0.3	0.4	–	–	–
22:6	2.5	3.0	2.1	2.7	2.7	2.0	1.2	0.8	0.8	0.7	0.7	1.6	0.8	0.3	0.5

Values are area % (directly related to concentration) as determined by integrating the peaks observed by gas chromatography. The animals used in this study were alloxan diabetics (35 mg/kg), killed after 2 months of diabetes. Blood glucose values exceeded 500 mg/dl throughout. Insulin control was initiated within 24 hr of alloxan injection by implanting an osmotic pump subcutaneously in the midscapular region. Fasting blood glucose values were maintained continuously below 120 mg/dl which was the average value for our normal, age-matched controls. The values reported in the table represent analysis of a pool of tissue from 4 animals.

TABLE 6

Fatty Acid Content of Phospholipids from Normal, Diabetic, and Insulin-Treated Diabetic Glomeruli

Fatty acid	Phosphatidylcholine			Phosphatidylethanolamine			Phosphatidylinositol			Phosphatidylserine			Diphosphoglycerol		
	Normal	Diabetic	Insulin Rx	Normal	Diabetic	Insulin Rx	Normal	Diabetic	Insulin Rx	Normal	Diabetic	Insulin Rx	Normal	Diabetic	Insulin Rx
16:0	32.7	30.8	31.6	11.9	8.6	9.5	8.9	8.6	7.9	3.0	2.5	2.9	4.4	4.4	5.4
16:1	1.7	0.2	0.7	0.8	0.4	–	0.1	1.2	0.6	0.9	0.2	–	–	1.7	1.5
18:0	12.2	12.5	14.3	20.7	22.4	22.8	39.7	43.5	36.0	42.4	45.8	40.6	2.8	4.1	2.7
18:1	8.3	8.8	8.6	7.3	8.8	8.2	3.7	4.0	7.0	6.0	5.6	6.1	10.9	10.5	10.5
18:2	23.5	33.8	24.3	8.2	14.3	8.8	2.6	3.7	4.6	10.9	14.2	14.0	74.1	69.6	69.5
20:3	1.5	1.5	1.3	0.5	1.7	0.4	1.5	4.4	1.6	2.1	4.1	2.3	1.1	2.4	1.8
20:4	16.7	6.9	15.5	40.2	28.2	38.6	40.4	29.4	36.6	22.6	13.3	22.2	4.4	3.2	3.8
20:5	0.6	0.3	0.6	1.1	1.7	1.2	0.5	0.6	1.0	0.5	0.9	0.6	0.6	0.7	1.5
22:4	0.7	0.4	0.8	3.6	2.4	3.8	1.8	2.8	3.4	5.8	3.0	5.1	1.1	3.0	2.9
22:5	0.6	0.9	0.6	2.8	3.4	3.0	0.5	0.8	0.7	3.6	3.7	3.8	0.5	0.3	0.3
22:6	1.6	3.8	1.7	3.1	8.1	3.6	0.3	0.9	0.6	2.0	6.7	2.4	–	–	–

Values are mean area % (directly related to concentration) for duplicate determinations, as determined by integrating the peaks observed by gas chromatography. Glomeruli from 4 animals in each group were pooled for analysis. The animals used in this study were streptozotocin diabetics (65 mg/kg), killed after 4.5 weeks of diabetes and age-matched controls. Insulin-treated diabetics received insulin subcutaneously continuously by osmotic minipump. Controls and diabetics also had minipumps implanted but the pumps contained only carrier. Average blood glucose values for the animals used were 122 ± 2, 622 ± 16 and 210 ± 27 mg/dl for controls, diabetics and insulin-treated diabetics, respectively.

TABLE 7

Phospholipid Content of Glomeruli from Normal, Diabetic, and Insulin-Treated Diabetic Rats

Phospholipid	μg phospholipid/400 μg total lipid			% of total phospholipid		
	Normal	Diabetic	Insulin Rx	Normal	Diabetic	Insulin Rx
PC	97.0	94.6	108.4	36.8	37.9	35.0
PE	80.5	73.3	89.4	30.6	29.4	28.8
PS	34.6	33.8	40.3	13.1	13.5	13.0
PI	15.0	12.8	16.6	5.7	5.1	5.4
Sph	21.5	23.3	40.4	8.2	9.3	13.0
DPG	14.7	11.9	15.0	5.6	4.8	4.8

Phospholipids are analyzed after resolution on 2-dimensional TLC as described in Methods. Recovery of lipid phosphorus from the plates averaged 98%. Each sample represents glomeruli pooled from 4 animals. The lipid/protein ratios for the samples analyzed are 0.236 for normals, 0.262 for diabetics and 0.258 for insulin-treated diabetics. The samples analyzed were the same as those in Table 6.

TABLE 8

Unsaturation Indices for Fatty Acid Components of Phospholipids from Normal and Diabetic Rat Renal Cortex

Phospholipid	Unsaturation index[a]	
	Normal	Diabetic
Phosphatidylcholine	165 ± 2	147 ± 5
Phosphatidylethanolamine	232 ± 2	216 ± 6
Phosphatidylinositol	186 ± 9	182 ± 4
Phosphatidylserine	193 ± 6	186 ± 4

[a]Calculated as (no. of double bonds X area %) from individual samples whose means are summarized in Tables 1 and 5.

create a more rigid or less fluid membrane. We have not measured significant changes in fluidity as assessed by electron spin resonance in diabetic membranes (8). Since membrane fluidity is strongly influenced by membrane-bound proteins and by sterols, it is reasonable to conclude that the regulatory processes involved in membrane formation are capable of maintaining constant membrane fluidity even in the face of these changes in fatty acid composition.

Our results with the membrane-bound enzymes illustrate the dangers of generalizing from the properties of the bulk membrane to the microenvironment of any specific protein. Both Na^+,K^+-ATPase and adenylate cyclase are transmembrane proteins found on the basolateral surfaces of renal tubular cells. Both are thought to be intimately associated with membrane lipids. In rabbit kidney microsomes, Na^+,K^+-ATPase has been demonstrated to be closely associated with PI (21). In contrast, adenylate cyclase from rabbit liver is thought to require PC for productive interaction of the enzyme subunits (22). In our studies, PI was minimally altered in diabetes; we could demonstrate no consistent change in linoleic acid content, arachidonic acid content, or unsaturation index. The Arrhenius plot for Na^+,K^+-ATPase was also unaltered by diabetes in the temperature range tested. In contrast, Arrhenius plots for adenylate cyclase showed a transition at 22 C for normal but 18 C for diabetic samples, suggesting an alteration in the microenvironment of the protein. More stringent or definitive interpretation of these alterations is at present impossible, since the factors influencing

TABLE 9

Acetate Incorporation into Separate Classes of Lipids

Fractions from silicic acid	Normal		Diabetic	
	Acetate incorporated (nmol)	% of total	Acetate incorporated (nmol)	% of total
Neutral lipid	4.29 ± 0.61	39.7	8.93 ± 0.88[a]	42.9
Glycolipid	0.87 ± 0.27	8.1	1.01 ± 0.09	4.9
Phospholipid	5.64 ± 1.00	52.2	10.87 ± 1.16[b]	52.2

For normals, n=3; diabetics, n=5.
[a]Significantly different from normal, $p<0.001$.
[b]Significantly different from normal, $p<0.01$.

TABLE 10

Acetate Incorporation into Specific Lipids

	Normal	Diabetic
	(nmol acetate incorporatd)	
Cholesterol	1.79±0.36	4.40±0.44[a]
Phosphatidylcholine	1.26±0.11	2.49±0.37[b]
Phosphatidylethanolamine	0.36±0.07	0.81±0.23[c]
	n=3	n=5

[a]p<0.001.
[b]p<0.01.
[c]p<0.05.

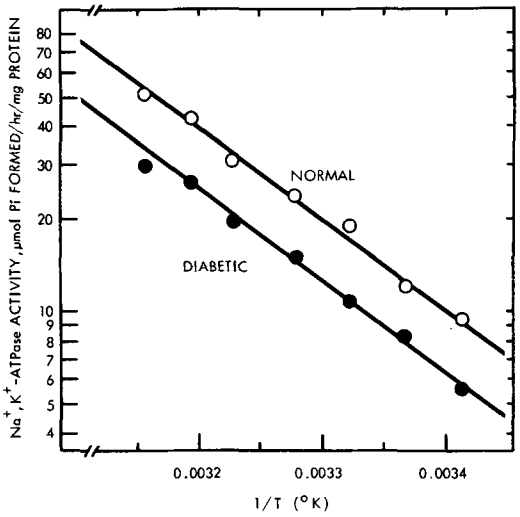

FIG. 1. Arrhenius plot for Na^+,K^+-ATPase. Membranes prepared from normal and diabetic rat renal cortices were incubated under standard conditions for the assay of Na^+,K^+-ATPase (see Materials and Methods), except that the temperature was varied. The data presented are means from separate measurements on 3 normal and 3 diabetic membrane preparations.

transition temperatures are complex and only imperfectly controlled or measured by current methodology. However, other workers have shown direct influences of phospholipid fatty acid composition on adenylate cyclase and/or Na^+,K^+-ATPase in mouse T lymphocyte tumor cells (23) and human platelets (24). These results as well as our own suggest that modulation of the action of significant membrane-bound proteins by membrane lipids is a significant regulatory process in several tissues.

Recently, other workers have analyzed fatty acids in phospholipids from a variety of tissues in streptozotocin-diabetic rats (25). Their results on whole kidneys are similar to the data we show in Tables 1 and 5. Although those workers conclude that the 5-desaturase is significantly impaired in most diabetic tissues, there

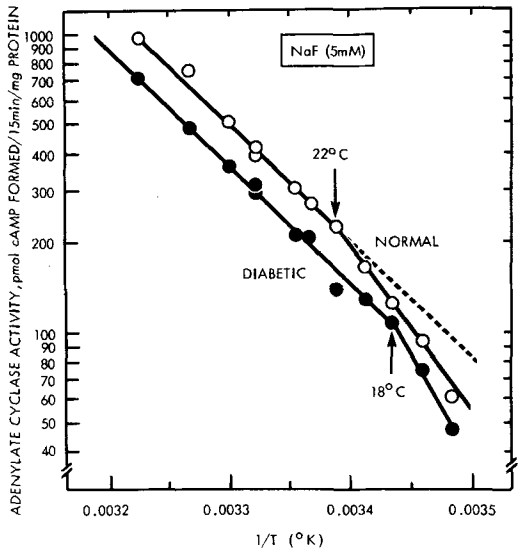

FIG. 2. Arrhenius plot for NaF-stimulated adenylate cyclase. Membranes prepared from normal and diabetic rat renal cortices were incubated under standard conditions for the assay of adenylate cyclase (see Materials and Methods), except that the temperature was varied. The data presented are means from separate measurements on 9 normal and 13 diabetic membrane preparations. .

was not a significant increase in 20:3ω6 in renal phospholipids (unlike other tissues); 18:2ω6 was increased and 20:4ω6 was decreased by factors similar to those in our experiments. Our data are entirely consistent with the results of Holman et al. (25) and extend their observations in greater detail in a single organ of interest. Hølman's study (25) analyzed all phospholipids as a pool rather than individually as we report, analyzed whole kidney only, and there were no data on the effects of insulin therapy.

Finally, our results support the conclusion that diabetes produces generalized metabolic effects in the kidney, in addition to the more well known effects on basement membrane. Rates of lipid metabolism were increased in slices from diabetic kidneys. The lipid composition of the kidney is altered in whole cortex, partially purified plasma membranes and glomeruli. There is evidence that these lipid changes may influence specific proteins and processes in the kidney. Another theoretical consideration is the effect the lowering of arachidonic acid content in specific renal phospholipids in diabetics might have on the ability of the kidney to autoregulate its function through local synthesis of prostaglandins. These questions await further studies.

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Dr. A.N. Siakotos graciously supplied advice on the lipid analyses and allowed the analyses to be performed in his laboratory on his equipment. This work was supported by a grant-in-aid to Dr. Queener from the American Heart Association, Indiana Affiliate.

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Autoxidation of Phenyl Linoleate and Phenyl Oleate: HPLC Analysis of the Major and Minor Monohydroperoxides as Phenyl Hydroxystearates[1]

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ABSTRACT

Phenyl linoleate was oxidized under different conditions. The monohydroperoxide products were isolated and subsequently hydrogenated. The isomers of phenyl hydroxystearate obtained were separated by high pressure liquid chromatography. On the basis of cochromatography with reference materials and mass spectroscopy, it was shown that the mixture was composed mainly (96%) of phenyl 9-hydroxy- and phenyl 13-hydroxystearates (9- and 13-HOPh) with 8-, 10-, 12- and 14-HOPh as minor compounds (4%). In the minor fraction, the 8- and 14-HOPh predominated in comparison to the 10- and 12-HOPh. The presence of α-tocopherol in the autoxidation experiment changed the proportion of the phenyl hydroxystearate isomers: the proportions of the 9- and 13-HOPh increased and those of the 8- and 14-HOPh decreased. After addition of 0.05% or higher concentrations of α-tocopherol, the minor fraction comprised approximately equal amounts of 8-, 10-, 12- and 14-HOPh. Autoxidation of phenyl oleate followed by hydrogenation of the monohydroperoxides resulted in the formation of a mixture of phenyl hydroxystearates containing approximately equal amounts of 8-, 9-, 10- and 11-HOPh.

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INTRODUCTION

A complex mixture of volatile aldehydes, which are very important for the flavor of fat-containing foods, results from the autoxidation of linoleic acid (1). The initial products of autoxidation are monohydroperoxides which react further, either by a β-scission of the OOH-group or by an oxidative cleavage of the double bond systems to yield an aldehyde mixture (2-6).

Different geometric isomers (*cis-trans* and *trans-trans*) of 2 monohydroperoxides (9- and 13-OOH) with a conjugated diene system are well known as primary products from the autoxidation of linoleic acid or its methyl ester (7-12). The initial step in the formation of these monohydroperoxides is a hydrogen abstraction from the *bis*-allylic carbon-11 of the fatty acid molecule (13,14). It had been proposed that a hydrogen atom is also abstracted to a lesser extent from the 2 monoallylic carbons-8 and -14 of the fatty acid (1,15). The 2 resultant allyl radicals, whose electrons are delocalized between either carbon-8 and carbon-10 or carbon-12 and carbon-14, could then oxidize to yield 4 monohydroperoxides (8-, 10-, 12- and 14-OOH), each containing an unconjugated diene system.

We were recently able to demonstrate (16, 17) that the monohydroperoxide fraction isolated from autoxidized methyl linoleate contained 1-1.3% of the 2 methyl esters of 8- and 14-hydroperoxy-*cis*-9,*cis*-12-octadecadienoic acid.

To detect these novel compounds, the monohydroperoxides were reduced to the corresponding alcohols. On the basis of the occurrence of 2 isolated *cis*-double bonds in the molecule, the minor compounds (8- and 14-OH) could be separated from the major compounds (9- and 13-OH) by high pressure liquid chromatography (HPLC) on a silica gel/$AgNO_3$ column (17). The other compounds (10- and 12-OH), which we expected to find as minor components of the mixtures, were not detectable when pure silica gel or silica gel impregnated with silver ions were used as stationary phases. However, it is possible that these minor isomers (10- and 12-OH) were not detectable in the column effluents because of a lack of separation from the major isomers (9- and 13-OH). Without a good separation, the weak UV-absorption of the unconjugated diene systems would be masked by the very strong UV-absorption of the conjugated diene system of the 9- and 13-hydroxy compounds.

To obtain an insight as to whether the 10- and 12-hydroperoxides are formed, the monohydroperoxides which result from the autoxidation of phenyl linoleate have been analyzed. The differences in the UV-absorption of unconjugated and conjugated diene systems were

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eliminated by hydrogenation of the double bonds. The phenyl esters were used so that after hydrogenation of the double bonds the products remained detectable, even at low concentrations, in the effluent from the HPLC by a UV-detector.

EXPERIMENTAL PROCEDURES

Materials

The following substances were purchased from the sources given: linoleic acid and oleic acid, both at least 99% pure (Sigma, München); Silica Gel HF254 palladium-charcoal and DL-α-tocopherol (Merck, Darmstadt); basic aluminia, activity I (Woelm, Eschwege) was deactivated with 6% water, Hypersil, 5 μm (Shandon, Labortechnik, Frankfurt), *meso*-tetraphenylporphine (EGA-Chemie, Steinheim).

Preparation of Pheny Oleate and Phenyl Linoleate

Oleoyl chloride and linoleoyl chloride were prepared by treatment of the corresponding fatty acid with phosphorus pentachloride in petroleum ether (18). Phenyl oleate and phenyl linoleate were prepared from the fatty acid chlorides dissolved in dry ethyl ether and phenol in the presence of pyridine (19). After a reaction time of 30 min, the mixture was filtered and, after addition of 50 ml of water, it was extracted twice with 50 ml diethyl ether. The combined diethyl ether extracts were washed with 1 M HCl, 50 ml, then with water, and finally dried over sodium sulfate.

After removal of the diethyl ether, a portion of the reaction product (ca. 350 mg) was dissolved in 3 ml of 5% diethyl ether in pentane and purified by chromatography at 10 C on a column packed with basic aluminia (20 × 1 cm). The phenyl ester was eluted with 80 ml 5% diethyl ether in pentane.

The phenyl esters were obtained in yields of ca. 50% (based on the fatty acid starting material). For phenyl linoleate, an elemental analysis (calc. $[C_{24}H_{36}O_2]$: 80.89% C, 10.11% H, 8.99% O; found: 81.00% C, 10.02% H, 8.84% O) gave satisfactory results. The UV spectrum of phenyl linoleate has a maximum at 214 nm (in hexane) with an extinction coefficient $\epsilon_{214} = 4800\ l \times mol^{-1} \times cm^{-1}$. The phenyl linoleate used for the autoxidation experiments was at least 99.9% pure, as shown by gas liquid chromatography (GLC) on a Silicone JXR-column (17) and by HPLC on a Hypersil column (4.6 mm id × 50 cm) with 0.05% acetonitrile in hexane (flow rate: 1.4 ml/min). Phenyl linoleate was eluted with 14 ml of the solvent. No peroxides were detected in the unoxidized phenyl esters by the Fe-test (20).

Photosensitized Oxidation

Photooxidations were performed using a specially constructed reaction vessel made of Duran 50 glass (21). Three mmol of phenyl linoleate were incubated with 2 μmol of *meso*-tetraphenylporphine in a total volume of 10 ml benzene. The stirred solution was irradiated for 70 min at 15 C using a 125 W mercury lamp under a positive pressure of 50 mbar of 99.9% oxygen. The solution was concentrated by distillation of the solvent in vacuo. The monohydroperoxides were isolated by preparative thin layer chromatography (TLC).

Autoxidation

A solution of 3 mmol fatty acid phenyl ester in 10 ml diethyl ether was pipetted into a 100-ml Erlenmeyer flask. The phenyl ester was freed from the solvent under a stream of oxygen. The sealed flask was stored, in the dark, for 72 hr at 37 C (phenyl linoleate) or at 80 C (phenyl oleate). The reaction mixture was dissolved in 1-2 ml diethyl ether and the monohydroperoxides were separated from the unreacted phenyl ester and from other products by preparative TLC.

Hydrogenation of Monohydroperoxides

The monohydroperoxides (50 μmol) were dissolved in 5 ml ethyl acetate and then hydrogenated for 6 hr with 10 mg palladium-charcoal as catalyst. The reaction mixture was filtered and concentrated by distillation of the solvent in vacuo. The phenyl hydroxystearates formed were purified by preparative TLC.

Thin Layer Chromatography

The reaction mixture, resulting either from the autoxidation, the photosensitized oxidation of fatty acid phenyl ester or from the hydrogenation of the monohydroperoxides, was applied to Silica Gel HF254 plates (500 μm thick). After development with hexane/diethyl ether (7:3, v/v), the phenyl esters were located by a UV lamp. The position of the monohydroperoxides was recognized by spraying the edges of the plate with solutions of potassium iodide and starch (22) and that of the phenyl hydroxystearates by cochromatography of a reference substance.

High Performance Liquid Chromatography

HPLC was performed using a stainless-steel column (4.6 mm id × 50 cm) packed with Hypersil. The column was coupled with a HPLC system (Beckman Instruments, München) and equilibrated at 22 C with 100 ml of the solvents detailed in the figure legends. The effluent was

monitored with a UV detector operating at 214 nm.

Mass Spectrometry (MS)

A mass spectrometer CH 7 (Varian MAT, Bremen) provided with a direct insertion probe was used. The inlet temperature was 80-90 C, the source temperature 200 C and the source potential 70 eV.

Elementary Analysis

Determination of carbon, hydrogen and oxygen were carried out by Labor Malissa, Gummersbach.

RESULTS

HPLC of the Isomers from Phenyl Hydroxystearate

In order to test the ability of our HPLC method to separate the isomers of phenyl hydroxystearate, we synthesized suitable reference compounds by hydrogenation of the monohydroperoxides produced from the autoxidation of phenyl oleate and from the photosensitized oxidation of phenyl linoleate.

From the autoxidation of phenyl oleate, the resulting phenyl hydroxystearates appeared as 4 HPLC peaks (nos. 1-4, Fig. 1), each of which was isolated and analyzed by MS. The MS of these compounds above m/e 110 show 5 characteristic peaks corresponding to the fragments A-E (Table 1). As shown in Figure 2, ions A, B and C allow the position of the hydroxy group in the phenyl hydroxystearate molecule to be determined. For all the isomers, the peak corresponding to ion B formed the base peak above m/e = 110 (Table 1). Ion D can be envisaged as forming from the phenyl hydroxy stearates via cleavage of the phenoxy radical and ion E by subsequent loss of water (Fig. 2). On the basis of the mass spectra, the compounds 1-4 (Fig. 1) were identified as the 11-, 10-, 9- and 8-hydroxy stearic acid phenyl esters (11-, 10-, 9- and 8-HOPh), respectively. From the areas of the peaks in Figure 1, the following composition was calculated: 8-OH (24.2%), 9-OH (23.6%), 10-OH (23.8%), 11-OH (28.4%). Since the phenyl hydroxystearates are simple derivatives of the corresponding monohydroperox-

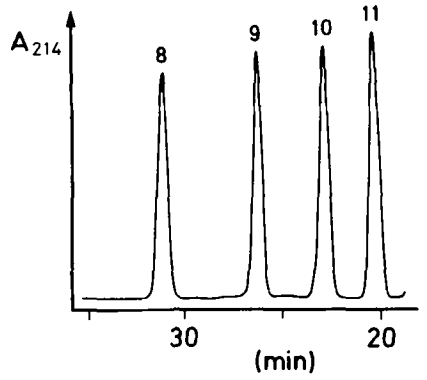

FIG. 1. Separation of phenyl hydroxystearates (produced by hydrogenation from phenyl oleate hydroperoxides) by HPLC. Sample: 0.6 μmol. Elution was performed with 0.3% ethanol in hexane (rate 1.4 ml/min). The isomers (11-OH, 10-OH, 9-OH and 8-OH) were identified by MS.

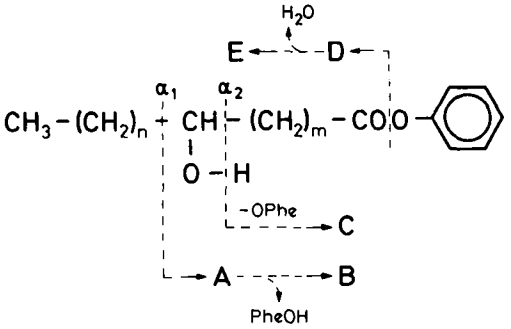

FIG. 2. Proposed reaction scheme for the interpretation of the MS of phenyl hydroxystearate isomers.

TABLE 1

Ions A-E[a] Found in the MS of Phenyl Hydroxystearate Isomers

Position of the OH-group	m/e (relative intensity, %) A	B	C	D	E
8	235 (17)	141 (100)	113 (29)	283 (24)	265 (30)
9	249 (13)	155 (100)	127 (31)	283 (24)	265 (26)
10	263 (11)	169 (100)	141 (37)	283 (23)	265 (42)
11	277 (15)	183 (100)	155 (50)	283 (42)	265 (80)
12	291 (13)	197 (100)	169 (75)	283 (45)	265 (68)
13	305 (10)	211 (100)	183 (73)	283 (74)	265 (63)

[a]The breakdown of the phenyl hydroxystearates with formation of the ions A-E is detailed in Figure 2.

ides, we can conclude that from the autoxidation of phenyl oleate the 4 isomeric hydroperoxides (8-, 9-, 10- and 11-OOH) are formed in similar amounts. That slightly greater amounts of the 8- and 11-hydroperoxides were found is in agreement with the regioselectivity which has been observed for the autoxidation of methyl oleate (23-27).

To obtain the 12- and 13-HOPh, the phenyl stearates, produced by hydrogenation of the monohydroperoxides formed from the photosensitized oxidation of phenyl linoleate, were chromatographed using the solvent given in Figure 1. With this solvent mixture, the 12- and 13-isomers eluted as a single peak (HPLC chromatogram not shown), although the 9- and 10-HOPh were separated. The material responsible for this peak was collected and rechromatographed using a solvent containing less ethanol. In this way, as is shown in Figure 3, the 2 isomers could be separated and subsequently identified by MS. The characteristic ions (m/e > 110) are given in Table 1.

Neither the 8- nor the 14-HOPh was detectable after a photosensitized oxidation of phenyl linoleate. From the areas of the separated phenyl hydroxystearates, the following composition was calculated for the mixture: 9-OH (37.8%), 10-OH (11.0%), 12-OH (11.3%) and 13-OH (39.9%). A predominance of the conjugated (9- and 13-OH) rather than the unconjugated (10- and 12-OH) isomers has also been observed in mixtures resulting from the photosensitized oxidation of methyl linoleate (28-30).

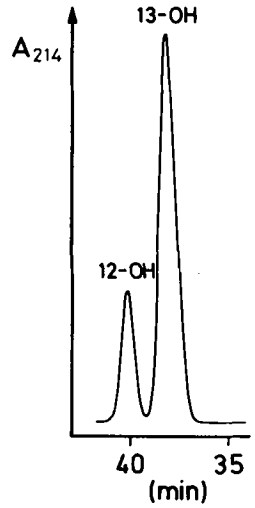

FIG. 3. Separation of the 12- and 13-hydroxystearic acid phenyl ester by HPLC. Sample: 0.6 μmol. Elution was performed with 0.12% ethanol in hexane (rate 3.6 ml/min).

Autoxidation of Phenyl Linoleate

Phenyl linoleate was autoxidized for 72 hr at 37 C and the monohydroperoxides, after separation by preparative TLC, hydrogenated for 6 hr. At the end of the hydrogenation, no hydroperoxides could be detected with the Fe-test (20) and the UV absorption due to the conjugated diene system (λ_{max} = 236 nm) had disappeared. After separating the resulting phenyl hydroxystearates by preparative TLC, their elemental composition was analyzed. (Calc. [$C_{24}H_{40}O_3$]: 76.59% C, 10.64% H, 12.76% O; found: 76.53% C, 10.74% H, 12.90% O.)

The agreement between the calculated composition and that found can be seen to be satisfactory. The UV maximum for the phenyl hydroxystearates (λ_{max} = 214 nm; hexane) was shown to have an extinction coefficient ϵ_{214} = 4400 l x mol^{-1} x cm^{-1}; which is somewhat smaller than that for the phenyl linoleate (see above). Possibly, a slight contribution to the absorption at 214 nm by the 2 *cis*-double bonds (31) of the phenyl linoleate molecule is responble for its larger absorption coefficient.

The phenyl hydroxystearates obtained were separated by HPLC (Fig. 4). The 2 major peaks

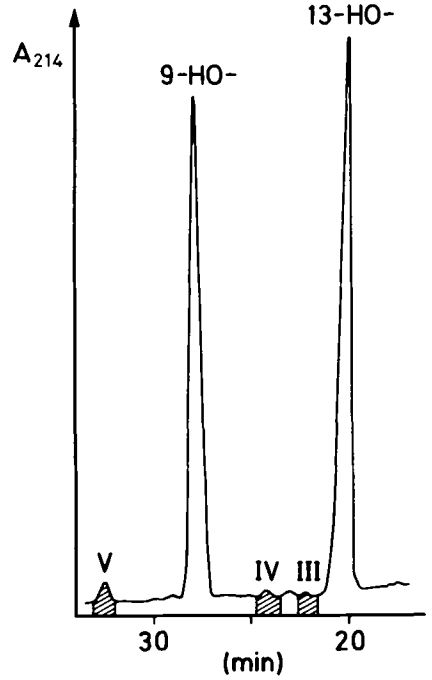

FIG. 4. Separation of phenyl hydroxystearates (produced by hydrogenation of hydroperoxides formed by autoxidation of phenyl linoleate). Sample: 0.6 μmol. Elution was performed with 0.3% ethanol in hexane (rate 1.4 ml/min). Hatching indicates that these portions of the effluent were collected.

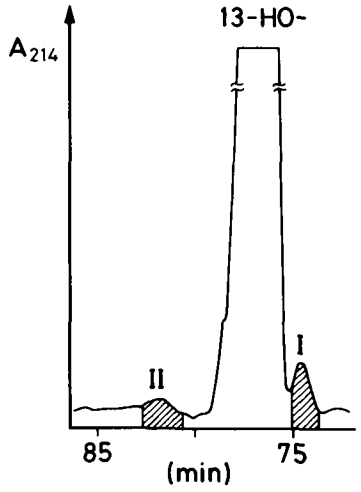

FIG. 5. Rechromatography (HPLC) of the fraction with the 13-hydroxystearic acid phenyl ester from Figure 4. Sample: 0.6 μmol. Elution was performed with 0.08% ethanol in hexane (rate: 4.2 ml/min).

were identified as the 9- and 13-HOPh on the basis of their mass spectra. In addition, a small peak (V) was clearly visible. Fractions III, IV and V (Fig. 4), whose elution volumes were identical with those of 11-, 10- and 8-HOPh (checked by cochromatography with reference materials), were also isolated.

Furthermore, the fraction containing the 13-HOPh was collected and rechromatographed using a solvent system containing less ethanol. As can be seen in Figure 5, 2 small peaks I and II, in addition to the major peak of the 13-HOPh, became visible in the chromatogram obtained. Cochromatography suggested that II had an elution volume identical with that of 12-HOPh.

Repetitive chromatography of a larger sample (ca. 10 μmol) of the phenyl hydroxystearates produced enough material of the fractions I-V to be collected for a MS analysis of each of the individual compounds.

The MS of compounds II and IV are given in Figure 6. Comparison of these spectra with those of reference substances (see Table 1) indicates that compound II is the 12- and compound IV the 10-HOPh.

The MS (m/e > 110) of compound I contained the ions at m/e 319 (14%), 225 (100%), 197 (82%), 283 (82%) and 265 (70%), which correspond to the fragments A-E (Fig. 2) of 14-HOPh. Both the elution volume and the MS of compound V indicated that this compound was 8-HOPh. Finally, although the elution volume for compound III suggested that it was 11-HOPh, the MS of compound III did not agree with that of the reference substance. It was not found possible to identify this compound positively.

To obtain a quantitative analysis, a defined amount of the phenyl hydroxystearate mixture was chromatographed as described in Figures 4 and 5. The individual isomers were isolated and their concentrations in the mixture calculated by using their UV-absorption at 214 nm.

With this method, the composition of monohydroperoxide isomers was determined in phenyl linoleate which was autoxidized under different conditions.

If one accepts that the proportion of the isomers is not altered during the hydrogenation of the mononydroperoxides and the work-up of the phenyl hydroxystearates produced, then the composition of the monohydroperoxide mixture resulting from the autoxidation of phenyl linoleate is given by the data in Table 2. Thus, in addition to the 9- and 13-hydroperoxides, a total of 3.6-5.0% minor hydroperoxides are formed in the experiments without addition of the antioxidant α-tocopherol (nos. 1-3 in Table 2). In the minor hydroperoxide fraction, the 8- and 14-hydroperoxides are the major components, whereas the 10- and 12-isomers are present only in smaller amounts. The composition of the minor hydroperoxides was independent of the reaction time (nos. 1-3 in Table 2) but was significantly changed when larger concentrations of α-tocopherol were present (nos. 5 and 6 in Table 2). The proportions of the 8- and 14-isomers on the fraction of monohydroperoxides decreases and those of the 10- and 12-isomers increases. Nearly equal amounts of these 4 isomers are formed.

To demonstrate the effect of the antioxidant on the composition of the minor monohydroperoxides, the individual solutions of compounds I, II, IV and V obtained in autoxidation experiments with and without 0.5% α-tocopherol (nos. 3 and 5 in Table 2) were combined and rechromatographed by HPLC. The resulting chromatograms (Fig. 7) reflect the data given in Table 2 for the change in the distribution of the minor monohydroperoxide isomers caused by the antioxidant.

DISCUSSION

The results given above suggest that, when phenyl linoleate is autoxidized, in addition to the major products (9- and 13-OOH), 4 hydroperoxides (8-, 10-, 12- and 14-OOH) are produced in small quantities. As was stated in the introduction, these compounds can be explained in terms of an H-abstraction from the 2 monoallylic carbons of the fatty acid radical.

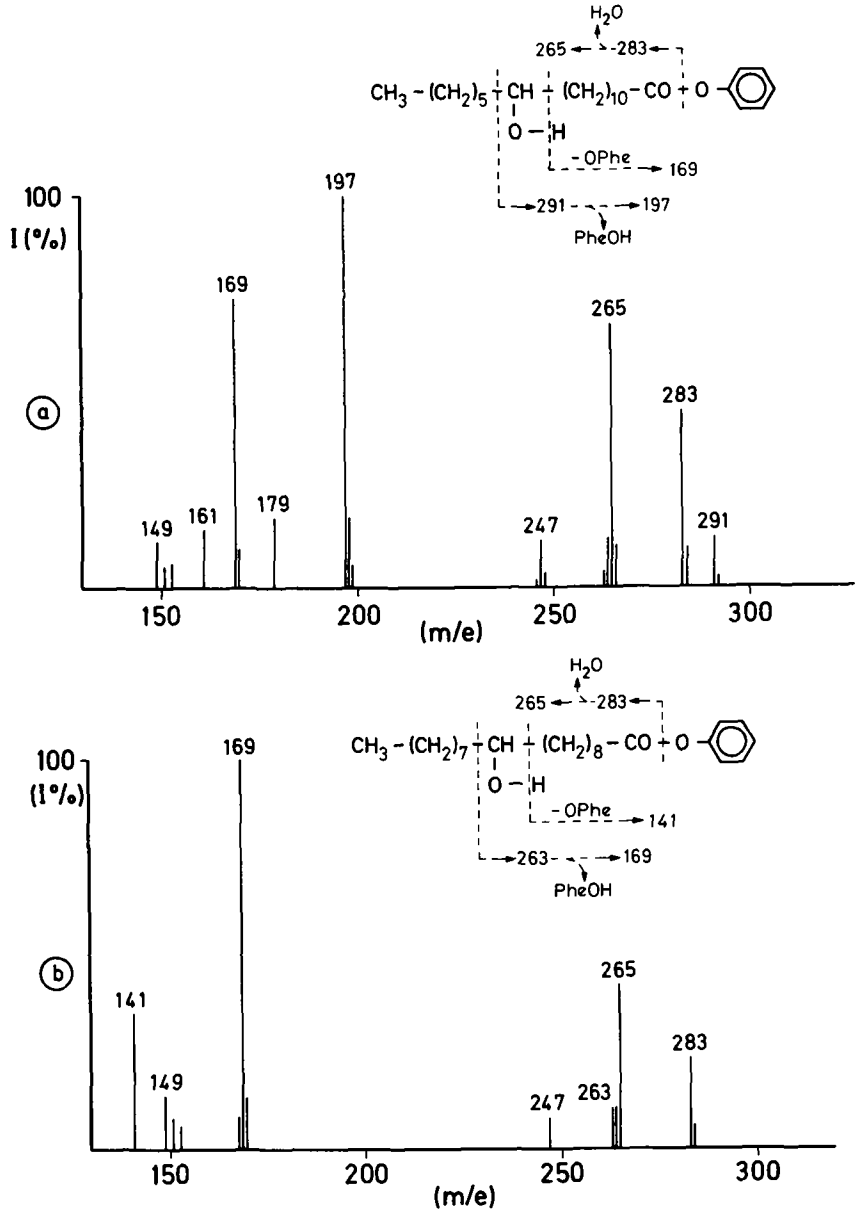

FIG. 6. MS of compounds II (spectrum a) and IV (spectrum b). Only the ions with m/e > 140 are given.

The identification of these minor products was achieved after converting them to the corresponding phenyl hydroxystearates and separation by HPLC using silica gel columns. This process allowed as complete a separation of the phenyl derivates as was achieved with the methyl hydroxystearates (24,25). However, the phenyl hydroxystearates could be monitored much more easily than the methyl derivatives because of their more intense UV-absorption.

Since the 10- and 12-monohydroperoxides, in addition to the 9- and 13-OOH, have been shown to be formed from the photosensitized oxidation of linoleic acid or its esters (28-30, 32-35), analysis of the samples was made to ensure that no monohydroperoxides were present before the start of the autoxidation process. The autoxidations were then carried out in complete darkness to exclude the possibility of formation of any photosensitized

TABLE 2

Monohydroperoxide Isomers from Autoxidized Phenyl Linoleate[a]

No.	α-Tocopherol[b]	Reaction time at 37 C	Yield[c] (μmol)	Monohydroperoxides					
				8-OOH	Weight-percent composition of isomers[d] 9-OOH	10-OOH	12-OOH	13-OOH	14-OOH
1	Without	24 hr	93	1.4	46.6	0.4	0.4	49.8	1.4
2	Without	48 hr	170	1.5	46.4	0.5	0.5	49.6	1.5
3	Without	72 hr	230	1.5	46.2	0.4	0.4	50.0	1.5
4	0.05	72 hr	80	1.1	46.9	0.4	0.4	50.1	1.1
5	0.5	72 hr	110	0.5	46.3	0.6	0.6	51.5	0.5
6	5.0	72 hr	180	0.7	46.6	0.6	0.6	50.8	0.7

[a]In each experiment, 3 mmol phenyl linoleate were oxidized in an excess of oxygen.

[b]α-Tocopherol (weight-percent related to the amount of phenyl linoleate) was added in the experiments nos. 4-6.

[c]After separation by preparative TLC, the yield of the monohydroperoxides was determined with the Fe-test (23).

[d]The composition of the monohydroperoxide isomers was determined by HPLC after hydrogenation to the phenyl hydroxystearates (see Experimental Section).

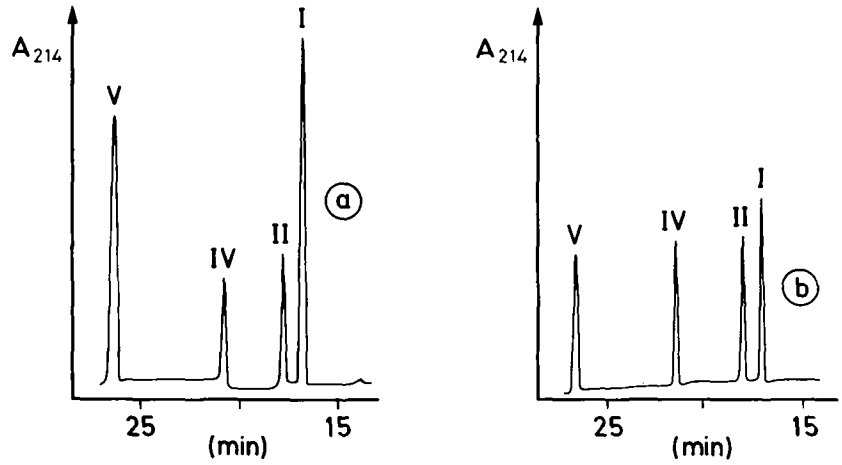

FIG. 7. Comparison of the minor compounds I, II, IV and V from autoxidized phenyl linoleate (a) and phenyl linoleate + 5% α-tocopherol (b) after 72 hr at 37 C. HPLC analysis of the phenyl hydroxystearate derivatives after separation from the major compounds (9- and 13-HOPh). Samples: 0.2 μmol. Elution was performed with 0.3% ethanol in hexane (rate: 1.9 ml/ min).

oxidation products.

In comparison to the 8- and 14-hydroperoxides, considerably smaller quantitites of the 10- and 12-hydroperoxides were found from the autoxidation of phenyl linoleate in the absence of an antioxidant. This difference in product concentrations can possibly be ascribed to the same effect which leads to a reduction in the amount of "inner" (10- and 12-OOH) relative to "outer" (9- and 13-OOH) monohydroperoxides during the photosensitized oxidation of linoleate (reaction with singlet-O_2). The origin of this effect is assumed to be the different reactivities of the precursors, the 10- and 12-peroxy radicals (30). These radicals are not only converted to hydroperoxides, as are the 9- and 13-peroxy radicals, but can also undergo intramolecular cyclization by reaction of the radical with the homoallylic *cis*-double bond to form hydroperoxy epidioxides (30,36). Such compounds can also be produced when the hydroperoxides from the photosensitized oxidation of methyl linoleate are stored (30).

We also assume that the reaction of 8- and 14-peroxy radicals via a similar intramolecular attack on the homoallylic double bond is not favorable since an unstable 7-membered cyclic peroxide would result.

Addition of α-tocopherol influenced the composition of the hydroperoxide isomers. The amount of the unconjugated hydroperoxides (8-, 10-, 12- and 14-OOH) decreased but they

were formed in approximately equal amounts. This result can be explained with the following 2 assumptions: the antioxidant partially inhibits the abstraction of a hydrogen atom from the 2 monoallylic carbons-8 and -14 of the linoleic acid molecule and, in addition, it accelerates the conversion of the peroxy radicals into monohydroperoxides. This accelerated conversion of peroxy radicals has been observed during the autoxidation of linoleic acid in the presence of hydrogen donating cosubstrates (11,12).

The inhibition effect would lower the amount of unconjugated (8-, 10-, 12- and 14-OOH) in comparison to the conjugated (9- and 13-OOH) hydroperoxide isomers. The activity of α-tocopherol as hydrogen donor for peroxy radicals would suppress the intramolecular cyclization of the 10- and 12-peroxy radicals on account of the formation of the corresponding monohydroperoxides, which therefore would increase in relation to the 8- and 14-isomers. This explanation agrees with the observation (37) that in linolenate autoxidation the proportion of the "inner" (12- and 13-OOH) increased in relation to the "outer" (9- and 16-OOH) monohydroperoxides and no hydroperoxy epidioxides were produced in the presence of 5% α-tocopherol.

Further experiments are under way to elucidate whether the hydroperoxy epidioxides, known from the photosensitized oxidation of linoleate (30,36), are also formed from the autoxidation of this unsaturated fatty acid. This question is of interest since such compounds can lead, on decomposition, to a variety of important aroma compounds such as hexanal, 2-heptenal and 3-octen-2-one (30).

ACKNOWLEDGMENT

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The Binding of Micellar Lipids to Chitosan[1]

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ABSTRACT

The lipid binding capacity of chitosan (partially deacetylated chitin) was determined with respect to micellar solutions of bile salts, dodecyl sulfate, natural ox bile and an artificial mixed microemulsion. The stoichiometry was determined following the separation of the solid phase by filtration or centrifugation. The major variables in the extent of binding were the pH and ionic strength, suggesting that the interactions are mainly of ionic nature. It is noteworthy that under optimal conditions chitosan could bind, i.e., coprecipitate, with 4-5 times of its weight with all the lipid aggregates tested. These results have a bearing on the nutritional and pharmacological applications of chitosan. The analyses of the components from the precipitates with microemulsion and ox bile show a significant selectivity of binding caused by hydrophobic interactions.

Lipids 18:714-719, 1983.

The interest in the polysaccharide chitosan (1), which is obtained from chitin by deacetylation, has recently spread into the field of nutrition and food technology. The patent of Yoshida and Yamashita (2) claims chitosan as a binder for pelletizing fertilizers and feeds. The powder of the commercially available free chitosan base was shown by Sugano et al. (3) to induce hypocholesterolemic properties in rats. Nagyvary and co-workers (4) have reported that the water-soluble chitosan acetate is superior to pectin as a general hypolipidemic agent. The promise of chitin and chitosan as an additive to bread was pointed out most recently by Knorr (5), who was concerned mainly with food functionality.

Although chitosan is believed to be of low toxicity (6), very little is known of its interactions with nutrients. Obviously, many properties of chitosan are predictable from its polycationic macromolecular structure. The rationale for fatty acid binding must be viewed as quite compelling. This is evidenced from a patent (7) being awarded for the application of chitosan as a lipid binder food additive in the absence of any biological or quantitative in vitro measurements. Since the major action of chitosan is expected to be on lipid absorption and metabolism, the interactions with individual lipids and their physiological combinations will have to be quantitated. In this paper, we report on the stoichiometry of the precipitation resulting by mixing chitosan acetate with bile salts and their micelles under a variety of conditions.

MATERIALS AND METHODS

The chitosan used in this study was donated by Dr. Q. Peniston, Kypro Co. It was a low viscosity grade preparation with an average molecular weight of 80,000. The chitosan was dissolved in 4% acetic acid, and then filtered through cheesecloth and freeze-dried. From elemental analysis, the average residue weight was established to be 219, i.e., the degree of deacetylation was ca. 90%. A sample of this preparation was dissolved in the appropriate buffer at 1.3 mg/ml, representing 1.0 mg/ml of free chitosan base.

The sodium salts of dodecyl sulfate (i.e., lauryl sulfate), cholic, glycocholic and taurocholic acids were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Lecithin from egg yolk was purified by alumina chromatography according to Singleton et al. (8). The readioactive compounds, [4-^{14}C]cholesterol, [24-^{14}C]taurocholate and glycerol tri[9,10(n)-^{3}H]oleate were purchased from Amersham-Searle Co. (Des Plaines, IL). [^{3}H]Oleic acid was prepared from the triolein by hydrolysis in alcoholic KOH, followed by acidification with dilute HCl and extraction with ether. Questran and cholestyramine were a gift of Dr. K. W. Wheeler (Mead Johnson Pharmaceuticals). Oleic acid was a product of Matheson, Coleman and Bell Co. (Norwood, OH). Bovine gall bladder bile was provided by Dr. T. R. Dutson, Texas A&M University.

The two buffer systems used in the binding experiments were 0.01 and 0.05 M imidazole-HCl (pH 6.8) and 0.05 M sodium acetate (pH 4.5). The ionic strength varied between 0.01

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and 0.25 M, and was adjusted by the addition of 3 M NaCl. Phosphate buffer caused a partial precipitation of chitosan, and so was avoided.

Lipid dispersions were composed of 2.0 mg taurocholate, 3.0 mg oleic acid, 0.5 mg monoolein, 0.5 mg triolein, 0.5 mg lecithin and 0.3 mg cholesterol. In some experiments, dodecyl alcohol was substituted for monoolein with the same results. An etheric solution of the above ingredients including one or two radioactive markers was added to the appropriate buffer solutions at the concentration of 6.6 mg total lipid/ml. The ether was evaporated in a stream of nitrogen under stirring. The resulting lipid dispersion was a fine microemulsion but it was stable for a few days. A freshly vortexed solution could pass through a 3-μm millipore filter with 95% of the radioactivity in the filtrate, but after 2 days 30% of the radioactivity was retained on the filter. For gravimetric determinations, the same composition was mixed without radioactive label at a total lipid concentration of 20 mg/ml.

Binding Experiments

One mg of chitosan was incubated with increasing amounts of bile salts (1 to 40 mg) in a total volume of 3 ml buffer at 20 C for 15 min. The solutions of the free bile salts were separated from the white flocculate by filtration through a 3-μm millipore filter. The bile salt concentration of the filtrates was determined by a modified Pettenkofer reaction (9). The amounts of bound bile salts were determined by subtracting the values obtained in the binding experiments from the amount of bile salts found in the control filtrates. The experiments were done in triplicate and the data were plotted as saturation curves.

The binding of the mixed lipid constituents to chitosan was carried out as described above except that some of the components were radioactively labeled for greater sensitivity. Also, it was preferable to use a combination of Whatman No. 24 and 3MM filter papers instead of millipore. Analytical tests for chitosan (ref. 1, p. 152) in the filtrate were negative up to a lipid/chitosan ratio of 10(w/w). More than trace amounts of chitosan were occasionally detected at higher ratios. The selectivity of binding for the various components was determined by using double label combinations such as [^{3}H] triolein and [^{14}C] cholesterol, or [^{3}H]-triolein and [^{14}C] taurocholate. Samples of the filtrates and controls, in triplicate, were assayed for radioactivity on a Beckman LS-250 counter using a scintillation fluid composed of 300 g naphthalene, 15 g PPO and 0.59 g POPOP in 3 l of dioxane. Corrections of quenching were made by the external standard channel ratio technique. The total amount of bound lipid mixture was obtained by direct gravimetric determinations. In these experiments, the binding was performed with larger amounts of chitosan (20 mg), and the precipitates were centrifuged at 800 rpm. The pellets were dried in a blow oven at 100 C and weighed on an analytical balance. The binding of sodium dodecylsulfate was measured in a similar manner.

RESULTS

This study of lipid binding to chitosan was initiated by measuring the binding of pure bile salts, cholate, glycocholate and taurocholate at different values of pH and ionic strength. Generally, saturation curves were obtained as shown in Figure 1 for cholate but the results were erratic at high detergent/chitosan ratios (above 40). The most meaningful comparison of 3 compounds under the various conditions can be done in terms of the saturation densities, i.e., maximum amount of bile salt bound per mg chitosan (Table 1). The binding of all 3 bile salts was considerable under all conditions tested.

The greatest binding at pH 6.8 was exhibited by the weakest acid, cholate, with 2.3 molecules of cholate bound per one glucosamine residue, or 7.3 molecules per one charged Glc-NH_3^+ residue. The Scatchard plot of cholate binding (Fig. 2) is biphasic with 2 slopes and corresponding intercepts of 2.3 and 6.5. The values may, in principle, denote the number of ligands at 2 different binding sites. The coincidence with the above-mentioned experimental values is intriguing. Because of the complexity of the plot, however, one cannot be sure about

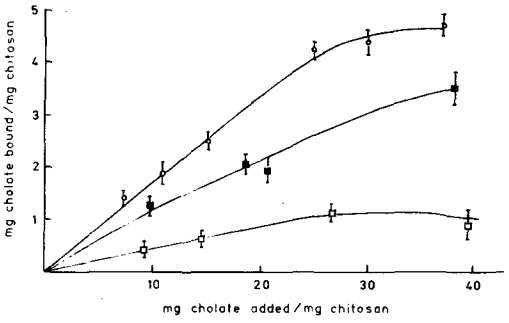

FIG. 1. Binding isotherm of cholate binding to chitosan; 0.01 M imidazole-HCl buffer, pH 6.8, o—o; 0.05 M imidazole-HCl, ■—■; 0.05 M imidazole-HCl, 0.2 M NaCl, □—□.

TABLE 1

The Binding of Micellar Lipids to Chitosan[a]

Lipid	pH	Buffer Ionic strength (M)	Free ligand (mg)	Bound ligand chitosan (mg/mg)	Bound ligand chitosan (mol/residue)
Cholate	6.8	0.01	36.9	4.8	2.5
		0.05	38.1	3.5	2.5
		0.25	26.7	1.2	0.6
Glycocholate	4.5	0.05	19.1	2.8	1.3
	6.8	0.05	20.3	1.7	0.8
Taurocholate	4.5	0.05	24.8	5.2	2.1
	6.8	0.05	18.4	1.6	0.6
		0.25	25.6	3.0	0.9
Dodecylsulfate	6.8	0.05	16.7	5.3	2.1
Ox bile	6.8	0.05	28.0	7.2	

[a]The data were obtained from saturation curves as shown in Figure 1; SD within 10-15%.

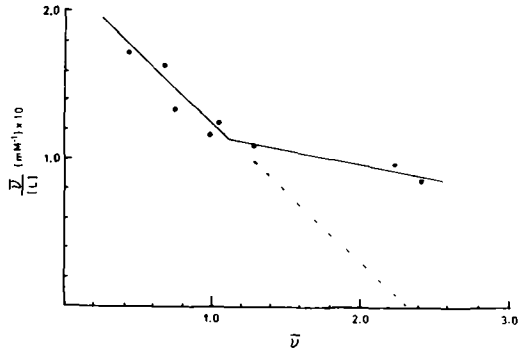

FIG. 2. Scatchard plot of cholate binding to chitosan. Experiments were performed in imidazole-HCl buffer pH 6.8 at 0.05 M ionic strength.

the meaning of the association constant (90 M^{-1}) calculated from the first slope. The same conclusions are valid for glycocholate and taurocholate binding which also exhibit a considerable degree of positive cooperativity and several binding sites. It is noteworthy that taurocholate binding increased with ionic strength from 0.05 to 0.25 M. No measurements were done in 1 M NaCl but the precipitation was visibly less significant.

To gain an understanding of the biological activity of chitosan, it appeared relevant to study the binding of larger micelles and aggregates containing the main ingredients found in the intestinal contents.

Experiments with dodecylsulfate micelles at pH 4.5 and 6.5 have shown a precipitation of the complex which contained optimally 4-5 mg micelle/mg chitosan according to a gravimetric assay (Table 1). Here we also observed an increasing solubilization of the precipitates at high micelle to chitosan ratios. To lend some physiological meaning to these studies, we also used a mixed composition relatively high in neutral lipids to yield a microemulsion of limited stability. The purpose of our inquiry was both to determine the amount of total lipid bound and to detect any changes in the composition of the mixture as the result of interaction with chitosan. Questran or cholestyramine was used for comparison because this resin has a clear preference for the anionic species.

The first set of experiments was designed to find out whether the anionic or a neutral component is found enriched in the complex. To this effect, a double label experiment was carried out using [^{3}H]triolein and [^{14}C]-taurocholate. Binding isotherms for both compounds are shown in Figure 3a. The ratios of triolein to taurocholate as a function of lipid concentration ranged from 1.5 to 2.0, representing a considerable increase from 0.25 found in the chitosan-free controls. Oleic acid also became enriched in the precipitate very much like triolein. According to a spot test, performed with 10 mg lipid added per mg chitosan, the oleic acid/taurocholate ratio increased to 9 from the original value of 1.5. In contrast, Questran exhibited a preference for taurocholate (Fig. 3b).

Little difference in the binding of neutral lipids to chitosan could be found. The molar ratios of labeled triolein and cholesterol remained close to one. When Questran was used with the same labels, the curve depicted a maximum of binding ca. 18 mg lipid added per 10 mg Questran, but the data were quite erratic at higher lipid concentrations. This phenomenon may be due to a precipitation and resolubilization of the neutral lipids as a result of changes in the amounts of the free bile salt.

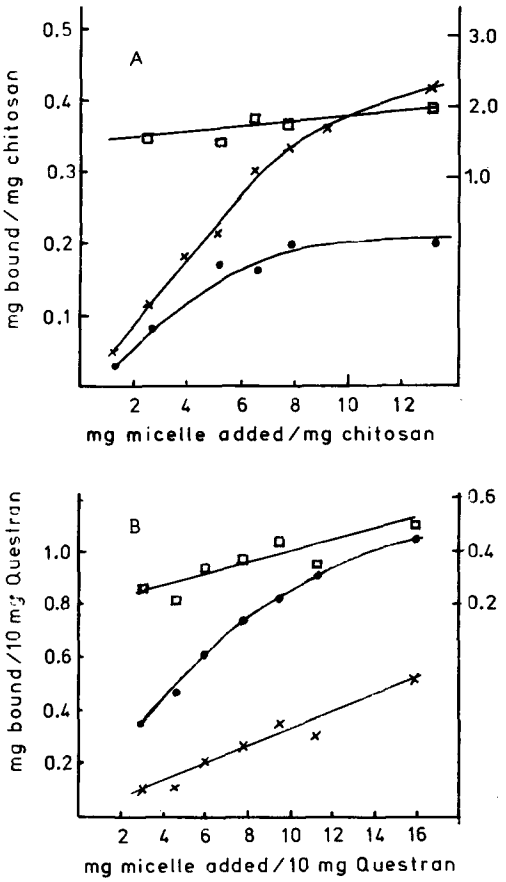

FIG. 3. The selectivity of binding to chitosan (A) and Questran (B) in imizazole buffer, pH 6.8, ionic strength 0.1 M. Triglyceride x—x; taurocholate •—•; the ratios of bound triglyceride/taurocholate (□—□) shown on the right ordinates.

The binding of total micellar lipids to chitosan was studied by both centrifugation and the filtration assays. As apparent from Figure 4, the latter method gave higher values and may provide the better conditions for binding. The low values obtained by centrifugation could have been due to the solubilization of the precipitate following the formation of a floating layer. Even the filtration method became erratic at high lipid/chitosan ratios when some chitosan complex could pass the filter.

The most complex natural micellar solution tested was bovine bile which gave a precipitate with chitosan composed of over 80% bile constituents. We have also found some selectivity in the binding of cholesterol, triglyceride and bile salts (Fig. 5). The data depict the fraction bound out of the total bile added. The neutral lipids were clearly represented in larger proportion in the bound fraction than the bile salts.

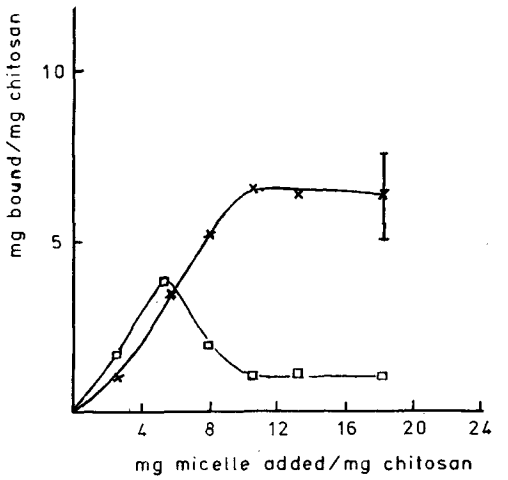

FIG. 4. Binding of artificial lipid aggregate at 0.1 M ionic strength, pH 6.8. Filtration and radioactive assay, x—x; centrifugation at 5000 rpm, □—□.

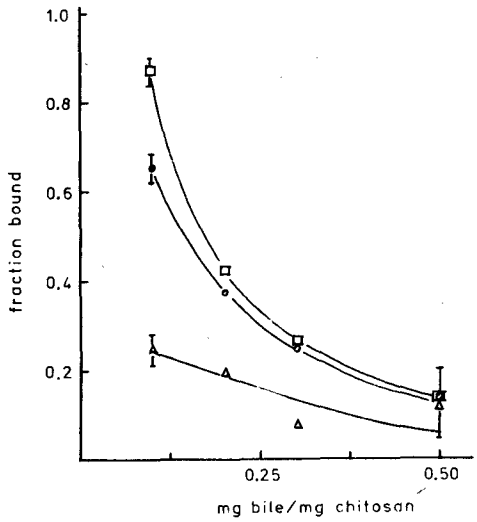

FIG. 5. Relative binding of ox bile constituents. Bile salts (△—△); cholesterol (□—□); and triglycerides (○—○) were measured by radioactive assays of added markers. The bile contained 51 mg/ml cholic acid equivalent.

DISCUSSION

The results presented above reveal that chitosan is a powerful bile salt sequestrant. In fact, no other natural or synthetic product is known to bind 4 times its own weight in bile salt under optimal conditions. The mechanism of binding appears to be quite complex, but the primary driving force is clearly determined by ionic interactions. The influence of the pH is expectedly strong, due to the respective pK

values of the components: chitosan 6.5, cholate 6.4, glycocholate 4.7, taurocholate ca. 1. Theoretically, the number of ionic binding sites in chitosan decreases from 100 to 33% as the pH is raised from 4.5 to 6.8, but, at the same time, the number of anions increases for glycocholate and cholate. Since more than one bile salt is bound per glucosamine residue, there must be hydrophobic interactions operating at the secondary binding sites. The greatest binding observed for cholate is in agreement with the least amount of electrostatic repulsion expected within cholate micelles. It is also possible that the unprotonated glucosamine residues may be involved in hydrogen bonding with some groups of the bile salt.

With regard to the hypolipidemic action of a polysaccharide, the measurement of pure bile salt binding has only a limited significance. According to Hofmann and Borgstrom (10,11), the total dietary lipid may be present in the duodenum in concentrations up to 35 mg/ml, but the amount of bile salt is 10 times less. Under these conditions, the lipids are present predominantly as a microemulsion which is in equilibrium with the smaller micelles. Therefore, chitosan could exert the most profound effect on lipid absorption through an interaction with the microemulsion and micelles.

The qualitative demonstration of interactions between chitosan and a microemulsion is a relatively simple matter. When a chitosan solution is shaken with oleic acid-triolein mixtures in a wide range of ratios, a stable viscous emulsion is formed. The consistency of this emulsion is such that it resists conventional physical-chemical attempts to quantitate the binding interactions. Nevertheless, it appears likely that the physiological action of chitosan is, to a major extent, due to the direct immobilization of the microemulsion. For practical reasons, our experimental system represents a compromise between the well characterized small micelles and a labile microemulsion. The polydisperse nature of a similar lipid aggregate was shown recently (12).

Of the two methods selected for the above studies, the equilibrium membrane filtration may be the more reliable one. The alternative approach used involved the separation of bound and free ligands by centrifugation. The latter was also the method recently employed by Vahouny et al. (13) for measuring the binding of various mixed micelle constituents to dietary fibers. These authors reported the pitfall of this method, which we have also experienced, the formation of a floating layer of largely neutral lipids. Accordingly, these results may not reveal the real extent of binding and the observed affinities of the various constituents that may not correlate well with the physiological conditions. Because of the forces operating in the centrifuge between particles of different buoyancies, centrifugation is not a suitable method to determine weak interactions which can be crucial in holding the fiber and micelles together. The suitability of the membrane filtration assay is evident from the successful application to the study of the elusive pectin-micelle interactions (12).

The extent of lipid micelle binding by chitosan as determined by the membrane filtration assay is impressive enough to be a major factor in the mechanism of its hypolipidemic action. Under physiological conditions of slightly acidic pH, chitosan could immobolize at least 4-6 times its weight in mixed micelles. However, the optimum binding of bile salts, dodecylsulfate nad mixed micelles cannot be assessed with sufficient certainty because of emulsification. It is possible, therefore, that chitosan could bind even more of the labile intestinal microemulsion which was not suitable for our study.

Of great theoretical interest is the observed disproportionation of the mixed aggregate as a result of binding to chitosan. The original anticipation of Sugano et al. (14) was the selective binding of bile salts to chitosan with the consequence of enrichment of neutral components in the solution. The formal analogy to cholestyramine would justify such an expectation. What we found, however, is the contrary: a precipitation rich in neutral lipids and a more stable micellar solution enriched in bile salt. As an interpretation of this finding, we may assume the engulfment of microemulsion droplets by chitosan chains. The result is analogous to the coacervation process in that two stable phases are formed. The capability of chitosan to surround and immobilize micelles of various composition is a unique property of this polycation. The crosslinked structure of cholestyramine, on the other hand, precludes the access of large aggregates.

In addition to its potential pharmacological use, the general lipid binding capability of chitosan described here suggests that soluble forms of chitosan could find application as emulsifying or binding agents in the food industry.

ACKNOWLEDGMENT

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(n-3) and (n-6) Polyunsaturated Fatty Acids in the Phosphoglycerides of Salt-Secreting Epithelia from Two Marine Fish Species

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ABSTRACT

Fatty acid analyses were carried out on phosphoglycerides isolated from microsomal fractions of the rectal gland of the dogfish, *Scyliorhinus canicula*, and gills of the cod, *Gadus morhua*. Ratios of (n-3)/(n-6) polyunsaturated fatty acids were ca. 10 for phosphatidylcholine, (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) from cod gills, reflecting high concentrations of 20:5 (n-3) and 22:6(n-3). The ratio for phosphatidylinositol (PI) from cod gills was 1.3, reflecting high concentrations of 20:4(n-6) as well as (n-3) polyunsaturates. PC, PE and PS from rectal glands all had much lower (n-3)/(n-6) ratios than in cod gills, reflecting higher concentrations of 20:4(n-6), but the lowest ratio was again present in PI. The latter phospholipid had high concentrations of 18:0 in both tissues. The relative constancy of the fatty acid composition of PI in the two salt-secreting tissues and its similarity to mammalian phospholipids is considered to reflect its specialized role in biomembranes.
Lipids 18:720-726, 1983.

INTRODUCTION

Osmoregulation in marine fish involves the elimination of excess ingested NaCl across specialized epithelia, specifically the branchial epithelium (gills) of teleosts and the rectal or salt gland of elasmobranchs. A similar organ is the salt gland of birds which is particularly well developed in marine birds.

The active cell in these "salt-secreting epithelia," the so-called chloride cell, is characteristically a large cell containing numerous mitochondria and extensively infolded basal and lateral plasma membranes (1). These plasma membranes are rich in (Na+K)-dependent ATPase which is intimately involved in salt secretion across the epithelium. The (Na+K)-dependent ATPase of salt-secreting epithelia is well characterized, the enzyme first having been isolated in a pure form from the rectal gland of the spiny dogfish, *Squalus acanthias* (2).

The turnover of the phosphate and inositol groups of plasma membrane phosphatidylinositol (PI) in response to cholinergic stimulus was first established in the avian salt gland (3). Subsequently it was shown that a similar turnover of the PI in the gills of seawater eels (*Anguilla anguilla*) was elicited by an α-adrenergic stimulus, a condition known to inhibit salt secretion by the gills, while conversely a β-adrenergic stimulus decreased PI turnover and stimulated salt secretion in gills (4-6). It is now known that salt secretion by both the rectal gland of elasmobranchs and the gills of seawater teleosts is stimulated by increasing intracellular levels of cyclic AMP either by addition of dibutyryl cyclic AMP or theophyline (7,8).

As well as being involved in the action of neural transmitters and/or hormones through the turnover of the phosphate and inositol groups, PI is also involved in prostaglandin formation by being the source of the arachidonic acid precursor used for PGE_2 synthesis (9-11). PGE_2 is known to inhibit salt secretion by marine teleost gills (12) and there is evidence that arachidonic acid, 20:4(n-6), rather than eicosapentaenoic acid, 20:5(n-3), is the preferred precursor of prostaglandins in marine fish (13). This contrasts with the fact that marine phospholipids in general are rich in (n-3) polyunsaturates and have in consequence high (n-3)/(n-6) ratios (14). Furthermore, it is established that (n-3) polyunsaturates are essential dietary factors in freshwater and marine fish but (n-6) polyunsaturates are generally held not to be essential (reviewed by Cowey and Sargent, 15). It is, of course, well established that (n-6) polyunsaturates are essential dietary factors in terrestrial mammals (16) and a critical role for (n-6) polyunsaturates in osmoregulatory (renal) physiology in these animals had long been known (17,18).

The present study seeks to further our understanding of the roles of (n-3) and (n-6) polyunsaturated fatty acids in osmoregulatory physiology by establishing the fatty acid com-

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positions of phosphoglycerides, especially PI, from plasma membranes from the chloride cells of two marine salt-secreting epithelia, the salt glands of the spotted dogfish, *Scyliorhinus canicula*, and the gills of the cod, *Gadus morhua*.

MATERIALS AND METHODS

Animals

Spotted dogfish (*S. canicula*) were caught off Millport in the Clyde Estuary, transported to Aberdeen and maintained in a running sea water aquarium at 13-15 C. Cod (*G. morhua*) were caught off Stonehaven, Kincardineshire and maintained in a running sea water aquarium at 10-11 C. They were used within 10 days of receipt.

Chemicals

All solvents were of HPLC grade and were obtained from Rathburn Chemicals Ltd., Walkerburn, Peebleshire, Scotland. Butylated hydroxytoluene (BHT), L-alanine and HEPES (N-2-hydroxyethylpiperazine-N-2- ethane sulfonic acid) were obtained from Sigma Chemical Co., Poole, Dorset, England. Trimethylamine-N-oxide dihydrate was from Fluka, obtained from Fluorochem Ltd., Glossop, Derbyshire, England. Dextran T40 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, England, and were of "Analar" grade, except the urea which was of "Aristar" grade.

Preparation of the Microsomal Fraction from the Rectal Glands of Dogfish

Fifteen dogfish were killed by transection of the spinal column posterior to the operculum and the spinal column pithed. The rectal glands were immediately removed and placed in ice-cold dogfish Ringer (composition 257 mM NaCl, 7 mM Na_2SO_4, 2.5 mM $NaHCO_3$, 4 mM KCl, 2 mM $CaCl_2$, 3 mM $MgSO_4$, 400 mM urea, 0.27 mM Na_2HPO_4, 0.01 mM NaH_2PO_4, 70 mM trimethylamine N oxide and 30 mM D-glucose, pH adjusted to 7.6 with 0.1 M HCl). All subsequent operations were carried out at 0-2 C. The capsule was stripped off the rectal glands which were chopped into small fragments and then homogenized with 25 ml of Ringer in a glass-in-teflon homogenizer (0.125-0.175 mm clearance, TriR model K43 drive, speed 6,500-11,000 rev/min range). The homogenate was diluted to 300 ml with a solution of 0.32 M sucrose containing 1 mM EDTA, adjusted to pH 8.0 with 0.1 M NaOH, and fractionated as follows. The suspension was centrifuged at 6500 gav-min to give a pellet of cell debris and a supernatant. The latter was then centrifuged at 300,000 gav-min to give a mitochondrial pellet and a further supernatant which was then centrifuged at 2.4×10^6 gav-min to give a microsomal pellet.

Preparation of the Microsomal Fraction from the Gills of Cod.

Four cod were stunned by a blow on the head and decapitated posterior to the operculum. The gills were immediately excised and placed in ice-cold Ringer (composition 161 mM NaCl, 2 mM K_2SO_4, 0.1 mM $(NH_4)_2SO_4$, 0.63 mM $CaCl_2$, 2 mM $MgSO_4$, 25 mM HEPES, 5 mM D-glucose, 5 mM L-alanine, 20 g/L Dextran 40, pH adjusted to 7.6 with 0.1 M NaOH). All subsequent operations were carried out at 0-2 C. The gill arches were blotted dry and the gill epithelium scraped off the underlying cartilage with a blunt scalpel. The gill scrapings (ca. 20 g wet wt) were suspended in 25 ml Ringer, stirred magnetically for 5 min and filtered through nyion gauze of rectangular mesh 106 μm square (Henry Simon, Stockport, England) to give a suspension of cells. The residue was resuspended in 25 ml of fresh medium, subjected to 2 strokes in a Dounce homogenizer, stirred for 5 min as before and again filtered through gauze.

The combined filtrates were then homogenized in a glass-in-teflon homogenizer (10 strokes), as detailed previously for rectal gland. The homogenate was then diluted to 300 ml with sucrose-EDTA medium and fractionated as before. At the stage of the mitochondrial pellet, a fluffy white layer could be swirled off the top of the dark tan mitochondrial pellet. This materila was rehomogenized in the 300,000 gav-min supernatant which was centrifuged to pellet the microsomal fraction as before.

Extraction of Phospholipids from Microsomal Fractions

The final microsomal pellets were resupended in 3 ml of 0.9% (w/v) NaCl and homogenized in 30 ml methanol containing 0.01% (w/v) BHT (6 strokes, details as before). Sixty ml of chloroform containing 0.01% (w/v) BHT was then added and the homogenate shaken. The solution was then filtered and 19.5 ml of water added to the filtrate which was shaken and then centrifuged (700 g, 5 min). The lower chloroform layer was filtered through phase separation paper (Whatman, England), concentrated under vacuum at 30 C and finally dried down under a stream of nitrogen at room temperature before dessicating in vacuo overnight.

Purification of Phospholipids

Phospholipids were chromatographed on thin layer Silica Gel G plates (Silica Gel 60, 0.25 mm, E. Merck, Darmstadt, West Germany) in two dimensions using chloroform/methanol/water/0.88 ammonia (130:70:8:0.5, v/v) in the first dimension and chloroform/acetone/methanol/glacial acetic acid/water (100:40:20:20:10, v/v) in the second dimension (19). After development, the plates were sprayed lightly with 0.1% (w/v) 2,7-dichlorofluorescein in 95% (v/v) methanol containing 0.1% (w/v) BHT, and visualized under ultraviolet light. Individual zones were scraped from the plates and the phospholipids eluted with 10 ml of chloroform/methanol/water (5:5:1, v/v) containing 0.01% (w/v) BHT. The solvent phase was removed under nitrogen and the phospholipids dessicated in vacuo for 3 hr.

Preparation of Fatty Acid Methyl Esters

The fatty acyl chains of the phospholipids were transmethylated for 16 hr at 50 C under nitrogen by treatment with 2 ml of 1% (v/v) sulfuric acid in absolute methanol (20). The methylation was stopped by the addition of 5 ml H_2O and the crude methyl esters extracted by 2 washes with 5 ml hexane/diethyl ether (1:1, v/v) containing 0.01% (w/v) BHT. The pooled organic phases were then extracted twice with 4 ml of 2% (w/v) $KHCO_3$ solution to remove the 2,7-dichlorofluorescein. The organic phase was dried with anhydrous Na_2SO_4, filtered through phase separation paper and dried under nitrogen. The crude methyl esters were then purified by thin layer chromatography on Silica Gel G using hexane/diethyl ether/glacial acetic acid (90:10:1, v/v) as the developing solvent. Methyl esters were eluted from the silica gel with 8 ml of hexane/diethyl ether (1:1, v/v) containing 0.01% (w/v) BHT, dried under nitrogen and stored in 100 μl of dichloromethane containing 0.01% (w/v) BHT at -20 C under nitrogen.

Analytical

Analysis of fatty acid methyl esters was performed in a Packard 429 gas chromatograph (Packard Instruments Ltd., Caversham, England) equipped with an open fused silica capillary, 50 m in length, 0.32 mm id, and coated with the liquid phase CP Wax 51 (Chrompack, Middelburg, The Netherlands). The injection port and flame ionization detector were operated at 250 C. The column was held at 160 C for 1 min after injecting the sample, after which the oven temperature was programmed to increase to 180 C at a rate of 4 C/min and then to rise by 0.5 C/min to a final temperature of 200 C which was retained for 20 min. Component peaks were identified by reference to known standards and to the data of Ackman and Eaton (21), and quantitated by a Hewlett Packard 3390A recording integrator (Hewlett Packard Instruments, Avondale, PA) attached to the gas chromatograph.

Dimethyl acetal derivatives of fatty aldehydes were confirmed by further chromatography on a Pye 104 gas chromatograph fitted with a 25 m x 0.32 mm fused silica column coated with Sil 5 (Chrompack), operated isothermally at 190 C with helium as carrier gas and coupled to a VG Micromass 16F single focussing mass spectrometer operated at an accelerating voltage of 4 kV and an ionization energy of 70 eV.

RESULTS

The rectal glands of elasmobranchs consist predominantly of chloride cells which have extensively infolded basal and lateral plasma membranes and a relative deficiency of endoplasmic reticulum (22,23). Consequently, the microsomal fraction isolated from homogenates of rectal glands consists predominantly of fragments of plasma membranes, reflected in its being highly enriched in (Na+K)-dependent ATPase (2). Chloride cells are also abundant in the gills of marine teleosts, although they are not the only or even the predominant cell type present in this tissue. Nevertheless, the microsomal fraction isolated from homogenates of "scrapings" of teleost gills by the methods used here is known to be highly enriched in (Na+K)-dependent ATPase located in plasma membrane fragments derived from chloride cells (24). Thus, the phosphoglycerides analyzed in the present study derive mainly from the plasma membranes of the cells actively involved in salt secretion in rectal glands and gills.

Table 1 details the fatty acid composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and PI isolated from the microsomal fraction of cod gills. The most notable finding in the table is that, whereas the major polyunsaturated fatty acid in PC, PE and PS is 22:6(n-3), the major polyunsaturate in PI is 20:4(n-6). 20:5(n-3) is a prominent polyunsaturate in all the phospholipid classes and, overall, the ratio (n-3)/(n-6) polyunsaturates is one order of magnitude less in PI than in PC, PE and PS. PC contains approximately equal concentrations of saturated and monounsaturated fatty acids, especially 16:0 and 18:1. PE differs from the other phosphoglycerides in that 17% of the products of

TABLE 1

Analyses of Fatty Acid Methyl Esters and Dimethyl Acetals Recovered from Phosphoglycerides Present in the Microsomal Fraction Isolated from the Gills of the Cod, *G. morhua*

	PC	PE	PS	PI
Fatty acid methyl esters				
14:0	1.6 0.4	tr	tr	0.8 0.4
anteiso 15:0	–	tr	tr	tr
15:0	0.6 0.1	tr	tr	tr
iso 16:0	tr	tr	tr	–
16:0	28.3 2.9	7.4 1.4	9.5 2.9	8.0 2.6
16:1(n-9)	1.4 0.2	0.6 0.2	tr	tr
16:1(n-7)	2.9 0.2	1.2 0.1	0.8 0.3	0.6 0.1
Unknown A	1.0 0.3	3.4 0.6	2.4 0.5	0.6 0.5
17:0	tr	0.6 0.1	0.7 0.1	0.7 0.1
Unknown 16,17	0.6 0.2(1)	1.0 0.9(3)	0.4 0.4(1)	1.1 1.1(3)
18:0	3.2 0.6	6.6 0.9	23.1 1.0	31.9 3.5
18:1(n-9)	18.4 0.2	6.4 0.6	6.9 1.3	6.0 0.7
18:1(n-7)	3.8 0.6	4.9 0.8	3.9 0.4	3.1 0.4
18:2(n-6)	tr	tr	tr	tr
18:3(n-3)	tr	tr	tr	tr
18:4(n-3)	tr	tr	tr	tr
Unknown 18,19	–	–	0.5 0.5(1)	–
20:0	tr	tr	1.0 0.2	tr
20:1(n-11)	tr	tr	tr	tr
20:1(n-9)	2.2 0.2	2.2 0.4	5.0 0.4	1.7 0.2
20:4(n-6)	2.7 0.8	3.7 0.9	3.2 0.1	15.3 2.8
20:5(n-3)	9.6 0.4	8.5 0.7	4.4 0.8	8.8 1.0
22:4(n-6)	tr	tr	tr	1.3 1.0
22:5(n-3)	1.1 0.2	1.6 0.3	1.0 0.7	1.5 0.8
22:6(n-3)	16.5 2.1	28.5 7.5	29.3 7.5	12.0 3.4
24:0	tr	–	tr	–
24:1(n-9)	–	–	–	0.6 0.9
Dimethyl acetals				
16:0	–	1.5 0.1	–	–
18:0	–	4.7 0.6	–	–
18:1(n-9)	–	7.4 1.8	–	–
18:1(n-7)	–	3.0 0.9	–	–
Total saturates	33.7	20.8	34.3	41.4
Total monounsaturates	28.7	25.7	16.6	12.0
Total(n-6) polyunsaturates	2.7	3.7	3.2	16.6
Total(n-3) polyunsaturates	27.2	38.6	34.7	22.3
Total polyunsaturates	29.9	42.3	37.8	38.9
(n-3)/(n-6)	10.07	10.43	10.84	1.34

Data are expressed as wt % and represent means ± standard deviations of three separate experiments each based on all of the individual gill arches from 4 fish; –signifies not detected and tr signifies present in at least 2 of the 3 experiments but less than 0.5%. Unknown A ran just before 17:0 methyl ester (9.84 min compared to 10.13 min) and after catalytic hydrogenation it moved to a position just behind 16:1(n-7) methyl ester (8.62 min compared to 8.48 min). Treatment with *bis*(trimethylsilyl) trifluoroacetamide failed to produce a trimethylsilyl ether derivative, indicating that the unknown was not a hydroxy fatty acid derivative. All unknowns were present at greater than 0.5% and are grouped on the basis of carbon numbers with the maximum numbers of individual unknowns present in a single experiment shown in parentheses after individual values.

acid-catalyzed transmethylation were recovered as dimethyl acetals implying that approximately one-third of the original ethanolamine phosphoglyceride zone was present as plasmalogens. Overall, however, PE has approximately equal concentrations of saturated and monounsaturated moieties. PS differs from PC and PE in having twice as much saturated as monounsaturated fatty acids with 18:0 being the major saturate. The high concentration of 20:4(n-6) in PI is accompanied by a ratio of saturates: monounsaturates of ca. 3.5:1.0 due to the presence of high concentrations of 18:0.

Table 2 details similar fatty acid compositions for PC, PE, PS and PI isolated from the microsomal fraction of *Scyliorhinus* rectal glands. In contrast to the situation in cod gills, all the phosphoglycerides in *Scyliorhinus* rectal glands have relatively high concentrations of 20:4(n-6) and only in the case of PC is the

TABLE 2

Analyses of Fatty Acid Methyl Esters and Dimethyl Acetals Recovered from Phosphoglycerides Present in the Microsomal Fraction Isolated from Rectal Glands of the Spotted Dogfish, *S. canicula*

	PC	PE	PS	PI
Fatty acid methyl esters				
14:0	1.0 0.7	tr	0.6 0.1	1.5 0.7
anteiso 15:0	tr	tr	tr	0.6 0.4
15:0	1.4 0.4	tr	tr	tr
Unknown 14,15	0.6 0.6(2)	–	–	–
iso 16:0	tr	tr	tr.	–
16:0	38.1 3.1	3.9 0.8	11.3 2.0	8.9 3.9
16:1(n-9)	1.2 0.2	0.6 0.3	0.4 0.3	0.6 0.4
16:1(n-7)	3.3 0.7	2.8 0.4	1.2 1.2	1.2 0.9
17:0	0.7 0.1	0.5 0.1	1.7 0.6	1.2 0.2
Unknown 16,17	3.5 1.1(6)	3.5 1.0(6)	2.6 1.0(4)	1.7 1.0(3)
18:0	2.2 0.4	8.3 1.0	26.2 0.6	30.6 4.7
18:1(n-9)	19.1 1.7	4.2 0.5	15.0 7.8	5.9 3.2
18:1(n-7)	5.5 0.5	2.7 0.5	2.1 0.9	2.2 0.2
18:2(n-6)	0.7 0.1	0.7 0.1	1.3 0.2	0.7 0.2
18:3(n-3)	tr	tr	tr	–
18:4(n-3)	–	–	tr	–
Unknown 18,19	–	0.6 0.6(2)	0.7 0.6(2)	1.3 1.4(2)
20:0	–	–	tr	tr
20:1(n-11)	tr	–	tr	–
20:1(n-9)	1.1 0.3	tr	1.8 1.9	1.8 1.2
20:4(n-6)	5.6 1.1	27.3 2.2	13.6 4.8	22.8 3.9
20:5(n-3)	2.9 0.5	8.2 1.6	3.5 0.8	3.3 0.6
22:1(n-11)	tr	–	0.7 1.0	1.0 0.4
22:4(n-6)	0.6 0.1	0.9 0.3	1.3 0.8	0.6 0.1
22:5(n-3)	0.9 0.2	0.7 0.2	1.0 0.5	1.4 0.7
22:6(n-3)	7.0 1.3	4.4 1.6	4.2 1.6	2.8 2.6
24:0	–	–	–	0.9 1.1
24:1(n-9)	–	–	tr	–
Unknown 20-24	–	–	–	1.4 1.4(2)
Dimethyl acetals				
16:0	–	3.8 0.6	–	–
17:0	–	2.0 0.6	–	–
18:0	–	2.3 0.3	–	–
18:1(n-9)	–	8.5 1.2	–	–
18:1(n-7)	–	6.8 1.4	–	–
Total saturates	43.4	20.8	39.8	42.4
Total monounaturates	30.2	25.6	21.2	12.7
Total(n-6) polyunsaturates	6.9	28.9	16.2	24.1
Total(n-3) polyunsaturates	10.8	13.3	8.7	7.5
Total polyunsaturates	17.7	42.2	24.9	31.6
(n-3)/(n-6)	1.56	0.46	0.54	0.31

Data are expressed as wt % and represent the means ± standard deviations of three separate experiments, each based on rectal glands from 15 fish; –signifies not detected and tr signifies present in at least 2 of the 3 experiments but less than 0.5%. All unknowns were present at greater than 0.5% and are grouped on the basis of carbon number with the maximum numbers of individual unknowns present in a single experiment shown in parentheses after individual values.

ratio (n-3)/(n-6) polyunsaturates greater than unity. PE was again the only phosphoglyceride which generated dimethyl acetals (some 23% of the total products) on acid-catalyzed transmethylation. This implies that approximately one-half of the original ethanolamine phosphoglyceride zone was present as plasmalogens. As in the case for cod gills, the PC and PE of *Scyliorhinus* rectal gland have essentially equal concentrations of saturated and monounsatrated fatty acids and PS has twice as much saturates as monounsaturates stemming from a high concentration of 18:0. PI in *Scyliorhinus* rectal glands has the same very high percentage of 18:0 as PI in cod gills. A further similarity in PI from the 2 tissues is that they both have the lowest ratio of (n-3)/(n-6) polyunsaturates of all the phosphoglycerides analyzed. However, a major difference between the 2 tissues is that, whereas in cod gills the highest percentage of 20:4(n-6) is found in PI, in *Scyliorhinus* rectal glands the highest percentage of 20:4(n-6) is

found in PE. Since both tissues contain ca. 4 times as much PE as PI (the present authors, unpublished data), it is clear that the bulk of 20:4(n-6) in *Scyliorhinus* rectal glands is associated with PE, whereas in cod gills it is associated very much more with PI.

DISCUSSION

The proposition that the polyunsaturated fatty acids of phospholipids from marine fish are dominated by (n-3) rather than (n-6) moieties (14,15) is based mainly on analyses of total phospholipid or major phospholipids such as PC and PE, often from whole fish or fish flesh (muscle). However, the proposition can apply to individual phospholipids from a discrete tissue since PC, PE and PS from cod (*G. morhua*) erythrocytes contain, respectively, 16.5%, 30.1% and 28.7% of their fatty acids as 22:6(n-3), the corresponding values for 20:4 (n-6) being 1.5%, 1.0% and 2.2% (25). Other situations exist, however, where marine fish phospholipids can be rich in 20:4(n-6). PC and PE from the olfactory nerve of the garfish (*Lepisosteus osseus*) contain, respectively, 9% and 26% of their fatty acids as 22:6(n-3), the corresponding values for 20:4(n-6) being 12% and 38% (26). PC and PE from the rectal gland of *S. acanthias* contain, respectively, 2% and 24% of their fatty acids as 22:6(n-3), the corresponding values for 20:4(n-6) being 4% and 15% (27). These findings, together with the present results (Tables 1 and 2), establish that wide differences can occur in the percentages of (n-3) and (n-6) polyunsaturated fatty acids in specific phospholipids from marine fish, even when tissues with physiologically similar functions are involved.

Dietary differences between species will obviously contribute to differences in the polyunsaturate compositions of phospholipids. Thus, the cod, *G. morhua*, is predominantly a pelagic fish linked firmly to pelagic food chains where lipids are characterized by high ratios of (n-3)/(n-6) polyunsaturates (28). Therefore, high concentrations of (n-3) polyunsaturates in cod phospholipids generally are to be expected. In contrast, *Scyliorhinus* is a bottom-living fish found in coastal, shallow waters where it feeds mainly on benthic invertebrates whose phospholipids can contain relatively high concentrations of 20:4(n-6) (29,30). Therefore, elevated levels of 20:4(n-6) in *Scyliorhinus* phospholipids generally are also to be expected. It is interesting in this context that the 20:4(n-6) in the phosphoglycerides of *Scyliorhinus* rectal gland are associated mainly with PE.

The results for PI in the present study are of special interest. PI accounts for ca. 6% of the total phospholipids in both cod gills and *Scyliorhinus* rectal gland (the present authors, unpublished data) and it differs from the other phosphoglycerides in both tissues in having (a) the highest percentage of 18:0, (b) the lowest percentage of monounstaturated fatty acids, and (c) the lowest ratio of (n-3)/(n-6) polyunsaturates stemming from high percentages of 20:4(n-6). That is, PI from both tissues is rich in 18:0 and 20:4(n-6) and is more akin to a typical terrestrial mammal phospholipid than a typical marine fish phospholipid. Relatively high concentrations of 18:0 and 20:4(n-6) have already been recorded for PI from the olfactory nerve of the garfish (26). PI from freshwater trout liver has 20% of its fatty acids as 20:4 (n-6), whereas the other phosphoglycerides from this tissue have 7% or less as 20:4(n-6) (31). More recently, Casteldine and Buckley (32) have shown that PI from total body phospholipids of the trout has 12% of its fatty acids as 20:4(n-6). Thus, PI from all fish tissues so far analyzed has a relatively low ratio of (n-3)/(n-6) polyunsaturates stemming from a high percentage of 20:4(n-6), and a relatively high percentage of saturates, especially 18:0.

Casteldine and Buckley (32) comment that the high concentration of 20:4(n-6) in trout PI is of special interest but suggest that PI is unlikely to have a special function since (n-6) polyunsaturates are considered not to be essential dietary factors in fish (33). We propose, in contrast, that the relative constancy of the composition of PI in fish tissues and the similarity of its composition to the PI of terrestrial mammals points firmly to fish PI playing highly specific roles in biomembrane function, entirely analagous to those established in terrestrial mammals. The implication is that (n-6) polyunsaturates are indeed essential dietary factors in fish but since they are involved with a quantitatively minor phospholipid, PI, the amounts required are likely to be very small, certainly much less than requirements for (n-3) polyunsaturated fatty acids and probably more akin to the requirements for the vitamin inositol. Studies are in progress in this laboratory to define the role of PI in the physiology of marine salt-secreting epithelia.

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Phospholipid Synthesizing Enzymes of Dermatophytes: II. Characterization of Choline Kinase

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ABSTRACT

Choline kinase was located in the cytosolic fractions of the filamentous, pathogenic fungi, *Microsporum gypseum* and *Epidermophyton floccosum*. A broad pH optima (6.0-9.0) was observed for the *M. gypseum* enzyme, but the *E. floccosum* enzyme was active at pH 8.4 as well as 10.5, the activity being higher at pH 8.4. Enzyme from both dermatophytes had Km value of 3.3×10^{-4} M for choline; however, for ATP, it was 6.6×10^{-4} M and 12.6×10^{-4} M for *M gypseum* and *E. floccosum*, respectively. Choline kinase of both dermatophytes showed SH-group requirement. The *M. gypseum* choline kinase was inhibited to a greater extent by Mn^{2+}, Ca^{2+} and Ba^{2+} than was the *E. floccosum* enzyme. In comparison to other nucleotides, ATP was the most effective phosphate donor for phosphorylating choline in both dermatophytes. Higher concentrations of ATP inhibited the enzyme in *M. gypseum* as well as *E. floccosum*. Phosphorylcholine inhibited the choline kinase activity from both fungi, whereas phosphoethanolamine and glycerol 3-phosphate were stimulatory.
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INTRODUCTION

Choline kinase (Mg ATP: choline phosphotransferase, EC 2.7.1.32) was initially described by Wittenberg and Kornberg (1) in yeast. It is the first enzyme of the cytidine pathway for de novo synthesis of *sn*-3-phosphatidylcholine. Several studies have been made on the choline kinase from various animal and plant sources (2-7), but among fungi it was partially characterized only in yeast (1). Phosphatidylcholine (PC) constitutes ca. 45% of the total phospholipids in dermatophytes (8) and its biosynthesis has not been elcuidated in these pathogenic fungi (9). Earlier attempts to study PC biosynthesis in *Microsporum gypseum* (10) suggested the presence of the cytidine pathway in this pathogen, as in other organisms (2-7). In continuation of our work to delineate the pathway of phospholipid synthesis in dermatophytes, choline kinase of *M. gypseum* and *Epidermophyton floccosum* was investigated and the results are reported in this communication.

MATERIALS AND METHODS

Chemicals

Methyl-^{14}C-choline (sp act 50 mCi/mmol) was obtained from New England Nuclear (Boston, MA) and Dowex 1 x 8 Cl^- (100-200 mesh) ion-exchange resin was obtained from Sigma Chemical Co. (St. Louis, MO).

Organisms and Growth Conditions

The source of *M. gypseum* and *E. floccosum* was as given earlier (11). Both dermatophytes were grown in Sabouraud's medium (4% glucose and 1% peptone, pH 5.4-5.6) at 27 C. In the growth medium of *E. floccosum*, glycerol was substituted for glucose, since glucose-grown cells showed no choline kinase activity.

Subcellular Fractionation

Preparation of cell homogenates and their fractionation was done as before (11) by assaying the marker enzymes cytochrome C oxidase and glucose 6-phosphatase for mitochondria and microsomes, respectively.

Enzyme Assay

Choline kinase activity was assayed according to the method of Weinhold and Rethy (6). Incubation mixture of the enzyme assay contained 60 mM Tris-HCl, pH 8.4, 15 mM $MgCl_2$, 10 mM ATP, 10 mM cysteine, 30 nmol of methyl-^{14}C-choline chloride and the enzyme preparation (0.05 mg protein), in a final volume of 0.1 ml. The mixture was incubated at 37 C for 20 min and the reaction was terminated by placing the tubes in a boiling water bath for 2 min. Controls contained no ATP. A chloride form of the Dowex resin was converted to the hydroxyl form by equilibrating with 2 N HCl, followed by 2 N NaOH. A column (0.5 cm x 4 cm) of this resin was made and an aliquot of the above reaction mixture (50 µl) was applied to it. Free choline was removed from the column by washing the column with 5 ml of water three times. Phosphorylcholine, produced during the choline kinase reaction, was adsorbed on to the column and was successively eluted with 0.5 ml 1.0 M NaOH, and 1.5 ml 0.1 M NaOH.

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Eluates from the column were counted in 10 ml Bray's scintillation fluid. Quenching correction was determined by treating a known amount of radioactive choline as above with the alkali. A unit of choline kinase activity was defined as nmol phosphorylcholine fromed/mg protein/20 min.

Product Identification

Products of enzyme reaction were characterized by autoradiography. An aliquot of the assay mixture was applied to a Whatman No. 1 paper strip and ascending chromatography was performed for 16-18 hr with *n*-butanol/ethanol/acetic acid/water (8:2:1:3, v/v/v/v) as the solvent system. The chromatogram was exposed to a Kodak X-ray film for 2 weeks and developed for visualization of radioactive spots. Detection of radioactive phosphocholine in the test sample and its absence in controls confirmed the presence of choline kinase in the dermatophytes.

Protein Estimation

Protein was estimated according to the method of Lowry et al. (12) using bovine serum albumin as standard.

RESULTS

Intracellular localization of choline kinase in the 2 dermatophytes was determined and its activity was detected in soluble fraction of the organisms (Table 1). The *M. gypseum* choline kinase was most active between pH 8.0 and 9.0 (Fig. 1), but the *E. floccosum* enzyme exhibited 2 pH optima of 8.4 and 10.5. However, the activity at 10.5 was less than at 8.4. The choline kinase reaction was linear for 30 min and therefore all assays were carried out for 20 min at pH 8.4.

As expected for kinases, choline kinase of both dermatophytes showed an absolute requirement for Mg^{2+} ions. Activity of the enzymes increased with increasing concentration of Mg^{2+}, reaching a plateau at 10 mM magnesium concentration (Fig. 2). Increasing the Mg^{2+} concentration beyond 10 mM inhibited the *M. gypseum* enzyme but not that of *E. floccosum*. Other metal ions inhibited the dermatophyte enzymes by varying degrees (Table 2).

Effect of different choline concentrations on the choline kinase of dermatophytes is shown in Figure 3a-d. The *M. gypseum* and *E. floccosum* enzymes were saturated with 0.6 (Fig. 3a) and 0.4 (Fig. 3c) mM choline, respectively. Enzymes of both the fungi had a similar Km of 3.3×10^{-4} M for choline; however, for ATP, the Km values were 6.6×10^{-4} M and 12.6×10^{-4} M for *M. gypseum* and *E. floccosum*, respectively. Choline kinase of *M. gypseum* was saturated by 10 mM ATP (Fig. 3b), whereas the *E. floccosum* enzyme was saturated by an ATP concentration of 5 mM (Fig. 3d).

Nucleotides other than ATP were not good substrates for choline kinase of either *M. gypseum* or *E. floccosum* (Table 3). Phosphorylation of choline in the presence of each nucleotide was only ca. 30% compared to that with the ATP control in both fungi. Addition of ADP to the assay system of *M. gypseum* and *E. floccosum* reduced the activity of choline kinase (Table 3).

TABLE 1.

Subcellular Distribution of Choline Kinase in *M. gypseum* and *E. floccosum*

Fractions	Specific activity (units)	
	M. gypseum[a]	*E. floccosum*[b]
Mitochondrial	1.116 ± 0.32 (15.14)	–
Microsomal	1.55 ± 0.13 (46.80)	2.14 ± 0.62 (17.34)
Cytosolic	16.55 ± 0.636 (1532.70)	13.84 ± 3.43 (841.72)

[a]Values are mean ± SD of 4 independent determinations.

[b]Values are mean ± range of duplicate determinations.

One unit of the enzyme is defined as the nmoles of phosphorylcholine formed/mg protein/20 min. Total activity of the enzyme is given in parentheses and one unit of total activity is defined as the nmoles of phosphorylcholine formed/20 min.

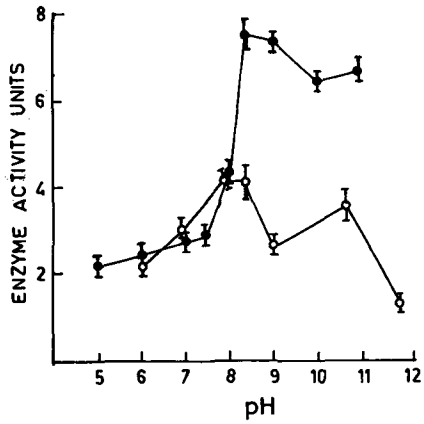

FIG. 1. Effect of pH on choline kinase activity of *M. gypseum* (•—•) and *E. floccosum* (○—○). Buffers of citrate (pH 5.5-6.5), Tris-maleate (pH 6.5-8.0), Tris-HCl (pH 8.0-9.0) and glycine-NaOH (pH 9.5-12.0) were used. Values are average ± range of duplicate determinations.

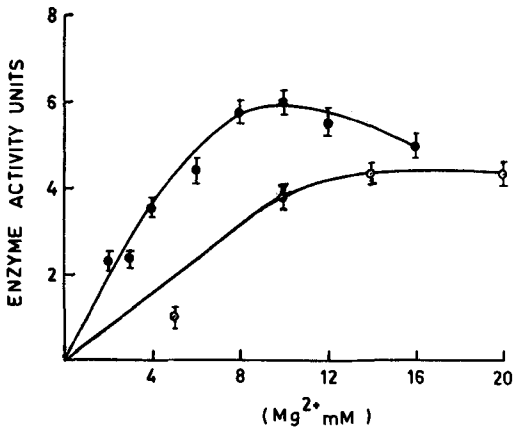

FIG. 2. Effect of Mg^{2+} on choline kinase activity of *M. gypseum* (•—•) and *E. floccosum* (○—○). Values are average ± range of duplicate determinations.

TABLE 2

Inhibition of *M. gypseum* and *E. floccosum* Choline Kinases by Metal Ions

Metal ion concentration (10 mM)	Specific activity (units)	
	M. gypseum	*E. floccosum*
Control[a]	4.4 ± 0.44	4.3 ± 0.90
Ba^{2+}	3.1 ± 0.10	1.2 ± 0.16
Co^{2+}	1.5 ± 0.17	0.8 ± 0.07
Mn^{2+}	0.6 ± 0.16	1.1 ± 0.40
Ca^{2+}	1.9 ± 0.27	1.3 ± 0.50

Values are mean ± SD of 4 independent determinations.

[a]Control contained only Mg^{2+}, whereas for other incubations, in addition to Mg^{2+}, the above-listed metal ions were added. The supernatant was dialyzed for 4 hr before examining the metal ions effect.

Effect of thiol reagents and thiol inhibitors on choline kinase activity of *M. gypseum* and *E. floccosum* was studied (Table 4). Increase in choline kinase activity of both dermatophytes was observed by the addition of either cysteine or β-mercaptoethanol. Activity of *E. floccosum* choline kinase increased ca. 2-fold; however, in *M. gypseum* the increase was only ca. 0.3-fold. Iodoacetate was inhibitory for the enzyme of both dermatophytes, but the inhibition was more for *E. floccosum* choline kinase than for the enzyme of *M. gypseum*.

Enzyme activities observed in the presence of various phospholipid substrates are given in Table 5. Phosphorylcholine reduced the choline kinase activity of both fungi, whereas phosphoethanolamine and glycerol 3-phosphate were stimulatory. Phosphoserine stimulated the *M. gypseum* enzyme, but was inhibitory for choline kinase of *E. floccosum*.

DISCUSSION

Presence of choline kinase in *M. gypseum* was earlier reported from our laboratory (13) and its activity in the fungus was increased when grown with glycerol as the carbon source and when choline or ethanolamine was supplemented into the growth medium. PC content of *M. gypseum* (13) and *E. floccosum* (14) increased when it was grown with glycerol as the carbon source. The present work was undertaken to compare the characteristics of choline kinase from 2 dermatophytes, *M. gypseum* and *E. floccosum*, in which we have earlier investigated phosphatidate phosphohydrolase (11).

Attempts to purify choline kinase from the dermatophytes were unsuccessful since the enzyme was active for only a short period (18 hr) and the activity could not be stabilized even with various stabilizing compounds viz. glycerol, choline, β-mercaptoethanol. etc.

Both the yeast (1) and dermatophyte choline kinases had an alkaline pH optimum. Also, the dermatophyte enzyme like that of yeast (1), higher plants (2) and mammals (15) showed an absolute requirement for Mg^{2+} ion and ATP. In the absence of either, phosphorylation of choline did not take place. Rapeseed (2) and spinach (4) choline kinases exhibited maximal activity when the Mg^{2+}/ATP ratio in the assay system was 1 and an increased Mg^{2+} concentration inhibited the enzyme activity in these systems. Maximal activity of *E. floccosum* was obtained at a Mg^{2+}/ATP ratio of 2; however, for the *M. gypseum* enzyme, this ratio was 1.

Activity of the dermatophyte enzyme was stimulated by cysteine or β-mercaptoethanol, similar to the yeast enzyme (1). The dermatophyte choline kinase activity appears to require endogenous thiol groups since iodoacetate inhibited its activity in *M. gypseum* as well as *E. floccosum*.

Enzymes from both dermatophytes exhibited a similar affinity for choline, but it was different for ATP. With higher concentrations of ATP, a reduced choline kinase activity was observed in these fungi.

Inhibition of enzyme activity by ADP suggests that product inhibition is responsible for reduced choline kinase activity at higher ATP concentrations. Choline kinase of filamentous fungi was inhibited in the presence of divalent cations other than Mg^{2+}, as reported earlier (16). Effect of phospholipid intermediates on choline kinase activity of the dermatophytes was investigated (Table 5). This study

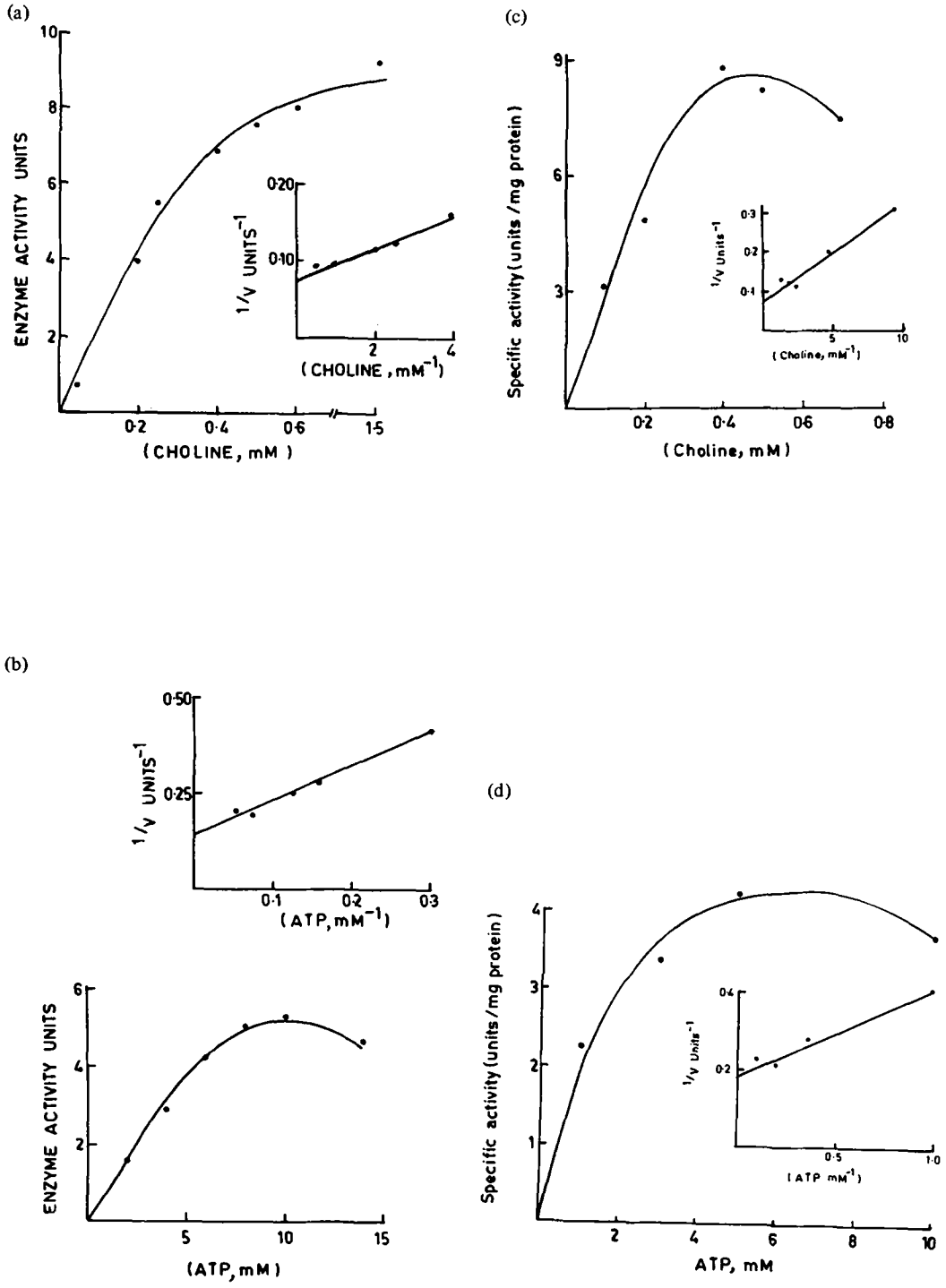

FIG. 3. Effect of choline (a, c) and ATP (b, d) on the choline kinases of *M. gypseum* and *E. floccosum*.

TABLE 3

Effect of Various Nucleotides on Choline Kinase Activity of *M. gypseum* and *E. floccosum*

Nucleotides	Specific activity (units)	
	M. gypseum	*E. floccosum*
ATP (control)	15.90 ± 0.60	3.12 ± 0.30
ADP: (5 mM)	7.95 ± 0.71	2.92 ± 0.30
(10 mM)	3.18 ± 0.12	2.51 ± 0.30
CTP	0.40 ± 0.01	1.46 ± 0.10
UTP	0.47 ± 0.02	1.07 ± 0.03
GTP	1.14 ± 0.04	1.55 ± 0.09
TTP	0.38 ± 0.02	1.08 ± 0.02
ITP	1.62 ± 0.09	1.18 ± 0.20

Values are mean ± SD of 4 independent determinations. Control and ADP assay system contained ATP at 10 mM concentration, whereas assay systems of other nucleotides contained only the nucleotide triphosphates at 10 mM concentration.

TABLE 4

Influence of Thiol Reagents and Sulfhydryl Inhibitors on *M. gypseum* and *E. floccosum* Choline Kinases

Thiol reagents and sulfhydryl inhibitors	Specific activity (units)	
	M. gypseum	*E. floccosum*
Control	4.48 ± 0.44	1.89 ± 0.26
Cysteine	5.90 ± 0.14	3.72 ± 0.37
β-Mercaptoethanol	5.78 ± 0.54	3.56 ± 0.11
Iodoacetate	0.85 ± 0.02	1.64 ± 0.02

Values are mean ± SD of 4 independent determinations. Assay system was the same as given in Materials and Methods. In addition to usual constituents, the sulfhydryl inhibitors were added in a final concentration of 10 mM.

TABLE 5

Effect of Phospholipid Intermediates on Choline Kinases of *M. gypseum* and *E. floccosum*

Phospholipid intermediates	Specific activity (units)	
	M. gypseum	*E. floccosum*
None	1.66 ± 0.17	4.30 ± 0.22
Glycerol-3-phosphate	2.77 ± 0.12	5.18 ± 0.05
Phosphocholine	0.36 ± 0.00	1.94 ± 0.10
Phosphoserine	2.38 ± 0.13	4.15 ± 0.05
Phosphoethanolamine	2.95 ± 0.10	4.57 ± 0.30

Values are mean ± SD of 4 independent determinations. Assay system was the same as given in Materials and Methods, except, in addition to the usual ingredients, the phospholipid intermediates were added in a final concentration of 10 mM.

was initiated to check possible regulation of enzyme activity by these cellular components as observed earlier with phosphatidate phosphatase of these pathogens (11). Change in the dermatophyte enzyme activity, produced by the addition of phospholipid intermediates, suggests that choline kinase is a regulatory enzyme for PC biosynthesis in dermatophytes, also indicated earlier from kinetic studies of this enzyme from rat liver (17).

ACKNOWLEDGMENTS

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METHODS

Quantitative Determination of Tri-, Di-, Monooleins and Free Oleic Acid by the Thin Layer Chromatography–Flame Ionization Detector System Using Internal Standards and Boric Acid Impregnated Chromarod[1]

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ABSTRACT

The separation conditions for hydrolysates of triglycerides by lipase and their quantitative determination are discussed for a thin layer chromatography-flame ionization detector system utilizing internal standards. The complete separation of glyceride hydrolysis mixtures (triolein, 1,3-diolein, 1,2-diolein, 1-monoolein and oleic acid) was achieved on a 3% boric acid-impregnated Chromarod S-II by development with benzene/chloroform/acetic acid (70:30:2, v/v/v) (mobile phase A) or hexane/ether/acetic acid (70:30:1, v/v/v) (mobile phase B). Mobile phase B had an advantage over mobile phase A in terms of free space to add internal standards for simultaneous quantitation and was employed. *p*-Hydroxybenzoic acid and *p*-carboethoxy benzyl alcohol, which appeared between 1,2-diolein and 1-monoolein, were adopted as the internal standards. The calibration curves relating internal standards to each glyceride were all approximated by the equations $Y=aX^b$ giving high correlations. The method was applied to hydrolysis of triolein by pancreatic lipase.
Lipids 18:732-736, 1983.

INTRODUCTION

Human sebum absorbed by worn garments is a complex lipid mixture consisting of 25% free fatty acid, 32% triglycerides, 12% mono- and diglycerides, 13% waxes, 9% hydrocarbons (mainly squalene), 3% cholesterol and a small amount of phospholipids (1).

During removal of such lipids by washing with an alkaline solution of surfactant, the free fatty acids are easily removed from fabrics by formation of water-soluble fatty acid soaps. Triglycerides are not saponified by the alkaline solution and remain on the fabric (2).

Studies on the application of a lipase, glycerol ester hydrolase, for removal of lipids have been undertaken and have proved that lipase promotes the removal of triglycerides (3,4). To clarify the mechanism of removal of triglycerides with the aid of lipase, a complete separation and quantitation of each component of the hydrolysate of triglycerides during washing is essential.

The hydrolysis of triglycerides by lipase has been analyzed by alkaline titration (5), electrical titration (6) or thin layer chromatography (TLC) (7) techniques. The two titration methods are effective only for the determination of free fatty acids derived by hydrolysis by lipase. On the other hand, a precise determination of the hydrolysate composition by TLC is possible but requires additional spectroscopic or gas liquid chromatography (8).

The application of a TLC-flame ionization detector (TLC-FID) method has recently been discussed for the quantitative analysis of neutral lipids and partial glycerides (9-14). Yoshizuka et al. (15) reported that separation and quantitative determination of each of the complex components of lipids are possible by the TLC-FID method by two-stage development using internal standards. This method, however, required some modification in regard to internal standards when we used it in our studies.

In this paper, the analytical conditions and the selection of internal standards for the purpose of separating and simultaneously quantitating the hydrolysis products of triglycerides by lipase with a high level of accuracy using a TLC-FID method are discussed.

EXPERIMENTAL PROCEDURES

Apparatus and Operating Conditions

The TLC-FID analyzer used was an Iatroscan TH-10 with Chromarod S-II rods (Iatron Lab. Inc., Tokyo). Chromatograms were recorded on

[1]Part of this investigation was reported at the annual meeting of JOCS in Tokyo, November 1982.

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Abbreviations: Triolein, TO; diolein, DO; monoolein, MO; oleic acid, OA, *p*-hydroxybenzoic acid, HB; *p*-carboethoxy benzyl alcohol, CEB; polyvinyl alcohol, PVA.

a Hitachi 056 2-pen recorder (Hitachi Ltd., Tokyo) and integrated by Chromatopack R-1B (Shimazu Co., Kyoto).

Operating conditions were: flow rate of hydrogen, 160 ml/min; flow rate of air, 2000 ml/min; scan speed, 40 sec/scan; chart-drive speed, 120 mm/min; voltage of detector, 50-100 mV; and voltage of recorder, 100-200 mV.

Materials

Oleic acid used for analysis (>99%) was obtained from Nippon Oils & Fats Co., Ltd. (Tokyo). 1-Monoolein was synthesized almost quantitatively as described by Daubert et al. (16), and purified (>99%) by several fractional recrystallizations from *n*-hexane. 1,3-Diolein was synthesized by monooleoylification of 1-monoolein synthesized as above and purified (>98%) by column chromatography on silica with benzene. Triolein was purchased from the Tokyo Kasei Kogyo Co., and purified (>99%) by distillation after column chromatography on silica with benzene.

p-Hydroxybenzoic acid (HB) and *p*-carboethoxy benzyl alcohol (CEB) were used as internal standards. Highly purified HB (mp 215.0-215.8 C) was obtained from Ueno Fine Chemical Ind. Ltd. (Osaka). CEB was synthesized by esterification of terephthaldehydic acid followed by reduction with sodium borohydride and distillation (130-132 C, 2 mm Hg).

To prepare the triolein emulsion, 75 ml of 2% PVA solution consisting of 1.85% PVA 117 (saponification value, 98.5% ± 0.5 mole %) and 0.15% PVA 205 (88.0 ± 1.5 mole %), both obtained from Kuraray Co., Ltd. (Osaka), and 25 ml of triolein were twice homogenized by Emulation (Teraoka Co., Osaka) at 5-10 C for 5 min each.

Porcine pancreatic lipase (activity, 2800 u/g) was purchased from Sigma Chemical Co. (St. Louis, MO).

Developing solvents were all of special grade and commercially available (Wako Pure Chemical Ind. Ltd., Osaka), and used without any further purification.

Method

The rods were cleaned, immersed in nitric acid overnight and rinsed with distilled water. They were impregnated with 3% boric acid for 5 min, dried at 110 C for 10 min and activated by scanning on the Iatroscan just prior to use. Diethyl ether solutions containing glycerides (5-20 mg/ml) were spotted at the origin with a glass capillary (ca. 1 μl). The rods were developed until the front was 10 cm from the origin. The rods were then dried at 120 C for 10 min, scanned on the Iatroscan, and cleaned for reuse.

Lipase hydrolysis was performed according to Yamada et al. (17). A mixture of 5 ml triolein emulsion, 3 ml phosphate buffer (0.1 M, pH 7.0) and 1 ml distilled water was incubated at 37 C for 10 min, and hydrolysis started by addition of 1 ml lipase solution (2.5 u). After 1 hr, 20 ml of ether containing internal standards was added to the reaction mixture to stop hydrolysis and extract the products. The extracts were dried over Na_2SO_4 and subjected to analysis as described above.

RESULTS AND DISCUSSION

Developing Solvents

Complete separation of a mixture of triolein, 1,3-diolein, 1,2-diolein, 1-monoolein and oleic acid was achieved by benzene/chloroform/acetic acid (70:30:2, v/v/v) (mobile phase A) or hexane/ether/acetic acid (70:30:1, v/v/v) (mobile phase B) (Fig. 1 (a) and (b)). Since the internal standards should migrate between 1,2-diolein and 1-monoolein in order to determine each component quantitatively, mobile phase B has an advantage over mobile phase A.

Internal Standards

As shown in Figure 2, the retention times of several acids, alcohols and phenols were examined to obtain adequate internal standards. *p*-Hydroxybenzoic acid (HB) and *p*-carboethoxy benzyl alcohol (CEB) migrated between 1,2-diolein and 1-monoolein (Fig. 1 (c) and (d)) without any overlapping.

Quantitative Determination of Glycerides

As seen in Tables 1 and 2, only small deviations caused by differences in rods and spotted volumes were found in the peak area ratio of glycerides to internal standards. Such results were observed at various weight ratios of glycerides to internal standards. Consequently, the method with these internal standards can be used to determine glycerides quantitatively with good accuracy.

It has been reported that the relationship between the peak area ratio and the weight ratios can be approximated as a straight line when the weight ratio is smaller than 1.0 (15). However, when the weight ratio becomes larger, such straight line approximations will become less accurate. In the early stages of lipase hydrolysis of triglycerides, it is necessary to estimate a large amount of triglyceride and a very small amount of products simultaneously, and therefore the straight line approximations

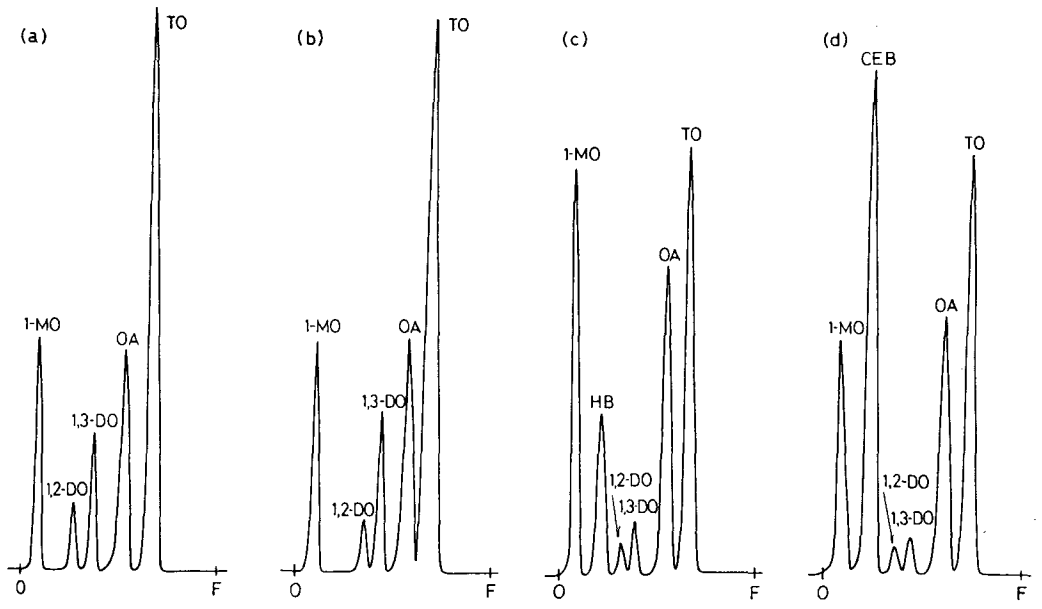

FIG. 1. Chromatograms of glyceride mixture on boric acid-impregnated Chromarod S-II. (a) Developed with benzene/chroloform/acetic acid (70:30:2, v/v/v) (mobile phase A); (b)-(d) developed with hexane/ether/acetic acid (70:30:1, v/v/v) (mobile phase B); (c) and (d) added of internal standards.

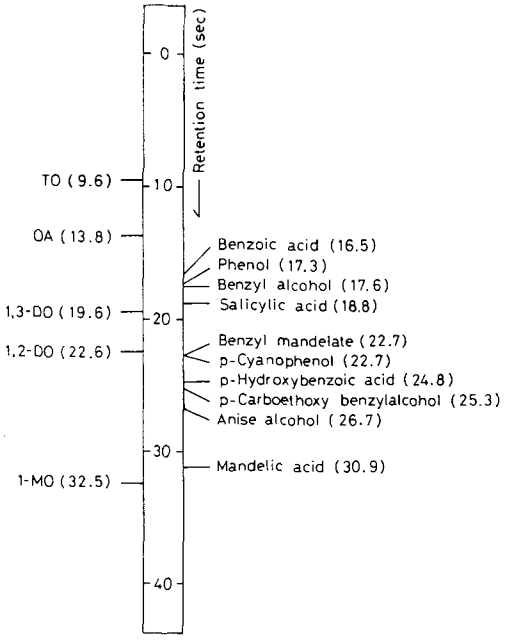

FIG. 2. Retention times of glycerides and several acids, alcohols and phenols on boric acid-impregnated Chromarod S-II developed with hexane/ether/acetic acid (70:30:1, v/v/v) (mobile phase B).

TABLE 1

The Peak Area Ratio of Glycerides to Internal Standard, CEB, on Boric Acid-Impregnated Chromarod S-II

Peak area ratio	Weight ratio 1.00	3.00
Triolein	1.20 ± 0.04	6.13 ± 0.28
1,3-Diolein	1.20 ± 0.05	6.96 ± 0.30
1-Monoolein	1.37 ± 0.04	6.38 ± 0.24
Oleic acid	1.51 ± 0.03	6.02 ± 0.16
Average	1.32 ± 0.13	6.37 ± 0.36

The figures of mean value and standard deviation were obtained from 10 rods.

TABLE 2

Effect of Spotted Volumes on Reproducibility of the Peak Area Ratio of TO to Internal Standard, CEB, at the Weight Ratio of 1.00

Spotted volume (μl)	Peak area ratio
0.5	1.17 ± 0.05
1.0	1.20 ± 0.04
3.0	1.21 ± 0.04

The figures of mean value and standard deviation were obtained from 10 rods.

were insufficient. A wide range of weight ratio of glycerides to internal standards were examined (Fig. 3). As expected, the relationships over a wide range cannot be expressed by straight lines but by curves. The calibration curves for the 4 glycerides are approximated the following equations with very high correlations.

For HB,

$$Y=0.6311\ X^{0.7213}\ (r=0.9600)$$

and for CEB,

$$Y=0.8435\ X^{0.6784}\ (r=0.9921)$$

These equations are quite useful for analysis of glycerides in mixture, but each glyceride would be determined more accurately by the following equations:

For HB to TO, $Y=0.6090\ X^{0.7476}$ (r=0.9983),
to 1,3-DO, $Y=0.5374\ X^{0.7757}$ (r=0.9972),
to MO, $Y=0.6989\ X^{0.6732}$ (r=0.9977),
and to OA, $Y=0.6403\ X^{0.7340}$ (r=0.9987),
and for CEB to TO, $Y=0.9163\ X^{0.6374}$ (r=0.9889),
to 1,3-DO, $Y=0.8625\ X^{0.6660}$ (r=0.9997),
to MO, $Y=0.7573\ X^{0.7175}$ (r=0.9989),
and to OA, $Y=0.8214\ X^{0.7130}$ (r=0.9930).

Application of the TLC-FID System Using Internal Standards to Analyze the Hydrolysates of Triolein by Lipase

As an example of the application of the method, the hydrolysis products of triolein by pancreatic lipase were analyzed. The reaction by lipase was performed as mentioned in the experimental section, and the products were directly spotted on the rods. It should be noted that the usual spotting with accurate volumes of sample is not necessary in this method. The amount of residual triolein and reaction products and the degree of hydrolysis are listed in Table 3. According to the Michaelis-Menten law, based on the relationship between the concentration of substrate and reaction rate, maximum velocity and Michaelis constant were calculated to be 280 μM/1. min and 4.6%, respectively.

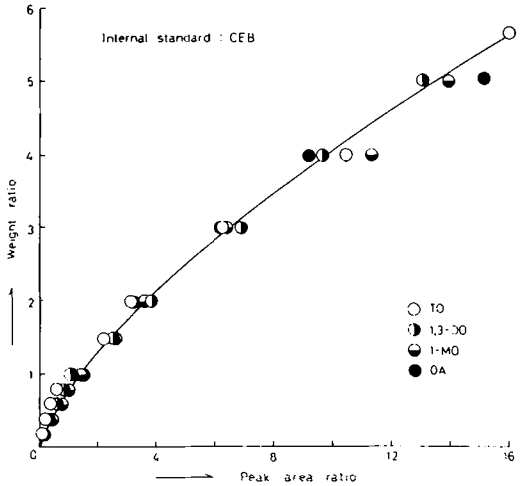

FIG. 3. The relationship between the peak area ratio and the weight ratio of four glycerides to internal standard, CEB, on boric acid-impregnated Chromarod S-II.

In summary, a convenient method for the simultaneous determination of partial glycerides with good accuracy has been established. This method should be very useful in studying the composition of glycerides, and in studying the kinetics of lipase hydrolysis on triglycerides.

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TABLE 3

Analytical Results of Lipase Hydrolysates of TO by the TLC-FID System Using Interal Standards

Conc of substrate (%)	Amounts in reaction mixture (X 10^{-6} M) TO	1,3-DO	1,2-DO	MO[a]	OA	Hydrolysis[b] (%)
100	1187.9	43.5	50.8	17.6	96.8	2.4
50	566.6	20.2	41.9	16.0	91.9	4.8
10	75.2	15.7	27.4	13.9	56.6	14.6
4	24.3	7.6	13.6	6.9	30.7	20.0
2	7.5	5.9	10.2	4.6	22.2	29.0

[a]Under our developing conditions, 1-MO and 2-MO could not be separated and so the representation "MO" is employed.
[b]Calculated by the formula (18):

$$\text{Hydrolysis (\%)} = \frac{\text{Amount of oleic acid produced by lipase}}{\text{Total amount of constitutive oleic acid of substrate}} \times 100$$

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COMMUNICATIONS

The Effect of Dietary α-Linolenic Acid in the Rat on Fatty Acid Profiles of Immunocompetent Cell Populations

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ABSTRACT

Analysis of diet-induced fatty acid changes in the major phospholipids of various immune cell populations has not been previously documented, particularly modifications induced by dietary α-linolenic acid. Rats were fed purified diets containing either 10% corn oil (CO), 10% linseed oil (LO) or 10% soybean oil-linseed mixture (SL) for 8 weeks. The α-linolenic to linoleic acid ratios of the diets were 1:32, 1:1 and 3:1, respectively. Fatty acid analysis of cell populations isolated from the spleen, thymus, thoracic cavity and peripheral blood phospholipids showed increases in ω3 fatty acids accompanied by decreases in the ω6 fatty acids when diets high in α-linolenic to linoleic acid ratios were fed. The extent of change observed was dependent on the magnitude of the α-linolenic to linoleic acid ratio. Both magnitude of change and the specific fatty acids altered varied with the cell population examined.
Lipids 18:737-742, 1983.

INTRODUCTION

The essentiality of α-linolenic acid is currently being reevaluated. In the case of α-linolenic acid, it is believed that one of its essential functions lies in its ability to alter the metabolism of other fatty acids, notably linoleic acid, via a preference of the Δ6 desaturase for the ω3 series fatty acid. Such a competitive inhibition can lead to alterations in the capacity of tissues for synthesis of prostaglandins (PG) and lipoxygenase products. Recently, we have shown with the rat that, the higher the level of α-linolenic relative to linoleic acid, the more PG synthesizing capacity is reduced (1). As would be expected, the higher linolenic acid diets lead to lower levels of ω6 fatty acids, notably arachidonic (20:4ω6), and increased ω3 fatty acids, notably timnodonic (20:5ω3). Among the tissues studied were those of the immune system. We have now selected the immune system as a model for illustrating the modulating effect of α-linolenic acid on PG synthesis. As a prelude to studies on the functional activities of immunocompetent cells, we studied the effects of various dietary levels of linolenic to linoleic acids on their major phospholipid fatty acid profiles.

MATERIALS AND METHODS

Animals and Diets

Male weanling Sprague-Dawley rats (Holtzman Co., Madison, WI) (50-60 g) were maintained on one of three purified diets, each adequate in all nutrients, for 8 weeks. The diets varied in the type of fat fed, i.e., 10% of the diet by weight contained either corn oil (CO), soybean-linseed mixture (SL) or linseed oil (LO). Diet composition was previously reported in full detail by this laboratory (1). All animals were individually housed in polypropylene cages with Sanicel^{R} bedding. A diurnal light cycle of 12 hr was maintained and food and water were available ad libitum. Body weights were recorded once a week throughout the feeding trial. The SL and LO diets were prepared fresh every day and all diets and oils were periodically checked for deterioration by determination of the peroxide number (2).

Cell Isolation

At the end of the feeding trial, the rats were lightly anesthetized with ether and bled by heart puncture. The blood was drawn into 2 ml of 10 mg/ml heparin-phosphate-buffered saline (pH 7.4) solution. The spleen and thymus were quickly excised and individually placed in 10 ml of ice-cold medium RPMI 1640 with L-glutamine (Grand Island Biological Company (GIBCO), Grand Island, NY) and 25 mM 4(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (pH 7.4). The cells were obtained by perfusion of the tissue with RPMI 1640. The cells were pelleted and washed twice by centrifugation at 400 × g for 10 min. Red blood cells were removed from the splenocytes by resuspending the cell pellet in 0.83% ammonium chloride for 10 min at 25 C. After centrifugation at 400 × g for 10 min, both

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the thymocyte and splenocyte pellets were stored under nitrogen at -20 C for lipid analysis. Trypan blue exclusion was used to demonstrate 95-99% cell viability after the isolation procedures.

The heparinized blood was added to 3 times its volume of RPMI 1640. The blood-RPMI mixture was layered over sterile lymphocyte separation medium (LSM) (Litton Bionetics, Inc., Kensington, MD) which has a density of 1.077-1.080 and centrifuged at 400 × g for 35 min. The peripheral lymphocytes at the interface between plasma-RPMI and LSM were removed. The isolated lymphocytes were washed in sterile RPMI 1640 medium, by successive centrifugations at 400 × g for 10 min. Assessment by light microscopy showed the peripheral lymphocytes to be 97-99% pure and >95% viable as determined by Trypan blue exclusion. At this point, the cells were pelleted and stored at -20 C under nitrogen for lipid analysis.

Mast cells were isolated as described by Sullivan et al. (3). Mast cells were harvested and purified using a mast cell medium (MCM) containing 150 mM NaCl, 3.7 mM KCl, 3.0 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 5.6 mM dextrose, 0.1% bovine serum albumin (BSA) (w/v), 0.1% gelatin (w/v) and heparin, 10 units/ml, pH 6.8. Thirty ml of ice-cold MCM was injected into the thoracic cavity of anesthetized rats, the area was gently massaged and the cell suspension was recovered by aspiration with a siliconized pasteur capillary pipette. The suspension was centrifuged at 50 × g for 7 min, the erythrocytes were lysed with 0.83% ammonium chloride and the cell suspensions from 4 rats were pooled. After centrifugation at 50 × g for 7 min, the pooled cell pellet was resuspended in 2 ml of MCM and layered onto 4 ml of a 38% BSA in MCM solution (w/v) in a 50-ml polycarbonate centrifuge tube. The cells settled for 25 min at 25 C and then were centrifuged at 450 × g for 20 min at 25 C. The MCM layer and the cells at the interface were removed by aspiration. The interface was washed twice with 4 ml MCM. Ten vol of MCM were then added to the BSA layer and the cells were collected by centrifugation at 50 × g for 10 min at 25 C. These cells were frozen for lipid analysis. Wright's differential staining and toluidine blue staining (4) showed the final cell pellet to be an essentially pure mast cell population.

Lipid Analysis

Lipid analysis of the splenocyte, thymocyte, peripheral lymphocyte and mast cell populations was carried out as previously described (5). After extracting the cell pellet with chloroform/methanol (2:1, v/v), the lipids were separated by two-dimensional thin layer chromatography (TLC) on Silica Gel H (6). The choline glycerophosphatide (PC) and ethanolamine glycerophosphatide (PE) fractions were collected and their derivatized fatty acids analyzed by gas liquid chromatography (GLC).

Statistical Analysis

Analysis of variance for balanced data (ANOVA), general linear model analysis for unbalanced data (GLM) and Student Newman-Keuls' Test (α=0.05) for variability were performed using Statistical Analysis System (SAS) (Cary, NC) computer programs.

RESULTS

The dietary treatments showed no significant effect on rat body weights which were 421 ± 8 g, 422 ± 14 g and 402 ± 11 g (mean ± SEM) for the CO, SL and LO diets, respectively.

Fatty Acid Analysis

The PC and PE fatty acid composition of cells isolated from the spleen were significantly altered by the higher ratio of α-linolenic to linoleic acid. In the PE fraction (Table 1), 18:3ω3 increased from 1.2 ± 0.3% in the CO group to 3.5 ± 0.8% in the LO group. This trend was also seen with 20:5ω3 which increased from nondetectable levels to 6.5 ± 0.3% ($p<0.005$). Docosahexaenoic acid (22:6ω3) also significantly increased, but to a lesser extent than the other ω3 derivatives. The ω6 fatty acids were shown to decrease on the SL and LO diets. Arachidonic acid significantly decreased from 29.6 ± 1.4% in the CO diet to 21.8 ± 0.7% ($p<0.005$) in the LO diet and 22:4ω6 decreased from 8.1 ± 0.4% to 1.7 ± 0.3% ($p<0.005$). The same trends were observed in the PC fatty acid (Table 1) response to the higher 18:3ω3/18:2ω6 diets. Archidonic acid and 22:4ω6 both significantly decreased. The ω3 derivatives, 20:5ω3, 22:5ω3 and 22:6ω3 all increased significantly when the CO diet was compared to the LO diet.

Due to insufficient sample number, PC results for the thymocyte population are not reported. The changes in thymocyte PE fatty acids observed were similar to those shown by the splenocyte PE fatty acid profile (Table 2). Arachidonic acid from rats fed the CO diet was 43 ± 2.7% and significantly different ($p<0.0011$) from SL and LO (37.3 ± 0.5%, 26.6 ± 0.9%). Timnodonic acid (20:5ω3) increased from nondetectable levels in the CO group to 7.2 ± 1.2% in the LO group.

TABLE 1

The Effect of Different Dietary Ratios of α-Linolenic Acid to Linoleic Acid on Splenocyte Ethanolamine Phosphoglyceride (PE) and Choline Phosphoglyceride (PC) Fatty Acid Composition

	PE				PC			
Diet[a]	CO (n=4)	SL (n=5)	LO (n=6)	p Value	CO (n=4)	SL (n=5)	LO (n=3)	p Value[c]
Fatty acid								
16:0	13.2 ± 0.9[b]	12.3 ± 1.2	11.1 ± 0.9	NS	37.6 ± 2.8	36.9 ± 1.2	39.1 ± 3.0	NS
18:0	20.8 ± 0.7	19.2 ± 1.9	16.7 ± 0.8	NS	8.5 ± 1.1	8.2 ± 0.6	7.8 ± 0.3	NS
18:1 ω9	7.3 ± 1.0	7.8 ± 0.2	9.6 ± 0.3	$p < 0.025$	9.7 ± 1.3	10.8 ± 0.8	13.5 ± 1.0	NS
18:2 ω6	10.4 ± 1.7	9.6 ± 0.6	11.6 ± 0.8	NS	17.0 ± 1.0	18.0 ± 1.8	16.3 ± 1.6	NS
18:3 ω3	1.2 ± 0.3	2.3 ± 0.6	3.5 ± 0.8	$p < 0.005$	0.5 ± 0.5	2.0 ± 0.5	4.7 ± 0.6	$p < 0.005$
20:2 ω6	2.2 ± 0.2	1.9 ± 0.6	1.4 ± 0.1	NS	4.7 ± 0.3	2.7 ± 0.1	1.8 ± 0.4	$p < 0.005$
20:3 ω6	2.5 ± 0.5	2.0 ± 0.3	4.0 ± 0.8	NS	1.7 ± 0.2	1.6 ± 0.3	2.3 ± 1.3	NS
20:4 ω6	29.6 ± 1.4	28.9 ± 2.2	21.8 ± 0.7	$p < 0.005$	15.7 ± 1.6	11.1 ± 1.5	9.3 ± 0.9	$p < 0.005$
20:5 ω3	ND[d]	3.0 ± 0.3	6.5 ± 0.3	$p < 0.005$	ND	1.6 ± 1.1	3.1 ± 1.0	$p < 0.1$
22:4 ω6	8.1 ± 0.4	2.7 ± 0.2	1.7 ± 0.3	$p < 0.005$	1.5 ± 0.3	1.8 ± 0.3	0.2 ± 0.2	$p < 0.02$
22:5 ω3	0.9 ± 0.2	7.0 ± 0.7	8.2 ± 0.3	$p < 0.005$	0.4 ± 0.2	1.7 ± 0.1	1.1 ± 0.1	$p < 0.005$
22:6 ω3	1.3 ± 0.2	2.5 ± 0.3	2.3 ± 0.3	$p < 0.05$	0.4 ± 0.1	1.2 ± 0.3	0.5 ± 0.1	$p < 0.025$
ω6/ω3[e]	15.4	3.1	1.9		32.0	5.4	3.2	

[a]CO=10% corn oil, SL=10% soybean-linseed oil mixture, LO=10% linseed oil.
[b]Mean ± standard error of the mean (SEM).
[c]p Values are a result of ANOVA, NS=not significant.
[d]ND=not detectable.
[e]ω6/ω3=total ω6 fatty acids/total ω3 fatty acids.

TABLE 2

Effect of Different Dietary Ratios of α-Linolenic Acid to Linoleic Acid on Thymocyte Ethanolamine Phosphoglyceride (PE) Fatty Acid Composition

Diet[a]	CO (n=3)	SL (n=3)	LO (n=3)	p Value[c]
Fatty acid				
16:0	8.6 ± 1.1[b]	8.4 ± 0.7	10.6 ± 1.0	NS
18:0	18.9 ± 2.8	19.8 ± 0.9	17.8 ± 0.6	NS
18:1 ω9	12.1 ± 0.8	18.5 ± 0.3	16.4 ± 0.7	0.008
18:2 ω6	7.2 ± 0.8	9.0 ± 0.9	10.6 ± 1.4	NS
18:3 ω3	ND[d]	0.9 ± 0.5	0.8 ± 0.1	NS
20:4 ω6	43.4 ± 2.7	37.3 ± 0.5	26.6 ± 0.9	0.001
20:5 ω3	ND	2.1 ± 0.1	7.2 ± 1.2	0.001
22:4 ω6	3.9 ± 0.4	2.2 ± 0.1	1.7 ± 2.4	0.009
22:5 ω3	0.4 ± 0.3	1.5 ± 0.2	2.3 ± 2.9	0.013
22:6 ω3	ND	0.4 ± 0.1	0.5 ± 0.0	0.001
Others	2.7	2.0	2.0	
ω6/ω3[e]	134.3	10.3	3.8	

[a]CO=10% corn oil, SL=10% soybean-linseed oil mixture, LO=10% linseed oil.
[b]Mean ± standard error of the mean (SEM).
[c]p Values are a result of ANOVA, NS=not significant.
[d]ND=not detectable.
[e]ω6/ω3=total ω6 fatty acids/total ω3 fatty acids.

TABLE 3

The Effect of Different Dietary Ratios of α-Linolenic Acid to Linoleic Acid on Rat Mast Cell Ethanolamine Phosphoglyceride (PE) and Choline Phosphoglyceride (PC) Fatty Acid Composition

	PE			PC		
Diet[a]	CO (n=5)	LO (n=5)	p Value[c]	CO (n=5)	LO (n=5)	p Value
Fatty acid			% of total			
16:0	9.9 ± 0.9[b]	10.7 ± 0.6	NS	23.1 ± 2.6	15.8 ± 1.5	NS
16:1 ω7	1.7 ± 0.3	1.7 ± 0.4	NS	5.6 ± 0.7	6.8 ± 0.6	NS
18:0	14.0 ± 1.6	14.1 ± 1.6	NS	9.6 ± 0.8	12.0 ± 0.9	NS
18:1 ω9	10.6 ± 0.5	13.3 ± 0.8	NS	11.5 ± 0.4	15.5 ± 0.2	0.001
18:2 ω6	12.2 ± 1.2	11.6 ± 1.4	NS	16.4 ± 0.5	17.4 ± 1.1	NS
18:3 ω3	ND[d]	2.3 ± 0.4	0.012	ND	4.0 ± 0.3	0.001
20:4 ω6	31.8 ± 2.5	20.2 ± 0.9	0.016	22.7 ± 2.1	12.1 ± 0.7	0.009
20:5 ω3	0.3 ± 0.2	9.5 ± 1.1	0.002	0.5 ± 0.3	5.6 ± 0.6	0.003
22:4 ω6	10.2 ± 0.7	2.3 ± 0.4	0.000	3.8 ± 0.3	1.5 ± 0.6	0.018
22:5 ω6	2.5 ± 0.2	0.4 ± 0.3	0.021	0.8 ± 0.1	0.2 ± 0.1	0.002
22:5 ω3	0.6 ± 0.2	9.0 ± 0.8	0.004	0.3 ± 0.0	4.5 ± 0.5	0.001
22:6 ω3	1.3 ± 0.3	3.0 ± 0.4	0.087	0.5 ± 0.1	1.1 ± 0.2	0.011
Others	2.4	3.1		4.7	3.0	
ω6/ω3[e]	45.2	1.6		33.6	2.1	

[a]CO=10% corn oil, LO=10% linseed oil.
[b]Mean ± standard error of the mean (SEM).
[c]p Values are a result of ANOVA, NS=not significant.
[d]ND=not detectable.
[e]ω6/ω3=total ω6 fatty acids/total ω3 fatty acids.

The same changes are noted in rat thoracic cavity mast cell PE and PC fatty acid profiles (Table 3). The most notable changes were shown in the PE fraction where 20:4ω6 decreased from 31.8 ± 2.5% in the CO group to 20.2 ± 0.9% in the LO group and 22:4ω6 was reduced to 2.3 ± 0.4% in the LO group from 10.2 ± 0.7% in the CO group. The ω3 fatty acids reciprocally increased, as shown by the large increases in 20:5ω3, 22:5ω3 and 22:6ω3 by mast cells from rats fed the LO diet. The fatty acid changes in mast cell PC were similar to those found in the PE fraction.

The peripheral lymphocytes PE and PC changes again reflect the major changes reported in the other cell populations (Table 4). Arachidonic acid, in the PE fraction of cells from rats fed the high α-linolenic to linoleic

TABLE 4

The Effect of Different Dietary Ratios of α-Linolenic Acid to Linoleic Acid on Peripheral Lymphocyte Ethanolamine Phosphoglyceride (PE) and Choline Phosphoglyceride (PC) Fatty Acid Composition

Diet[a]	PE				PC			
	CO (n=3)	SL (n=3)	LO (n=3)	p Value[c]	CO (n=4)	SL (n=2)	LO (n=4)	p Value
Fatty acid								
16:0	11.5 ± 0.9[b]	15.5 ± 3.0	14.6 ± 0.9	NS	56.7 ± 4.3	58.4 ± 0.2	58.7 ± 4.9	NS
18:0	16.9 ± 2.1	20.4 ± 2.5	16.9 ± 1.4	NS	10.0 ± 1.6	7.7 ± 0.6	9.2 ± 0.8	NS
18:1 ω9	5.5 ± 1.0	6.5 ± 0.5	8.0 ± 0.6	NS	7.7 ± 1.1	6.9 ± 0.2	7.3 ± 0.8	NS
18:2 ω6	6.6 ± 0.7	9.7 ± 0.8	11.6 ± 2.0	NS	10.6 ± 1.1	12.3 ± 0.4	8.8 ± 2.6	NS
18:3 ω3	2.9 ± 1.2	2.6 ± 1.6	2.5 ± 0.5	NS	1.4 ± 0.4	1.8 ± 0.1	2.9 ± 0.4	NS
20:2 ω6	4.4 ± 1.8	2.3 ± 1.3	2.1 ± 1.0	NS	2.3 ± 0.6	1.7 ± 0.2	1.5 ± 0.3	NS
20:3 ω6	2.3 ± 0.1	2.1 ± 0.3	2.1 ± 0.1	NS	0.7 ± 0.2	1.0 ± 0.3	0.9 ± 0.1	NS
20:4 ω6	33.2 ± 1.9	26.9 ± 3.7	21.4 ± 2.2	$p < 0.05$	12.4 ± 1.7	5.9 ± 0.05	4.5 ± 0.9	$p < 0.01$
20:5 ω3	ND[d]	2.9 ± 0.3	8.7 ± 0.8	$p < 0.005$	0.3 ± 0.3	1.6 ± 0.6	2.4 ± 0.6	$p < 0.005$
22:4 ω6	14.2 ± 1.4	5.8 ± 0.8	2.3 ± 0.5	$p < 0.005$	2.3 ± 0.3	1.3 ± 0.7	1.1 ± 0.6	NS
22:5 ω3	ND	4.1 ± 0.8	4.7 ± 0.9	$p < 0.005$	0.3 ± 0.3	0.8 ± 0.2	1.0 ± 0.2	$p < 0.005$
22:6 ω3	ND	0.6 ± 0.4	0.3 ± 0.2	NS	0.4 ± 0.2	0.8 ± 0.8	0.4 ± 0.4	NS
ω6/ω3	20.8	4.6	2.4		11.7	4.4	2.5	

[a]CO=10% corn oil, SL=10% soybean-linseed oil mixture, LO=10% linseed oil.
[b]Mean ± standard error of the mean (SEM).
[c]p Values are a result of ANOVA, NS=not significant.
[d]ND=not detectable.
[e]ω6/ω3=total ω6 fatty acids/total ω3 fatty acids.

acid ratio, was significantly ($p<0.005$) reduced to 2/3 the value observed in the CO group. The 20:5ω3 and 22:5ω3 increased from non-detectable levels in the CO group to 8.7 ± 0.8% and 4.7 ± 0.9% in the LO group. The PC fraction of the peripheral lymphocytes was characterized by a high 16:0 value equally represented in all three diet treatments. The fatty acid changes observed in this fraction due to high α-linolenic to linoleic acid intake were similar to those found in PE but lower in magnitude.

The ω6/ω3 ratio of all cell types, in both PE and PC fractions, were severely reduced by increasing the α-linolenic to linoleic acid rato.

DISCUSSION

Only a few studies have been conducted examining the effect of dietary fats on immunocompetent cell function (7,8). Functional changes have been observed, but they are rarely accompanied by reports of cellular fatty acid compositional changes. Tsang and coworkers (9) reported changes in total fatty acids of splenocytes from rats fed diets varying in linoleic acid content for up to 28 weeks. The accumulation of ω9 fatty acids such as 18:1ω9 and 20:3ω9 was observed in splenocytes of rats fed a diet devoid of linoleic acid. Similar results were observed in this laboratory (10) when isolated peripheral blood lymphocytes PE and PC fatty acid profiles were examined from rats fed a 5% hydrogenated coconut oil (HCO) diet for ca. 2 months.

Here we report the fatty acid changes induced by feeding fats containing high α-linolenic to linoleic acid ratios. After only a 2-month feeding period, the peripheral blood lymphocyte, thymocyte, splenocyte and thoracic cavity mast cell populations all showed altered fatty acid compositions in response to dietary lipid manipulation. Essentially the ω3 class of fatty acids reciprocally replaced the ω6 class of fatty acids in both PE and PC lipid fractions when higher ratios of 18:3ω3 to 18:2ω6 were fed. The magnitude of fatty acid change increased as the 18:3ω3/18:2ω6 ratio increased. These results are in agreement with our earlier findings on whole tissue analysis of animals fed similar dietary treatments (3).

The various immune cell populations also exhibited individual characteristics. The splenocytes and mast cells from rats fed the LO diet accumulated greater percentages of 22:5ω3, whereas peripheral lymphocytes and thymocytes incorporated 20:5ω3 as the major ω3 fatty acid. Rat thymocyte 20:4ω6 concentration in the CO group was ca. 25% higher than in the other immune cell populations. The differences most likely reflect the fatty acid profile of the dominant cell species making up the respective immune cell population.

Differences in the relative contribution of these fatty acids to the component phospholipids may lead to variations in the respective metabolism of these cell populations. This would be relevant in studies manipulating immune cell prostaglandin (PG) synthesis by increasing the relative concentrations of membrane fatty acids which compete with 20:4ω6 for the PG synthetase. Studies examining the influence of these fatty acid changes on immune activities in which PG play a reglatory role are presently being conducted.

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Australian Fish — An Excellent Source of Both Arachidonic Acid and ω-3 Polyunsaturated Fatty Acids

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ABSTRACT

The fatty acid methyl esters obtained by the esterification of total lipids extracted from 24 species of fin fish and 4 species of invertebrates caught in the rivers and coastal waters of southern Australia were analyzed by gas chromatography. The lipids of most species contained significant levels of arachidonic acid (0.7-15.8%) as well as the more common marine polyunsaturate, eicosapentaenoic acid (0.7-15.9%). The major ω6 fatty acid present in most species was 20:4; however, other fatty acids of this series, including 18:2, 22:4 and 22:5, were present. The level of total ω6 fatty acids ranged from 3.9 to 22.3% of the total lipid. In general, the level of total ω3 polyunsaturates was higher than the total ω6 fatty acids with levels of ω3 fatty acids ranging from 9.6 to 48.2%. Only 2 fish (barramundi and gurnard perch) had ω6/ω3 ratios greater than 1.0. Most of the Australian species examined contained low levels of fat (0.5-7.8% of fresh weight). Two species examined, callop (freshwater) and blue groper (marine) contained sufficient quantities of both fat (7.7 and 7.8%) and arachidonic acid (4.8 and 9.3%) to warrant consideration for commercial exploitation.

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INTRODUCTION

The resurgence of interest in the nutritional properties of fish has been based chiefly on the fact that fish oils are known to be a rich source of ω3 fatty acids (1-3). Recent research has indicated that these fatty acids, particularly eicosapentaenoic acid (20:5ω3), either interfere with the production of the thrombotic prostaglandin, thromboxane (4-6), or alternatively are readily converted to antithrombotic prostaglandins in vitro (7,8). In vivo support of this is provided by a number of studies which have shown increased bleeding times and changes in platelet function in subjects fed either fish (9-12) or fish-based products.

So entrenched is the idea that fish oils are rich in ω3 fatty acids that the two expressions often are used interchangeably, a fact which has caused some concern (13). In part, this is a consequence of the fact that northern hemisphere species such as cod, menhaden, pilchard and herring (3,6) and anchovies from the corresponding southern latitudes, all rich in ω3 fatty acids, constitute the world's major supply of fish oil and hence research into these oils is well supported. Reports of ω6 fatty acids in fish are available (6,14) but, in general, the levels listed are low. The marine and freshwater turtles provide almost the only known example of 20:4ω6 and 22:4ω6 being important in depot fats. The phospholipid fatty acids of seals and whales also are reported to be rich in 20:4ω6 (6).

In this brief survey of table fish commonly eaten in an Australian city, we have obtained evidence that most of the fish sampled contained significant levels of ω6 fatty acids and a few were a rich source of arachidonic acid.

MATERIALS AND METHODS

All fish were obtained at local shops and markets in the Adelaide area and identified by an officer of the State Department of Fisheries. Duplicate samples were taken from fresh fish fillets by removing a piece of ca. 5 g, consisting of flesh and skin, from the center of the fillet. The tissue was cut into small pieces and immediately homogenized in 10 ml of chloroform/methanol (1:2, v/v) according to the method of Bligh and Dyer (15) which was specifically developed for the extraction of lipids from fish. The resulting lipid fraction was weighed and a fraction removed for analysis.

Lipid samples were converted to their constituent fatty acid methyl esters by heating the lipids in a solution of 1% H_2SO_4 in methanol in a sealed tube at 70 C for 3 hr under nitrogen. The esters were extracted with petroleum spirit (bp 40-60 C). Routine analysis of methyl esters was performed by gas liquid chromatography (GLC) on 1.5 m columns (2 mm id) packed with 5% SP2310 on Chromosorb W AW (Supelco, Inc., Bellefonte, PA). The chromatographic conditions were as follows; injection port temperature 200 C, flame ionization detector temperature 300 C, initial oven temperature 125 C, rising to 225 C at 4C/min and holding for 20 min. The carrier gas used was

nitrogen, set at a flow rate of 16-20 ml/min. To confirm the identity of peaks and to separate 22:1 from 20:5ω3, samples were routinely injected onto 1.5 m × 2 mm id columns packed with 15% OV275 on Chromosorb P AW-DMCS (Supelco, Inc.) under chromatographic conditions similar to those just described, except that the column temperature was maintained at 200 C initially, then increased at 2 C/min to 220 C, where it was maintained for 30 min.

One sample of each fish was also analyzed by capillary gas chromatography using a 50 m wall-coated open-tubular glass column, 0.5 mm id., coated with OV275. Nitrogen was used as the carrier gas at a velocity of 20 cm per sec. The column temperature was maintained at 160 C for 4 min initially, rising to 220 C at 2 C/min, where it was maintained for 40 min.

Additional separation of fatty acid methyl esters was achieved by thin layer chromatography (TLC) on plates coated with Silica Gel H containing 5% $AgNO_3$ according to methods described in detail elsewhere (16). Bands containing saturates, monoenes, dienes, trienes and polyenes were scraped from the TLC plates into vials containing 0.5 ml H_2O and 2 ml diethyl ether. After vigorous shaking, the ether layer was removed for direct injection into the GLC. Identification of compounds was based on the retention times of standards obtained from Nu-Chek Prep, Elysian, MN.

All organic solvents used in this study were redistilled reagent grade and contained the antioxidant butylated hydroxyanisole at a concentration of 5 mg/l.

RESULTS

Identification of FAME

Normal separation of fatty acid methyl esters (FAME) obtained from fish lipid samples is illustrated in Figure 1. The SP2310 liquid phase, which proved ideal for FAME derived from human milk (16) and membrane lipids (17), gave excellent separation of fish FAME, with the important exception that 20:5ω3 cochromatographed with 22:1 (Fig. 1a). This problem was overcome by chromatographing the samples on the more polar phase, OV275, which clearly separated these 2 FAME (Fig. 1b). Chromatography on OV275 also gave confirmatory retention times for the major ω6 fatty acids, 18:2, 20:4, 22:4 and 22:5.

To obtain further evidence for the identity of the major polyunstaurated fatty acids, methyl esters were subjected to analysis by argentation TLC and the results are illustrated in Figure 2. This method confirmed the presence of saturates and also monoenes which

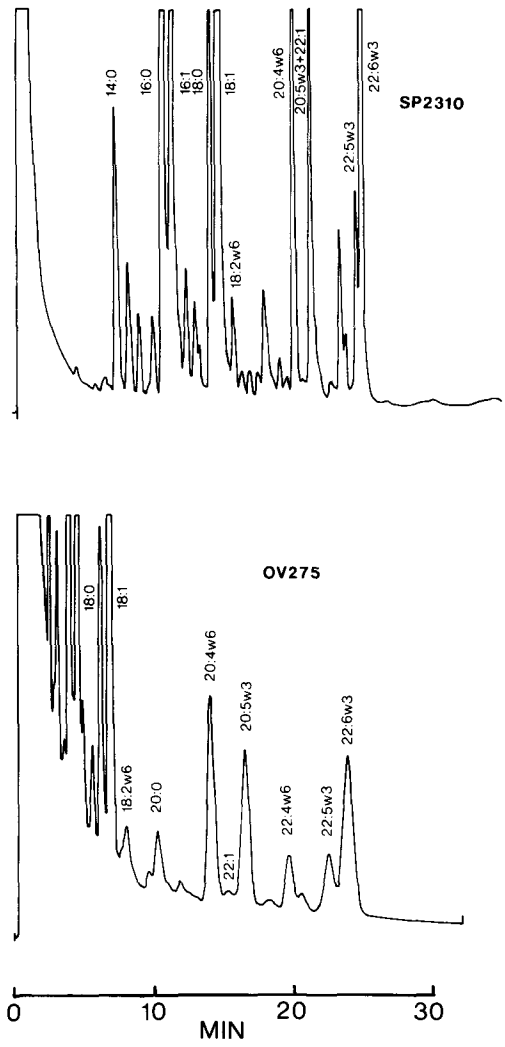

FIG. 1. Separation of fatty acid methyl esters derived from King George whiting lipids on columns packed with either 5% SP2310 on Chromosorb W AW (upper) or 15% OV275 on Chromosorb P AW-DMCS (lower). Note the clear separation of 22:1 and 20:5ω3 on the OV275 column.

were chiefly composed of 16:1 and 18:1 with only trace amounts of 22:1. The method also confirmed that the peaks earlier identified as 20:4ω6, 20:5ω3, 22:4ω6, 22:5ω3 and 22:6ω3 all appeared in the polyene fraction where they were expected. The peak tentatively identified as methyl arachidonate was found in the tetraene band and not in the triene band where 20:3ω3, which cochromatographs with 20:4ω6 on some packed columns, was found as a trace component.

Additional proof that the arachidonate peak had been correctly identified was supplied

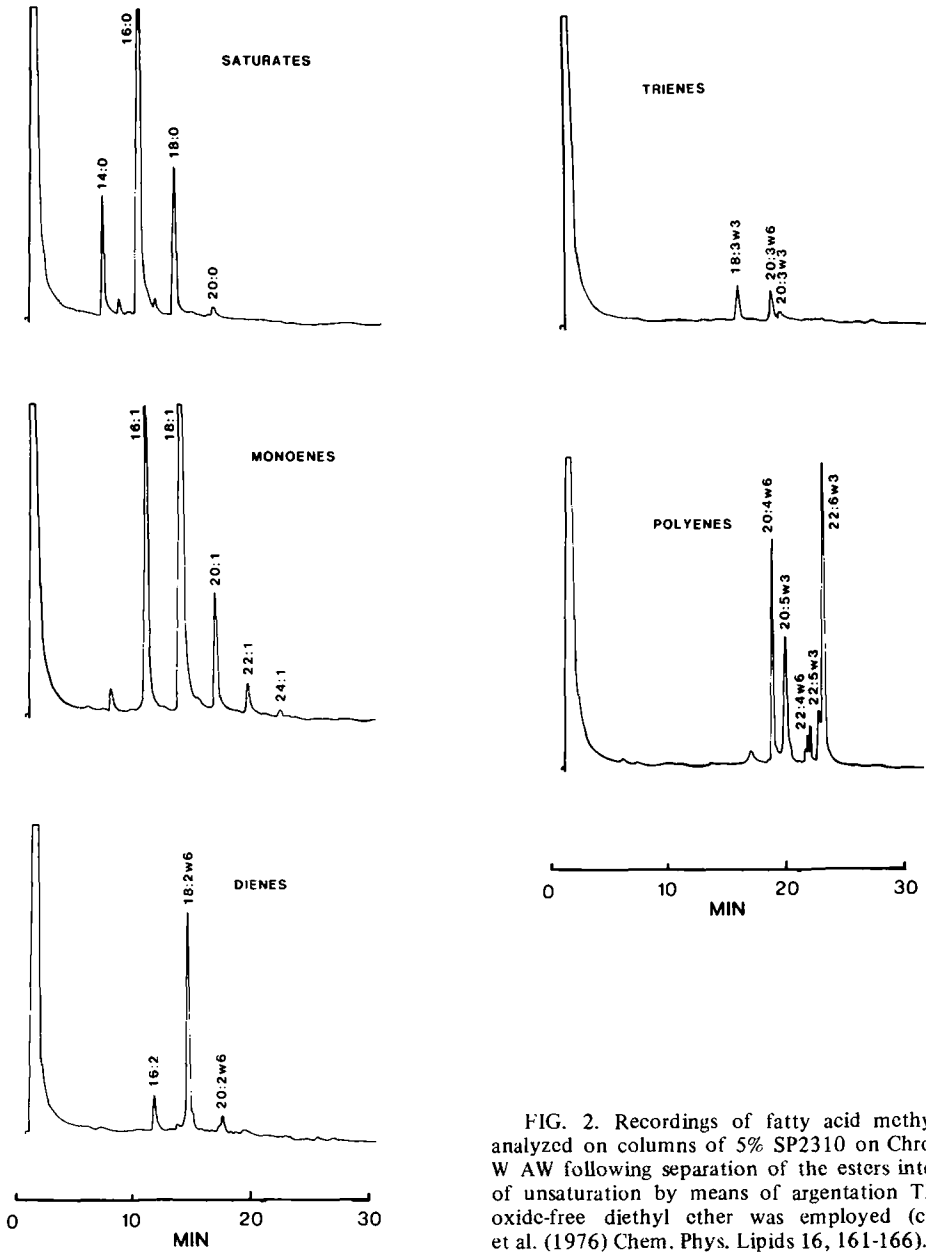

FIG. 2. Recordings of fatty acid methyl esters analyzed on columns of 5% SP2310 on Chromosorb W AW following separation of the esters into classes of unsaturation by means of argentation TLC. Peroxide-free diethyl ether was employed (cf. Chen et al. (1976) Chem. Phys. Lipids 16, 161-166).

from capillary GLC. Standards injected on a 50 m column coated with OV 275 demonstrated that good separation of 20:3ω3 from 20:4ω6 (Fig. 3a) was possible in this system. Fish lipids analyzed by this method (Fig. 3b) showed a major peak corresponding in retention time 20 20:4ω6 and only trace levels of 20:3ω3.

Fatty Acid Composition of Fish Lipid

Most of the fin fish examined contained low levels of fat and only 7 of the 29 species contained more than 2% fat (Table 1). The fat contents of the freshwater fish known locally as callop (8%), and of 2 local marine varieties, blue groper (8%) and jumper mullet (5%),

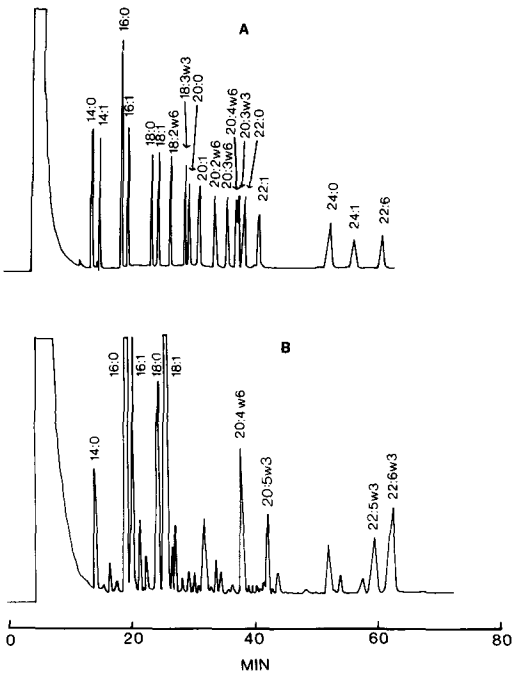

FIG. 3. Separation of fatty acid methyl esters by capillary GLC. A column 50 M in length and coated with OV275 was used to separate either a mixture of standards to demonstrate the separation of 20:3ω3 from 20:4ω6 (A) or a mixture of esters obtained from fish lipids (B).

were relatively high. The species consumed in large amounts by the community in "fish and chips" include several species high in fat such as mulloway (4.4%), imported South African hake (6%), turbot (4.5%), blue groper and callop. In contrast, the most expensive local fish which is much prized for its delicate flavor, King George whiting, is very low in fat (1%).

The saturated fatty acids in the lipids of Australian fish were found to be qualitatively similar regardless of species, with palmitic acid averaging ca. 20% of the total fatty acids followed by lesser amounts of stearic and myristic acids (Table 2). Most species contained ca. 30% of their total fatty acids as saturates, but jumper mullet (50%) and gurnard perch (4%) were important exceptions.

The monoenoic fatty acid arrays were also similar in Australian fish fats (Table 2). Generally, 18:1 was present in the greatest concentration followed by 16:1; jumper mullet was an exception to this rule, however, as the order was reversed. Monoenes of the C22 group were present only in very low concentrations (<2%) in all the species tested, with the exception of gurnard perch (8%) and South African hake (6%). There was a large variation in the proportion of monoenes in the fish samples assayed, with values ranging from 12 to 68% of the total fatty acids.

A majority of the fish caught in local waters contained significant levels of ω6 fatty acids (Table 2). Arachidonic acid was the major ω6 fatty acid in most species (1-11%); its precursor, linoleic acid, was also present in detectable quantities (1-6%), as were the products of elongation and desaturation of arachidonic acid, namely, 22:4ω6 and 22:5ω6.

The ω3 fatty acids (18:3, 20:5, 22:5, 22:6) were present in high concentrations in most of the species examined (Table 2). The ω3 fatty acid present in highest concentration generally was 22:6, but there were important exceptions where 20:5 exceeded 22:6, most notably in yellowfin whiting, leather jacket, yelloweye mullet, jumper mullet and turbot. The other long-chain member of the ω3 series, 22:5, was present in concentrations ranging from 0.5 to 4.5%, whereas the precursor of all these compounds, linolenic acid, was generally present in amounts ranging from 0.1 to 5.7%. Gurnard perch was an exception where 18:3ω3 (5.7%) was the major ω3 fatty acid.

Despite high concentrations of ω6 fatty acids present in many of the fish assayed, only 3 species contained levels of total ω6 acids equal or greater than the amount of ω3 acids. Gurnard perch and barramundi both had ω6/ω3 ratios higher than 1.0, but in most of the fish caught locally the ratios ranged from 0.1 to 0.9. Callop and blue groper, 2 species shown to be rich in oil, also had ω6/ω3 ratios in excess of 0.3.

DISCUSSION

The present study emphasizes that lipids of a large number of fish caught in Australian coastal waters are rich in ω6 fatty acids, unlike lipids of most marine fish of the northern hemisphere. Concentrations of ω6 acids ranging from 4 to 22% of the total fatty acids were detected in Australian fish (Table 2) and, although the major constituent was arachidonic acid, levels of 18:2ω6 (0.6-5.9%), 22:4ω6 (0.3-4.4%) and 22:5ω6 (0.3-3.1%) were also detected.

Although fatty acids of the ω6 type have been reported in fish in trace levels on numerous occasions (3,6,18), occasional reports have appeared indicating high levels of these acids. For example, ω6/ω3 fatty acid ratios ranging from 0.28 to 0.85 with 20:4ω6 levels as high as 16% of the total lipids have been reported

TABLE 1

Common and Scientific Name, Habitat, Origin and Lipid Content of Fish

Common name		Description	Origin	Scientific name	Fat content
Fin fish	N				
Barramundi	5	Estuarine, marine coastal carnivore	North coast	*Lates calcarifer*	0.8
Callop	5	Freshwater, carnivore	Local	*Macquaria ambigua*	7.7
Cod, English smoked	1	Marine, carnivore	North Atlantic	*Gadus morhua*	0.6
Cod, red	1	Marine, carnivore	New Zealand	*Physiculus bacchus*	0.5
Garfish	3	Marine, coastal, herbivore planktivore	Local	*Hyporhamphus melanochir*	1.0
Gemfish	1	Marine, carnivore	East coast	*Rexen solandri*	–
Grenadier, blue	3	Marine coastal, carnivore	Local east coast	*Macruronus novaezealandiae*	0.5
Groper, blue	2	Marine, carnivore	Local	*Achoerdus gouldi*	7.8
Hake, South African	1	Marine, carnivore	South Africa	*Merluccius carpensis*	5.7
Leather jacket	3	Inshore, marine, herbivore	Local	Family Aluteridae	0.5
Morwong	1	Marine, coastal	East coast, New Zealand	*Nemadactylus macropterus*	1.0
Mullet, jumper	3	Marine, coastal, detritus, feeder	Local	*Liza argentea*	4.9
Mullet, yelloweye	2	Marine, coastal, detritus, feeder	Local	*Aldrichetta fosteri*	1.9
Mulloway	1	Estuarine, marine carnivore	Local	*Argyrosoma holdepidotus*	4.4
Perch, gurnard	2	Marine, carnivore	East coast, Tasmania	Family Scorpaenidae	–
Perch, redfin	1	Freshwater, carnivore (introduced)	Local	*Petca fluriatilis*	–
Ruff, tommy	3	Inshore marine Zooplanktivore, carnivore	Local	*Arripis georgianus*	0.8
Salmon, Australian	1	Coastal marine, carnivore	Local	*Arripis trutta*	1.8
Shark, school	1	Coastal marine, carnivore	Local	*Galeorhinus australis*	1.1
Snapper	1	Coastal marine, carnivore	Local	*Chrysophrys auratus*	2.1
Snapper, red	1	Coastal marine, carnivore	Local	*Trachichthodes gerrardi*	0.8
Snook	2	Coastal marine, carnivore	Local	*Australuzza novaehollandiae*	0.9
Turbot	1	Marine, carnivore	Northern hemisphere	*Scophthalmus maximus*	4.5
Whiting, King George	5	Coastal marine, carnivore	Local	*Sillaginodes puctratus*	1.0
Whiting, yellowfin	3	Inshore marine, carnivore	Local	*Sillago schomburgkii*	1.0
Whiting, sand	2	Inshore marine, carnivore	Local	*Sillago ciliata*	1.0
Invertebrates					
Octopus	1	Marine, carnivore	Local	*Octopus* sp.	–
Scallop, queen	5	Marine, filter, feeder	Tasmania	*Equichlamys bifrons*	–
Shrimp (king prawn)	3	Marine, detritus, feeder	Local	*Penalus latisulcatus*	–
Squid	3	Marine, carnivore	Local	*Sepioteuthis australis*	–
Squid	3	Marine, carnivore	Queensland	Family Loliginidae	–
Squid	3	Marine, carnivore	Malaysia	Family Loliginidae	–

N = Number of fish sampled.

for North American freshwater fin fish (19), and 13% 20:4ω6 was reported in the lipids of the freshwater roach from Scotland (20). More recently, results have been presented which indicate that marine organisms common to the tropical waters of Northern Australia are a rich source of ω6 acids (21); results obtained in the present study confirm these findings (Table 2).

The origin of ω6 fatty acids in Australian fish is somewhat obscure due to a paucity of information on both the local food web and the fatty composition of local species at the beginning of the food chain. High concentrations of 20:4ω6 have been reported in 4 species of Rhodophyta and two species of Phaeophyta marine algae harvested in southern coastal waters of Australia (22), while 2 local varieties of seagrass are reported to contain 18:2ω6 (23). The fish in this study found to contain high levels of ω6 fatty acids in their lipids include both herbivorous and carniverous species (Table 1).

The role of ω6 acids, in general, and arachidonic acid, in particular, in the metabolism of Australian marine and freshwater organisms is difficult to ascertain. Certainly the mean temperature of coastal waters is never lower than 12 C and fats with a high level of unsaturation would not be necessary for cold water survival. Although specific or subspecies differences may complicate the issue, the 3 samples of squid tested in this study seem to indicate a trend in the level of ω6 acids; the ω6 concentration is lowest in the local squid caught in temperate waters and highest in the Malaysian squid caught in tropical waters, with the semitropical Queensland squid containing intermediate levels. Whether this trend relates to decreased oxygen availability, due to its lower solubility in warmer water, or whether other mechanisms are involved (24), is debatable. There is no correlation between the ratio of ω6 to ω3 fatty acids and the calculated unsaturation index, and furthermore there appears to be no constant inverse relationship between the concentration of ω6 and ω3 fatty acids as occurs in animal membrane phospholipids (25).

Many of the fish tested in this study would appear to be excellent sources of dietary arachidonic acid (Table 2). Indeed, it has been recently shown that plasma arachidonic acid levels increased markedly in a group of Australian aboriginals who consumed a diet rich in seafood from northern Australian waters for a period of 2 weeks (21). A number of clinical conditions have been associated with low levels of arachidonic acid and dietary supplementation using evening primrose oil, containing 7% 18:3ω6, has been proposed as a remedy (26,27). Two of the fish sampled, callop and blue groper, contained both a high proportion of fat and sufficiently high levels of arachidonic acid to warrant consideration as a commercial source of 20:4ω6 (Tables 1,2).

Many of the other fatty acid groups detected in Australian fish require comment. Saturated fatty acids ranged in concentration from 4 to 50% of total fatty acids but more commonly constituted ca. 30% of the fatty acids (Table 2). The saturated fatty acid found in highest concentration was palmitic acid with smaller amounts of stearic acid and some myristic acid. These figures are consistent with levels found in fish lipids by other workers (3,14, 18-20).

The monoenes are of special interest since they constitute a large proportion of the total fatty acids of fish from the northern hemisphere (3,18-20). The monoenes of Australian fish ranged from 13 to 68% of the total fatty acids but only 4 species contained levels in excess of 40% (Table 2). The major fatty acid of this class was found to be 18:1 followed by 16:1 (Table 2). Only trace levels of 22:1 were detected in all of the fish caught in local waters. Marine algae from Australian coastal waters are reportedly low in 22:1 (22). However, the imported species gurnard perch and South African hake contained 22:1 levels of 8 and 6%, respectively. No attempt was made in this study to identify the position of the double bond in 22:1, although the ω11 isomer is reported as the major 22:1 isomer of fish oils (28).

The ω3 fatty acids, normally associated with oils of marine origin, were found to be major contributors to the total polyunsaturated fatty acids (PUFA) of most of the species examined and only gurnard perch contained less than 10% ω3 PUFA. The precursor of the 3-series prostanoids, 20:5ω3, was present in the oils of local species in levels ranging from 3 to 16% which is in the same range as that reported from commercial species from the northern hemisphere (3). Although 22:6ω3 was present in all the species sampled, the levels varied considerably (3-37%).

Fish oils have been proposed as antithrombotic dietary supplements (4-6,13). The rationale is that oils rich in 20:5ω3 would provide substrate for the production of platelet antiaggregatory factors such as TXA_3 and PGI_3, but concern has been expressed over the wisdom and efficacy of such proposals (29,30). While the 3-series prostanoids may be antithrombotic, there is no doubt that the 2-series prostag-

TABLE 2

Fatty Acid Analysis of the Total Lipid Obtained from Table Fish (results are expressed as a percentage of the total fatty acids present)

Fatty acid	Barramundi	Callop	Cod, English smoked	Cod, Red	Garfish	Gemfish	Grenadier, blue	Groper, blue	Hake, South African	Leatherjacket	Morwong
14:0	2.0	3.0	1.0	1.0	0.9	0.8	1.7	0.8	3.5	1.9	2.3
15:0	0.8	1.1	0.2	0.4	0.4	0.3	0.2	0.5	0.4	0.4	0.5
16:0	22.9	19.9	20.0	19.7	21.2	22.8	22.0	19.8	18.2	18.0	19.1
18:0	9.0	4.9	5.7	5.8	5.6	9.4	7.3	7.4	2.9	7.2	6.5
20:0	0.6	0.9	0.3	0.6	0.5	0.1	1.4	0.1	–	0.7	0.4
22:0	–	0.8	–	–	0.8	0.3	0.5	0.2	–	0.7	0.5
Σ saturates	35.3	30.6	27.2	27.5	29.4	33.7	33.1	28.8	25.0	27.9	29.5
16:1	7.0	12.8	3.4	5.7	3.7	4.4	4.1	2.4	7.7	4.5	6.6
18:1	23.9	18.6	14.8	16.8	11.5	13.9	13.1	9.3	29.9	12.6	17.7
20:1	0.9	0.9	3.3	–	1.1	0.6	1.2	1.1	11.1	0.8	4.8
22:1	0.3	0.6	1.1	1.2	0.5	0.4	1.0	0.7	5.9	1.0	1.0
Σ monoenes	32.1	32.9	22.6	23.7	16.8	19.3	19.4	13.5	54.6	18.9	30.1
18:2	5.9	3.4	1.8	2.2	4.4	1.4	2.0	1.2	2.4	2.2	2.7
20:4	8.8	4.8	1.9	2.6	6.1	8.3	2.2	9.3	1.1	15.8	3.1
22:4	1.3	2.1	0.3	0.4	1.2	1.7	0.3	0.9	0.4	2.2	0.8
22:5	2.6	2.3	0.4	0.4	6.1	1.2	0.3	2.2	0.3	2.1	0.8
Σ ω6 acids	18.6	12.6	4.3	5.6	17.8	12.6	4.8	13.6	4.2	22.3	7.4
18:3	2.0	2.8	0.5	0.7	3.3	0.4	1.0	0.3	0.8	1.1	0.6
20:5	2.0	4.1	8.5	12.7	4.5	5.3	13.3	6.1	4.8	15.9	7.4
22:5	1.5	4.1	1.2	1.0	3.2	3.7	1.1	1.2	1.1	3.3	3.3
22:6	8.5	13.1	35.7	28.7	24.7	25.1	27.5	36.6	9.5	10.8	21.7
Σ ω3 acids	14.0	24.1	45.9	43.1	35.7	34.5	42.9	44.2	16.2	31.1	33.0
Σ unsaturates	62.1	67.2	72.4	72.2	64.0	65.2	66.7	69.1	74.7	70.0	69.7

TABLE 2 (continued)

Fatty acid	Mullet, jumper	Mullet, yelloweye	Mulloway	Perch, gurnard	Perch, redfin	Ruff, tommy	Salmon, Australian	Shark, school	Snapper	Snapper, red	Snook
14:0	8.7	4.7	3.6	0.5	1.9	1.7	0.8	0.8	2.1	1.8	1.6
15:0	3.9	1.2	2.9	0.3	0.7	0.6	0.6	0.3	0.5	0.5	0.4
16:0	26.8	18.5	18.8	2.3	18.2	20.8	19.2	22.8	22.7	19.1	20.7
18:0	4.3	7.1	4.9	0.9	3.2	6.4	6.1	9.4	8.3	7.3	8.5
20:0	5.2	1.2	0.9	–	0.6	0.6	0.5	0.1	0.4	0.5	0.4
22:0	1.3	0.6	0.4	–	0.7	0.7	0.5	0.3	0.5	0.5	0.4
Σ saturates	50.0	33.3	31.5	4.0	25.3	30.7	28.3	33.7	34.5	29.6	31.9
16:1	17.1	9.2	15.5	6.0	13.0	5.1	7.1	4.4	6.8	4.0	3.8
18:1	8.6	20.3	22.2	38.4	17.2	14.1	16.7	13.9	24.5	14.7	12.7
20:1	–	2.4	1.7	16.2	0.7	2.4	1.6	0.6	2.5	1.7	0.6
22:1	1.4	0.6	0.5	7.8	0.4	0.7	0.4	0.4	0.6	0.7	0.3
Σ monoenes	27.1	32.5	39.9	68.4	31.3	22.3	25.8	19.3	34.4	21.1	17.4
18:2	2.3	3.4	2.3	0.7	3.2	2.0	2.9	1.4	1.6	1.9	1.6
20:4	1.7	4.2	2.7	10.3	5.9	5.0	5.1	8.3	5.5	2.5	4.9
22:4	0.9	1.5	0.8	4.0	1.2	1.4	1.2	1.7	2.0	0.3	0.8
22:5	–	1.3	0.8	3.1	2.3	1.5	1.6	1.2	1.2	1.3	1.6
Σ ω6 acids	4.9	10.4	6.6	18.1	12.6	9.9	10.8	12.6	10.3	6.0	8.9
18:3	2.0	2.2	1.1	5.7	2.7	0.9	0.8	0.4	0.5	0.5	0.6
20:5	7.8	9.3	5.6	0.7	6.8	7.0	6.6	5.3	3.4	5.9	4.7
22:5	3.6	4.5	5.2	–	2.1	2.6	3.2	3.7	3.8	2.1	1.6
22:6	4.9	7.7	10.2	3.2	19.3	26.5	24.6	25.1	13.0	34.8	34.9
Σ ω3 acids	17.9	23.7	22.1	9.6	30.9	37.0	35.2	34.5	20.7	43.3	41.8
Σ unsaturates	50.0	65.4	67.7	93.0	72.4	67.8	70.1	65.2	64.3	69.1	66.5

TABLE 2 (continued)

Fatty acid	Turbot	Whiting, King George	Whiting, silver	Whiting, yellowfin	Octopus	Scallop, queen	Shrimp (king prawn)	Squid, local	Squid, Queensland	Squid, Malaysian
14:0	5.6	2.4	2.9	1.3	1.4	1.8	1.3	2.0	3.5	2.2
15:0	0.4	0.7	0.7	0.7	0.3	0.8	1.6	0.6	0.5	1.1
16:0	12.9	19.0	20.0	20.8	21.3	20.5	15.7	28.5	27.6	29.1
18:0	2.2	6.1	4.9	6.8	8.7	9.3	10.8	4.0	5.9	3.2
20:0	–	0.3	0.5	0.3	0.6	1.0	0.6	0.2	0.2	0.4
22:0	–	0.5	0.7	0.7	–	0.2	0.6	–	0.3	0.4
Σ saturates	20.9	28.8	29.6	30.6	32.4	33.7	30.5	35.3	37.9	36.4
16:1	10.8	8.5	17.2	6.6	1.4	4.0	8.0	2.4	3.1	3.2
18:1	28.9	15.3	21.0	14.1	10.0	9.1	14.7	6.3	8.1	6.6
20:1	16.0	1.7	1.9	1.3	4.8	2.0	1.9	3.4	2.0	1.6
22:1	1.9	0.6	0.5	0.3	1.7	–	0.6	0.4	0.6	0.8
Σ monoenes	57.6	26.1	40.6	22.3	17.9	15.1	25.2	12.5	13.8	12.2
18:2	2.2	1.9	3.8	1.9	1.0	1.5	2.2	0.6	1.0	0.6
20:4	0.7	10.8	6.2	11.4	9.8	4.7	6.4	2.0	3.9	7.2
22:4	0.6	2.8	4.3	4.4	1.7	2.5	0.8	0.6	0.4	0.7
22:5	0.5	1.5	–	1.5	0.6	0.5	0.9	0.7	1.1	2.5
Σ ω6 acids	4.0	17.0	14.3	19.2	14.8	9.2	10.3	3.9	6.4	11.0
18:3	0.7	0.4	0.8	0.4	0.3	0.2	0.9	0.2	–	0.1
20:5	11.8	9.2	7.4	13.2	13.9	14.8	14.3	12.5	10.3	8.3
22:5	1.5	3.7	2.8	4.6	2.5	1.1	1.0	0.9	0.5	0.9
22:6	3.6	14.8	4.6	9.8	20.0	25.8	17.7	34.6	31.0	31.2
Σ ω3 acids	17.6	28.1	15.6	28.0	36.7	41.9	33.9	48.2	41.8	40.5
Σ unsaturates	78.9	69.7	70.4	67.9	67.0	65.8	68.6	64.0	61.0	61.1

landins, derived from 20:4ω6, also have a diverse and essential role to play in the processes of the body (6,31). The use of dietary supplements rich in both 20:5ω3 and 20:4ω6, such as the oils of fish discussed herein, may provide a more balanced approach.

Lipids of fish obtained from tropical waters are also rich in ω6 fatty acids (21). We have recently analyzed a number of fish obtained from Malaysia (results not presented), including both invertebrates and fin fish, with similar results. Since, for a large proportion of Asian people, fish products are a major source of protein, a study on the effect of such a diet on the incidence of coronary thrombosis and related biochemical events seems justified.

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Diet- and Hormone-Induced Lipid Deposition in Rat Kidney: Correlation with Systolic Blood Pressure

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ABSTRACT

The influence of estradiol on deposition of cholesterol in tissues of ovariectomized rats on normal and high lipid diets was studied. Concomitantly the influence of a contraceptive steroid combination was studied in a similar manner in intact rats. It was found that the high lipid diet resulted in increased deposition of cholesterol in aorta, heart, liver and kidney. The presence of either endogenous or exogenous hormones accentuated the deposition of cholesterol in the kidney and resulted in significantly higher systolic blood pressures in these rats. In the rats on a high lipid diet, the concentration of cholesterol in the kidney correlated positively with systolic blood pressure. It is concluded that estrogen and high lipid diet exert a synergistic effect on deposition of cholesterol in kidney. The positive correlation between kidney cholesterol concentration and systolic blood pressure suggests a possible role for kidney lipid deposition in the hypertensive effect of estrogens.

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The hyperlipidemic effects of female sex steroids have been studied at length by various investigators. Estrogen was once thought to be hypocholesterolemic (1,2) but studies in recent years have demonstrated the hypercholesterolemic effect of estrogen and contraceptive steroid combinations in humans (3-7) and avian species (8). In rats, estrogen has a bimodal effect (9,10), high doses depressing plasma cholesterol and lower doses given over a prolonged period of time elevating plasma cholesterol. Contraceptive steroid administration to rats, in various dosages, may not elevate or may even depress plasma cholesterol (10-12). The effect of these steroids on concomitant tissue deposition of cholesterol, on the other hand, has not been studied to any extent. Since women before menopause have lower incidence of atherosclerotic heart disease than men of the same age, estrogen has been thought to be protective, but the mechanism is not known. In this regard, the effect of estradiol on deposition of cholesterol in vascular tissue would be relevant information to obtain.

We recently reported the development of a diet which would produce hypercholesterolemia in rats without the use of antithyroid drugs (12). We were thus able to avoid the inanition and depressed metabolism associated with these drugs, maintaining the rats on this diet for 20 weeks without loss of appetite or weight. We treated these rats with sex steroids and reported a synergistic increase in plasma cholesterol produced by the combination of diet and hormones (12).

We are now reporting the effect of this diet and hormone treatment on deposition of cholesterol in cardiovascular tissue, liver and kidney, with particular reference to the kidney, which in a number of cases showed pathological changes and in which increased deposition of cholesterol was associated with increased systolic blood pressure.

EXPERIMENTAL PROCEDURES

Female rats aged 3 months, half of them ovariectomized, were obtained from Charles River Breeding Laboratories, Wilmington, MA, (Crl:CD-SD-BR strain) divided into 4 groups and treated as described below. The experimental design essentially consisted of 2 separate studies, the first involving the ovariectomized rats and the second the intact rats.

Ovariectomized. Group I: cottonseed oil, 0.1 ml weekly; group II: estradiol, 10 μg weekly (depoestradiol cypionate, Upjohn Co., Kalamazoo, MI).

Intact. Group III: cottonseed oil, 0.1 ml weekly; group IV: mestranol, 5 μg-norethynodrel, 250 μg weekly (kindly supplied by T. Martinez, Searle Laboratories).

All injections were administered in 0.1 ml volume, with cottonseed oil as the vehicle for all groups. The groups were further subdivided into 2 groups each, one subgroup in each case being maintained on Purina laboratory rat chow and the other subgroup in each case on Purina laboratory rat chow to which cholesterol (4%),

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[1] Deceased.

coconut oil (4%) and cholic acid (1%) were added, the latter subgroups being designated groups IA, IIA, IIIA, and IVA.

The groups were chosen to represent clinically relevant situations: (a) ovariectomized women with and without estrogen replacement therapy, and (b) women with intact ovaries with and without contraceptive steroid administration.

The rats were maintained on this regimen for 20 weeks, at the end of which time the rats were killed by cervical dislocation after an overnight fast.

Systolic blood pressure was measured by means of the tail cuff method (Narco Biosystems), as previously described (13). Measurements were taken on each rat repeatedly until 2-3 good records were obtained, free of artifacts from movement. These 2-3 values were then averaged to get the individual blood pressure value for each rat. Rats which had been ovariectomized one week before we obtained them already showed lower blood pressure than intact rats. We concluded that we could not use these pressures in the ovariectomized animals as control pressures. However, since all rats, both intact and ovariectomized, were from the same supplier pool, the intact untreated rats were taken as a control pool from which to judge baseline blood pressure. Blood pressures were again measured on all rats at the end of the 20-week period.

Aortic Cholesterol Deposition

Aortas were carefully dissected from the level of the aortic valves to the bifurcation into iliac arteries, trimmed of loose adventitia, dried and stored. Dried specimens were subsequently ground with a mortar and pestle; cholesterol was extracted by the method of Folch et al. (14). Total cholesterol and percentage esters were determined by the method of Sperry and Webb (15).

Organ Cholesterol Deposition

Whole kidney, heart and liver specimens were dried, ground in a mortar, extracted, and cholesterol concentrations determined on a weighed aliquot, as above.

Histological Studies

Kidney slices were removed immediately after death and fixed in 10% buffered formalin. Sections were embedded in paraffin, cut at 4-5 micron intervals and stained with hematoxylin and eosin (H&E). Selected sections were stained with periodic acid schiff (PAS) and counterstained with Alcian blue, and Van Giesen stain.

Stained sections were examined and photographed using a Zeiss Ultra Phot II equipped with a camera.

The Student's t-test was used to compare means between groups in each of the 2 studies. Linear regression analysis was used to compute correlation coefficients between systolic blood pressure and tissue cholesterol deposition.

RESULTS

There were no lesions in the aortas of rats after 20 weeks on the high cholesterol diet. Concentrations of total cholesterol in the aortas of these rats are shown in Table 1, along with significance of differences between groups. The feeding of the high lipid diet increased the concentration of cholesterol in aorta, although in the intact oil treated animals the difference was not significant, probably due to the unusually large standard error in the group on a high lipid diet. The percentage of cholesterol esters in the tissue was slightly increased in most cases by the high lipid diet (not shown) but the change was significant in only 2 groups, increasing from 8.1 ± 2.8 to 14.6 ± 2.5% ($P < 0.05$) of total cholesterol in group II-IIA and from 6.1 ± 2.3 to 15.7 ± 3.1% ($P < 0.025$) in group III-IIIA. Ovariectomized rats given estradiol, when compared to ovariectomized rats given oil, demonstrated increased aortic lipid deposition on a normal diet ($P < 0.025$) but not on the high lipid diet.

Concentrations of cholesterol in the heart are also shown in Table 1. The high lipid diet resulted in significant increase in tissue concentration of cholesterol in all groups of animals ($P < 0.01$ to 0.0005). There was no significant increase in percentage esters (not shown).

Consistent with the expected finding of markedly increased cholesterol concentration in the liver of lipid-fed animals (Table 1), the livers were grossly enlarged and yellowish in color. In the liver, the percentage of cholesteryl esters was dramatically increased in all groups on the high lipid diet from means of less than 20% of the total cholesterol on the normal diet to greater than 90% on the high lipid diet ($P < 0.005$ to 0.0005).

Concentrations of total cholesterol in kidney are shown in Table 1. At the time of sacrifice, the kidneys of many of the rats on the high cholesterol diet were enlarged and yellowish in color as compared to kidneys of rats on the normal diet. This was particularly marked in the estradiol-treated rats. The presence of exogenous or endogenous hormones accentuated the deposition of cholesterol in the kidney of rats on a normal diet. Likewise, the rats on

TABLE 1

Concentration of Cholesterol in Tissue (mg/g)

Tissue	Diet	Ovariectomized rats		Intact rats	
		Control (oil)	Estradiol	Control (oil)	Mestranol-norethynodrel
Aorta	Standard	3.59 ± 0.14[a,b]	4.05 ± 0.14[a,c]	3.93 ± 0.14	3.88 ± 0.14[d]
N = 8-12	High lipid	4.79 ± 0.31[b]	5.10 ± 0.30[c]	5.02 ± 0.62	4.99 ± 0.17[d]
Heart[e]	Standard	4.61 ± 0.07	4.80 ± 0.09	4.77 ± 0.10	4.66 ± 0.07
N = 6	High lipid	5.39 ± 0.08	5.61 ± 0.06	5.38 ± 0.09	5.64 ± 0.32
Liver[f]	Standard	6.0 ± 0.4	7.0 ± 0.3	6.9 ± 0.6	7.1 ± 0.2
N = 4	High lipid	135.6 ± 19.3	146.1 ± 18.4	129.7 ± 25.2	167.4 ± 38.5
Kidney	Standard	15.3 ± 0.3[g,h,i]	16.9 ± 0.5[g,j]	16.4 ± 0.2[h]	16.2 ± 0.3[i,k]
N = 5-7	High lipid	16.0 ± 0.4[l,m]	20.5 ± 1.1[j,l]	18.1 ± 1.1	18.4 ± 0.4[k,m]
Systolic blood pressure	Standard	117 ± 2	130 ± 3[n]	135 ± 4[n]	132 ± 4[n]
N= 5-7	High lipid	120 ± 3	144 ± 9[n]	143 ± 4[n]	141 ± 5[n]

Superscripts within table denote pairs of values being compared.

[a] $P < 0.025$.

[b] $P < 0.005$.

[c] $P < 0.005$.

[d] $P < 0.0005$.

[e] In all groups, the high lipid diet resulted in significant ($P < 0.01$ or better) increase in concentration of cholesterol over that of rats on standard diet.

[f] In all groups, the high lipid diet resulted in significant ($P < 0.005$ or better) increase in concentration of cholesterol over that of rats on standard diet.

[g] $P < 0.025$.

[h] $P < 0.01$.

[i] $P < 0.05$.

[j] $P < 0.01$.

[k] $P < 0.0005$.

[l] $P < 0.005$.

[m] $P < 0.005$.

[n] Significantly higher ($P < 0.05$ or better) than the mean blood pressure (123 ± 2) of the pool of intact rats before diet or treatment was instituted.

high lipid diet, with one exception, had significantly more cholesterol deposition when hormones were present. The percentage esters was slightly increased in the lipid-fed rats.

Histopathological Changes in the Kidney

Some glomeruli were enlarged, devoid of blood cells and had capillaries with very narrow lumen. Kidney sections stained with Alcian blue and PAS revealed deposition of PAS positive homogeneous material on the capillary wall, presumably in the basement membrane. The Bowman's capsules of some glomeruli were filled with proteinaceous material (Fig. 1).

The kidney also showed diffuse tubular degeneration. The epithelial cells of the renal tubules, particularly the convoluted tubules, showed swelling and deposition of oil red 0 positive lipid droplets in their cytoplasm. In some cases, the tubules showed severe necrosis (Fig. 2). Several urinal tubules were dilated and filled with hyalin casts (Fig. 3). The interstitium showed mild infiltration of mononuclear cells and atrophy of the tubules in some areas.

The renal arteries appeared normal, except that in some arteries there was an increase in the elastic fibers in the media.

Systolic Blood Pressure

At the beginning of the experimental period when the rats were 90 days old and no treatment or special diet had yet been started, the intact rats had a mean systolic blood pressure of 123 ± 2. These rats entered groups III-IIIA and IV-IVA. This mean was calculated for the 23 rats in this pool in which renal cholesterol was measured. No initial pressures were taken on the animals in groups I-IIA and II-IIA, the ovariectomized rats. After 20 weeks, the rats in groups II, IIA, III, IIIA, IV and IVA had significantly higher blood pressures than the intact rats at the beginning of the period ($P < 0.05$ or

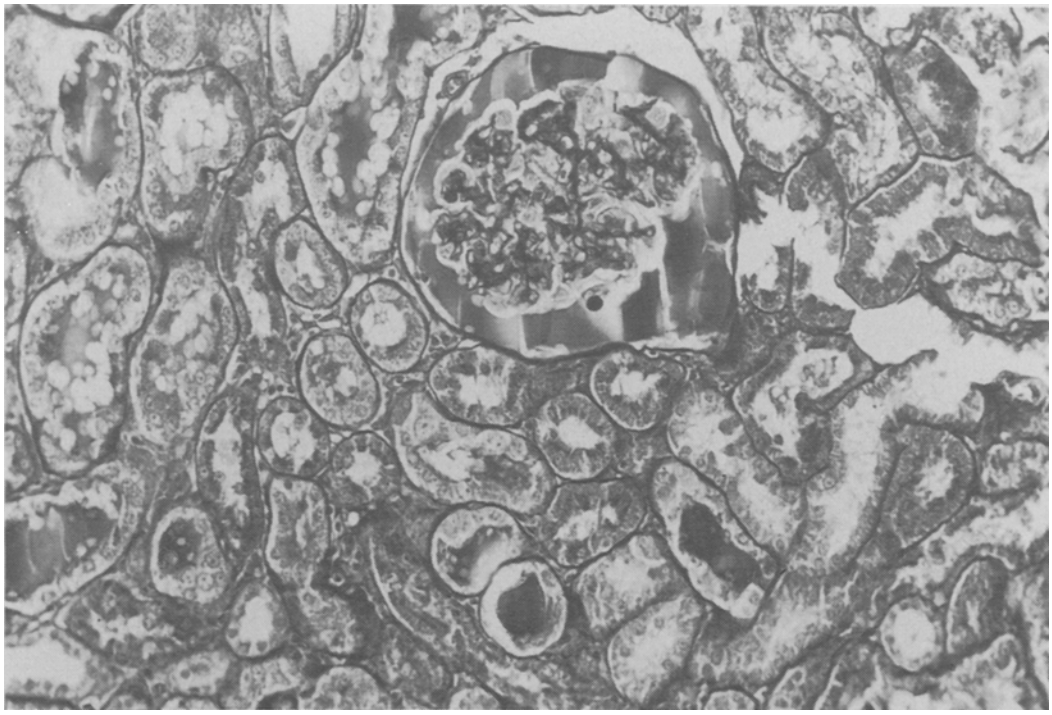

FIG. 1. Photomicrograph (16×) of kidney from rat from group IIA. Bowman's capsule is filled with PAS positive homogeneous material.

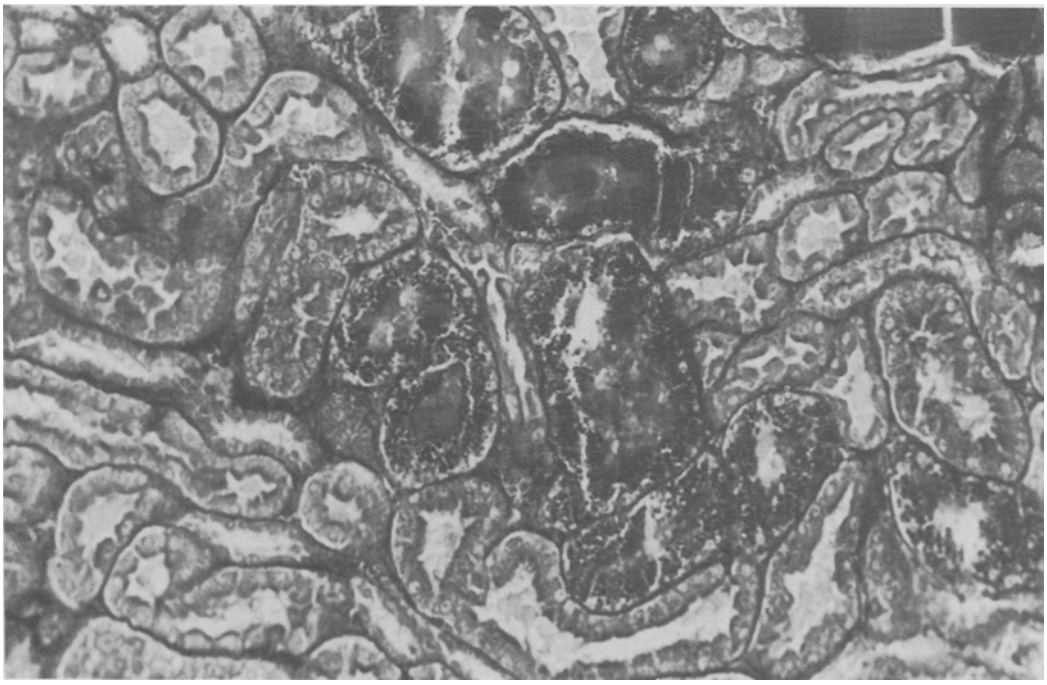

FIG. 2. Photomicrograph (16×) of kidney from rat from group IIA (estradiol-treated ovariectomized rat on high lipid diet). Groups of convoluted tubules revealed severe necrosis of the lining epithelial cells. The necrotic tubules are found adjacent to normal tubules. Alcian blue-PAS stain.

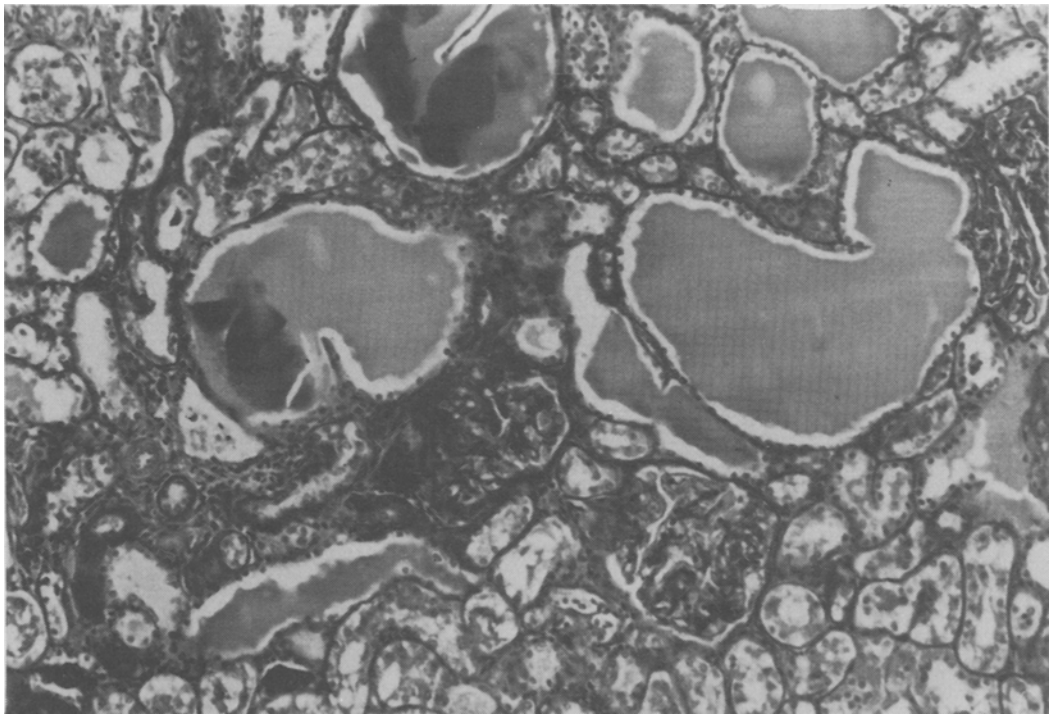

FIG. 3. Photomicrograph of kidney from rat from group IIA. A large number of tubules are dilated and filled with proteinaceous material.

better), whereas groups I and IA, which had no endogenous or exogenous hormone, did not have increased blood pressure (Table 1). It was noted that in many cases the rats which had abnormal-appearing kidneys and high concentrations of cholesterol in their kidneys had relatively high blood pressure. The mean systolic blood pressure of all of the groups are presented in Table 1 and Figure 4, in which the mean pressures are plotted against mean kidney cholesterol concentrations of the groups. A linear regression analysis revealed a significant positive correlation ($r = 0.842$, $P < 0.01$) between systolic blood pressure and kidney cholesterol concentration. A weaker correlation between cholesterol concentration and blood pressure was observed if all animals on the high lipid diet were considered without averaging the groups. The blood pressures in the rats of group IIA exhibited wider scatter (higher standard error, Table 1). However, if this group was omitted from the calculation of correlation coefficient, an even higher correlation coefficient was obtained ($r = 0.888$). There was no significant correlation between systolic blood pressure and any of the other tissue concentrations of cholesterol, the kidney thus being unique in this respect.

DISCUSSION

These studies indicate that estradiol administration accentuated deposition of cholesterol in the kidney. In this respect, the results were similar to those for plasma cholesterol (12) where a synergistic effect was seen between diet and estradiol in raising plasma levels of cholesterol. Marked cholesterol deposition in the kidney was accompanied by gross and microscopic pathological changes.

It would appear that undue deposition of cholesterol in the kidney leads to pathological changes which could possibly affect kidney function. The correlation of systolic blood pressure with concentration of cholesterol in the kidney but not in the other tissues would suggest that the pathological changes induced by the cholesterol might be contributing to the elevated blood pressure. However, blood pressure was increased in the hormone-treated rats on a normal diet, although not as much as in those on the high lipid diet. Such correlations cannot be conclusive of cause and effect, but they suggest the need for further studies as to the role of the kidney and diet in the hypertensive effects of hormones. It is possible that estrogen exerts its hypertensive effect through a

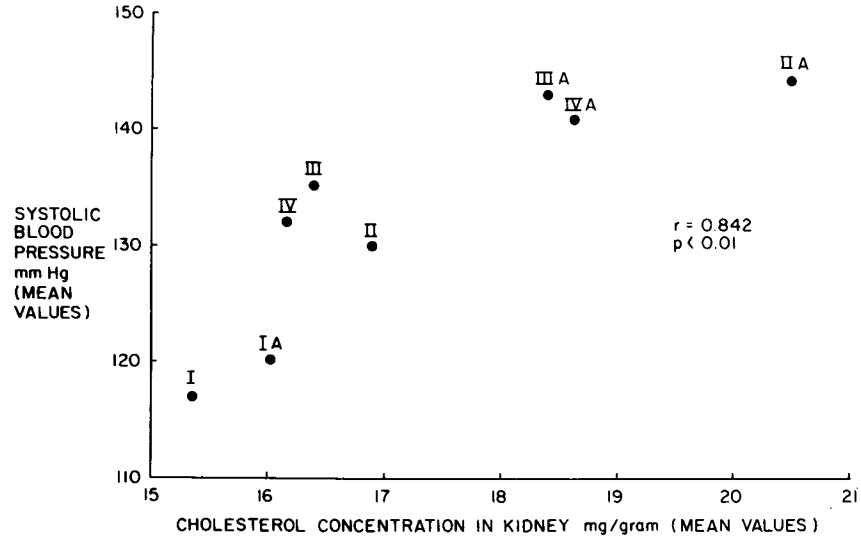

FIG. 4. Mean values of systolic blood pressure of rats in each group are plotted against mean concentrations of cholesterol in kidneys of the same rats. N = 5-7 for each group.

kidney mechanism regardless of cholesterol, but that the mechanism is enhanced by large amounts of cholesterol, whether through a biochemical effect or anatomical damage. Activity of certain enzymes in the kidney has been shown to be changed by estradiol (16). There may be multiple mechanisms. For example, it has been shown that renin substrate is increased by estrogen (17). Most of the studies in which the hypertensive effect of estrogens and contraceptive steriods in humans has been documented have not contained data on cholesterol content of diet.

Although conclusions as to the human situation cannot be drawn from these rat studies, there are several counterparts which suggest that the pursuit of further studies might be warranted. (a) Estrogen receptors have been found in human kidneys (18,19) as well as in the rat (20,21). (b) Many American diets are high in cholesterol. (c) Obesity in humans has long been associated with increased incidence of hypertension.

These studies indicate that estrogen does not protect against the deposition of cholesterol in normal vascular and cardiac tissue in the rat. It should be emphasized that there were no lesions in aortas or heart. We have shown previously that mestranol-norethynodrel administration to rabbits on an atherogenic diet retards the development of atherosclerotic plaques and concomitant deposition of cholesterol (22). In those studies, the steroid combination inhibited collagen synthesis in the atherosclerotic vessels, a mechanism which might protect against atherosclerosis. Since the rat is resistant to atherosclerosis, we were able in the current study to determine the effect of long-term cholesterol feeding without the development of lesions. Thus, the effect of estrogen on aortas without atherosclerotic lesions could be determined. In this case, estrogen did not protect against cholesterol deposition, although estrogen-treated rats had presumably higher concentrations of cholesterol in plasma than did the other rats (12).

In summary, the results of this study indicate a synergistic effect of high cholesterol diet and estradiol on cholesterol deposition in the kidney and raise the question as to whether this increased deposition may be related to an elevated systolic blood pressure. Further studies correlating renal changes to elevated systolic blood pressure will be required.

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Acyl Group Distributions in Tissue Lipids of Rats Fed Evening Primrose Oil (γ -Linolenic Plus Linoleic Acid) or Soybean Oil (α-Linolenic Plus Linoleic Acid)

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ABSTRACT

Three groups of rats were fed diets with either 10 weight percent (wt%) of evening primrose oil, safflower oil or soybean oil for 11 weeks. Diets contained 7.1 wt% linoleic acid + 0.8 wt% γ-linolenic acid, 7.6 wt% linoleic acid, or 5.3 wt% linoleic acid + 0.7 wt% α-linolenic acid, respectively. In liver mitochondria as well as in heart, dietary γ-linolenic acid did not affect the fatty acid profiles of phosphatidylcholines (PC), phosphatidylethanolamines (PE) or cardiolipins (CL), whereas dietary α-linolenic acid caused an increased formation of (n-3) polyunsaturated fatty acids (PUFA). The liver Δ6- and Δ5-desaturase activities determined in vitro were not affected by the dietary fats. In brain PE, which are rich in C22- and C20-(n-3) PUFA, as well as in testes PC and PE, which are rich in (n-6) PUFA, no effects were found from a partial replacement of dietary linoleic acid with γ-linolenic acid or α-linolenic acid. In kidney PC, PE, phosphatidylinositol (PI) and CL, 20:3(n-6) was moderately elevated to ca. 1% following intake of γ-linolenic acid, whereas partial replacement of linoleic acid with α-linolenic acid was followed by increased deposition of 22:6(n-3) in PC and PE of testes and kidney. Thus, no general effect of evening primrose oil on the content of (n-6) PUFA in rat tissue phospholipids was observed, whereas a significant incorporation of γ-linolenic acid into liver and adipose tissue triglycerides was found.
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INTRODUCTION

The rate-limiting step in the conversion of dietary linoleic acid into arachidonic acid is the formation of γ-linolenic acid, *cis*-6,*cis*-9,*cis*-12-18:3, by Δ6-desaturation of linoleic acid (1,2). Previously, it has been attempted to increase the formation of long-chain polyunsaturated fatty acids of the (n-6)-series by administration of γ-linolenic acid or di-homo-γ-linolenic acid, *cis*-8,*cis*-11,*cis*-14-20:3, in order to bypass the Δ6-desaturation. Hassam and Crawford (3) found that in the rat, maternal dietary γ-linolenic acid increased the levels of 18:3(n-6) and 20:3(n-6) in the stomach contents as well as in the liver triglycerides and the total phospholipids of the sucklings pups. In the stomach contents of the pups, the level of 18:3(n-6), however, was only 0.48% of the total fatty acids. Hassam et al. (2,4) also showed, using suckling rats given a single oral dose of radiolabeled 18:3(n-6) or 20:3(n-6) together with 18:2(n-6), that 18:3(n-6) and 20:3(n-6) were incorporated into liver and brain phospholipids and converted into 20:4(n-6) to a higher extent than was 18:2(n-6). They demonstrated that a part of the administered radioactivity was retained as 20:3(n-6) in liver and brain. In neither of these papers, based on experiments with rats fed semisynthetic diets, were data presented on the fatty acid composition of the individual phospholipid classes of the tissues investigated.

The effect of γ-linolenic acid on renal lipids has not been investigated, but Danon et al. (5) have demonstrated that dietary 20:3(n-6) increases the content of 20:3(n-6) in rat renal phospholipids. Danon et al. reported a level of 3-4% 20:3(n-6) in the total phospholipids of rat renal medulla and cortex following administration of 300 mg ethyl di-homo-γ-linolenate a day for 23 days to adult rats, but the composition of heart, brain or testes lipids was not investigated. Knapp et al. (6) demonstrated an augmented renal formation of PGE_1 in the rabbit following dietary intake of 20:3(n-6).

The effect of feeding 0-2 cal% γ-linolenic acid for 7 days to essential fatty acid (EFA)-deficient rats has been studied (7). No deposition of 18:3(n-6) was found, but up to 3.7% 20:3(n-6) was detected in total liver phospholipids.

This paper describes an experiment in which rats were fed vegetable oils containing 18:3 (n-6) + 18:2(n-6), 18:2(n-6), or 18:2(n-6) + 18:3(n-3), respectively, for 11 weeks. The

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effects of the dietary regimen on the fatty acid composition of various phospholipds from liver, heart, brain, testes and kidney, and of triglycerides from liver, kidney and adipose tissue as well as the in vitro activity of liver microsomal Δ6- and Δ5-desaturases are reported.

EXPERIMENTAL PROCEDURES

Animal Experiments

Twenty-four weanling male Wistar rats (specific pathogen-free; Møllegaards Avlslaboratorium Aps., Ll. Skensved, Denmark) were divided into 3 groups; each group contained 8 rats with similar average weight. The rats received a diet of the following composition (wt%): casein (Holstebro Maelkekondenseringsfabrik, a.m.b.a., Holstebro, Denmark), 20%; sucrose, 64%; vitamin mixture, 0.5%; salt mixture (including trace elements), 5.0%; choline chloride, 0.5%; and fat, 10%. The compositions of the vitamin mixture and the salt mixture were as previously described (8). The dietary fats were seed oils from evening primrose (EPO), *Oenothera biennis* ("Naudicelle," Bio Oil International Ltd., Surrey, England); safflower (SAF) (Nutana, Fuglebaek, Denmark); and soybean (SOY) ("Manchurex," Aarhus Oliefabrik A/S, Aarhus, Denmark). The dietary fatty acids were shown by thin layer chromatography (TLC) to be present only in triglycerides. The fatty acid compositions of the dietary fats are given in Table 1.

The rats were caged in pairs at 25 C and a relative humidity of 45%. Diets and water were supplied ad libitum. The rats were examined and weighed weekly. During the 9th week of the feeding period, the absorption of fat was determined.

Absorption of fat was calculated from total fat ingested and the amount excreted via feces. The feces were collected daily from each cage, lyophilized, and an aliquot of 4 g was refluxed with 120 ml 3 N HCl for 1 hr to neutralize calcium soaps and extracted for 6 hr with diethyl ether on a Soxhlet apparatus. After 11 weeks, the rats were anaesthetized with diethyl ether, and a blood sample was drawn from the heart. Then, after killing the animals, liver, heart, testes, kidneys, brain and a sample of the adipose tissue were immediately excised.

Preparation of Liver Mitochondria and Liver Microsomes

The livers from each group of rats were divided into 4 pools of 2 livers each. From each liver, 5 g of tissue was used for preparation of mitochondria as previously described (9), and 3 g from each liver was used for the preparation of a microsomal fraction, which included the "fluffy layer" just above the microsomal pellet, according to Hølmer et al. (10). The protein content of the organelle fractions was quantitated according to Lowry et al. (11). The remaining liver material was used for preparation of phosphatidylinositols (PI) and triglycerides.

TABLE 1

Fatty Acid Composition of Dietary Fats

	Dietary fat		
	EPO	SAF	SOY
Fatty acids	(%)[a]	(%)	(%)
16:0	6.1	7.3	10.7
16:1	0.2	0.1	0.2
18:0	1.7	2.5	4.0
18:1	11.7	13.0	24.3
18:2(n-6)	71.0	76.1	52.8
18:3(n-6)	8.4	–	–
18:3(n-3)	–	0.3	7.0

EPO: Evening primrose oil; SAF: safflower oil; SOY: soybean oil.

[a]Weight percentages determined by GLC.

Analyses of Lipids

Total lipids were extracted from liver, liver mitochondrial and microsomal membranes, and from heart, brain, testes and kidney, according to Folch et al. (12). Phospholipids from membrane fractions were separated by TLC, either for quantitation of phospholipids (13), or for methylation followed by gas liquid chromatography (GLC) (9,14). The applied procedure assured separation of cardiolipins and free fatty acids. To obtain pure fractions of liver PI, phospholipids from total liver were separated by TLC on prewashed 0.5 mm Silica Gel H plates (E. Merck, Darmstadt, West Germany), using chloroform/methanol/acetic acid/water (170:25:26:6, v/v/v/v). A fraction containing sphingomyelins (SPH) + PI was scraped off. The phospholipids were then extracted with chloroform and rechromatographed using chloroform/acetone/methanol/acetic acid/water (30:40:10:7:5, v/v/v/v/v) as solvent, which separated PI from SPH. Authentic standards of phosphatidylcholines (PC), phosphatidylserines (PS), PI and SPH were cochromatographed. PI was methylated as previously mentioned.

Triglycerides were isolated from total liver lipids, kidney lipids and depot fat by TLC using hexane/diethylether/acetic acid (80:20:1) as solvent. Quantitation of liver triglycerides was performed by TLC, followed by fluorometric determination as described by Lykkelund and Damgaard-Pedersen (15).

Determination of Microsomal Desaturase Activity

Δ6- and Δ5-desaturase activities were determined essentially according to Larsson and Brimer (16); 685 pmol [1-^{14}C]18:2(n-6) or 0.96 pmol [^{3}H]20:3(n-6) (Radiochemical Centre, Amersham, England) were incubated with 10 mg or 5 mg, respectively, of microsomal protein for 20 min at 37 C. The incubation mixture was saponified, free fatty acids were extracted and methylated. Methyl esters were separated according to unsaturation on Silica Gel G plates containing 12.5% $AgNO_3$ with solvent systems of hexane/diethyl ether (55:45, v/v, for separation of 18:2/18:3; and 50:50, v/v, for 20:3/20:4). Appropriate bands were scraped off and counted in a liquid scintillation counter. After quench correction, percentage conversion of exogenous fatty acids was calculated.

RESULTS

Dietary Fats

The use of evening primrose oil (EPO), safflower oil (SAF) and soybean oil (SOY) as dietary fats in this experiment allowed us to study the effect of substituting part of the dietary linoleic acid with 18:3(n-6) while keeping the dietary level of (n-6) fatty acids constant and maintaining an adequate supply of linoleic acid (EPO vs SAF) (Table 1). It was also possible to compare the effects of feeding 18:3(n-6) and 18:3(n-3) at the same level with a high supply of 18:2(n-6), albeit not exactly the same (EPO vs SOY).

Animal Experiments

No differences in growth or general performance were observed during the experimental period. The absorption of dietary fat was similar in the 3 dietary groups (92.6-94.5%).

Liver

In liver mitochondria, no significant differences in phospholipid content were observed. The incorporation of 18:3(n-6) into the dietary fat at the expense of 18:2(n-6) (EPO vs SAF) did not affect the fatty acid composition of PC, PE or CL of liver mitochondria (Table 2). No significant deposition of 18:3(n-6) or 20:3 (n-6) was observed, except for cardiolipin (CL). When 18:3(n-3) was incorporated into the diet (SOY), the 22:4(n-6) and 22:5(n-6) were replaced by 22:6(n-3), and the content of 20:4 (n-6) was lowered, while 18:2(n-6) was unchanged.

The PI of rat liver (Table 3) were unaffected by dietary 18:3(n-6), whereas dietary 18:3(n-3) reduced the content of 20:4(n-6) and increased 18:2 and 20:3(n-6) moderately, while the formation of 22:5(n-3) and 22:6(n-3) was augmented.

In the liver microsomes (Table 4), a small but significant elevation of 18:3(n-6) and 20:3 (n-6) was found in the EPO group, but the total levels of PUFA were not affected. In the SOY group, the PUFA of the (n-6) series were reduced compared to the SAF group, but the content of 18:2(n-6) was unaffected. The Δ6- and Δ5-desaturase activities measured in vitro did not reveal any significant differences between the 3 dietary groups. The triglycerides of rat liver contained the highest levels of 18:3 (n-6) and 18:3(n-3) of any liver lipid, but the contents were lower than in adipose tissue (Table 5).

Heart

In the heart, the levels of PE and CL were reduced in the EPO group compared with the SAF group (Table 6). Also a minor reduction of 18:2 and a slight increase in 22:4(n-6) in the EPO group were observed. In the SOY group, an increased formation of 22:6(n-3) and 22:5 (n-3) was parallelled by a decrease in 22:4(n-6) and 22:5(n-6).

Brain

In the PE of rat brain, no differences were observed (Table 7) between the EPO and the SAF, indicating that a partial substitution of 18:2(n-6) with an equal amount of 18:3(n-6) on a weight basis does not affect the fatty acid composition of PE. Inclusion of 18:3(n-3) (group SOY) in the diet led to considerably smaller alterations of the fatty acid profile than observed for liver and heart, in agreement with the finding of Sinclair (17) that dietary influence on the brain fatty acid profile decreases with age.

Testes

When a high dietary level of 18:2(n-6) (SAF) is used, the fatty acids of testicular phospholipids are extremely unsaturated (Table 8) and dominated by (n-6)-series PUFA, primarily 20:4(n-6) and 22:5(n-6), whereas 18:2(n-6) is low in accordance with reports by Jensen (18) and Takatori et al. (19). Dietary 18:3(n-6) is not deposited in the testicular phospholipids and does not affect the formation of PUFA (EPO vs SAF). Only a minor effect on the fatty acid composition of phospholipids follows from incorporation of 18:3(n-3) in the diet (SOY vs SAF), namely, a small deposition of 22:6(n-3) instead of 22:5(n-6).

TABLE 2

Phosphatidylcholines, Phosphatidylethanolamines and Cardiolipins of Rat Liver Mitochondrial Membranes

Phospholipid	PC			PE			CL		
Dietary group[a]	EPO	SAF	SOY	EPO	SAF	SOY	EPO	SAF	SOY
Fatty acid[b]									
16 ald[c]	–	–	–	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	–	–	–
16:0	20.1 ± 0.5	18.3 ± 0.4	20.3 ± 0.7	15.9 ± 1.1	14.0 ± 0.3	15.6 ± 0.7	3.7 ± 0.3	2.9 ± 0.7	3.1 ± 0.4
16:1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	–	0.1 ± 0.0	0.2 ± 0.0	3.2 ± 0.2	3.2 ± 0.5	3.4 ± 0.3
18 ald	–	–	–	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	–	–	–
18:0	24.8 ± 0.8	24.0 ± 0.8	25.6 ± 0.5	26.9 ± 0.6	26.9 ± 0.2	27.4 ± 0.4	1.1 ± 0.1	0.7 ± 0.2	0.7 ± 0.1
18:1	3.9 ± 0.2	4.0 ± 0.1	4.7 ± 0.1	4.2 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	14.3 ± 0.5	15.0 ± 0.7	16.6 ± 0.8
18:2	7.7 ± 0.5	8.0 ± 0.2	8.8 ± 0.5	4.8 ± 0.2	4.5 ± 0.1	4.6 ± 0.2	67.7 ± 1.9	69.4 ± 2.5	68.4 ± 2.0
18:3(n-6)	0.2 ± 0.0^{B}	0.1 ± 0.0^{A}	0.1 ± 0.0^{A}	0.2 ± 0.0	0.1 ± 0.0	–	0.6 ± 0.1	0.5 ± 0.0	–
18:3(n-3)	–	–	–	–	–	–	–	–	0.6 ± 0.1
20:2	–	0.4 ± 0.0	–	–	0.4 ± 0.0	–	3.1 ± 0.4	3.2 ± 0.6	2.3 ± 0.3
20:3(n-6)	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.3	1.0 ± 0.7	0.1 ± 0.0	0.1 ± 0.0	2.3 ± 0.1	2.8 ± 1.0	2.3 ± 0.3
20:4(n-6)	37.6 ± 0.7^{B}	37.0 ± 0.7^{B}	32.9 ± 0.7^{A}	33.0 ± 0.8^{B}	33.9 ± 0.9^{B}	30.6 ± 0.2^{A}	3.1 ± 0.2	2.9 ± 1.0	2.1 ± 0.2
22:4(n-6)	0.8 ± 0.1	0.7 ± 0.0	–	1.8 ± 0.1^{C}	1.3 ± 0.0^{B}	0.5 ± 0.3^{A}	–	–	–
22:5(n-6)	3.8 ± 0.4	4.1 ± 0.1	–	7.7 ± 0.7	8.1 ± 0.4	–	–	–	–
22:5(n-3)	–	–	–	–	–	1.1 ± 0.1	–	–	–
22:6(n-3)	0.3 ± 0.2^{A}	1.7 ± 0.1^{B}	6.2 ± 0.3^{C}	3.3 ± 0.8	4.2 ± 0.3	14.6 ± 0.3	–	–	–
μg Pi/mg protein	4.3 ± 0.2	4.4 ± 0.1	3.9	3.1 ± 0.1	3.0 ± 0.1	2.9	0.8 ± 0	0.6 ± 0	0.8

$A < B < C$; $p < 0.05$ by Student's t-test.

[a]Averages of 4 different pools.

[b]Weight percentages determined by GLC.

[c]Dimethyl acetals from plasmalogens.

TABLE 3

Phosphatidylinositols of Rat Liver

Dietary group[a]	EPO	SAF	SOY
Fatty acid	(%)[b]	(%)	(%)
16:0	4.4 ± 1.0	4.1 ± 0.3	3.8 ± 0.3
18:0	47.2 ± 1.4	44.6 ± 0.5	44.3 ± 0.4
18:1	1.7 ± 0.3	1.5 ± 0.1	2.0 ± 0.2
18:2	3.0 ± 0.3	2.9 ± 0.1	4.2 ± 0.1
18:3(n-6)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
18:3(n-3)	–	–	–
20:3(n-6)	0.5 ± 0.1	0.4 ± 0.1^{A}	1.1 ± 0.2^{B}
20:4(n-6)	39.2 ± 2.9	42.2 ± 0.9^{B}	38.5 ± 0.6^{A}
22:4(n-6)	0.9 ± 0.1^{B}	0.7 ± 0.1^{B}	0.3 ± 0.1^{A}
22:5(n-6)	1.1 ± 0.1^{B}	0.8 ± 0.1^{B}	0.2 ± 0.0^{A}
22:5(n-3)	–	–	0.6 ± 0.1
22:6(n-3)	0.6 ± 0.1^{A}	0.8 ± 0.2^{A}	2.8 ± 0.3^{B}
μg Pi/g tissue	98.2 ± 3.1	95.9 ± 2.2	95.1 ± 3.1

A < B < C; $p < 0.05$ by Student's t-test.

[a]Averages of 4 different pools.

[b]Weight percentages determined by GLC.

TABLE 4

Rat Liver Microsomes: Fatty Acid Composition and Desaturase Activity

Dietary group[a]	EPO	SAF	SOY
Fatty acid[b]	(%)	(%)	(%)
16:0	18.3 ± 0.4	17.5 ± 0.2	18.5 ± 0.4
16:1	0.7 ± 0.0	0.7 ± 0.1	0.8 ± 0.0
18:0	23.5 ± 0.3	23.8 ± 0.3	22.8 ± 1.0
18:1	5.0 ± 0.2^{A}	5.4 ± 0.2^{A}	7.5 ± 0.7^{B}
18:2	12.0 ± 0.8	13.1 ± 0.3	14.7 ± 1.5
18:3(n-6)	0.4 ± 0.0^{B}	0.2 ± 0.0^{A}	0.2 ± 0.1
18:3(n-3)	tr^{A}	tr^{A}	0.5 ± 0.1^{B}
20:1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:2	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:3(n-6)	0.3 ± 0.0^{C}	0.1 ± 0.0^{A}	0.2 ± 0.0^{B}
20:4(n-6)	32.5 ± 0.3^{B}	31.6 ± 0.6^{B}	27.0 ± 1.0^{A}
22:4(n-6)	1.1 ± 0.0^{B}	0.8 ± 0.0^{B}	0.3 ± 0.0^{A}
22:5(n-6)	4.1 ± 0.5^{B}	3.6 ± 0.2^{B}	0.3 ± 0.0^{A}
22:5(n-3)	–	–	0.4 ± 0.1
22:6(n-3)	1.1 ± 0.0^{A}	1.8 ± 0.1^{B}	5.9 ± 0.4^{C}
Δ6-Desaturase[c]	7.3 ± 0.4	8.2 ± 0.4	7.6 ± 0.6
Δ5-Desaturase[d]	72.0 ± 2.4	66.0 ± 2.7	69.3 ± 6.5

A < B < C; $p < 0.05$ by Student's t-test.

[a]Averages of 4 different pools.

[b]Weight percentages determined by GLC.

[c]Percentage conversion of [^{14}C]18:2/10 mg protein/20 min.

[d]Percentage conversion of [^{3}H]20:3/5 mg protein/20 min.

Kidney

In the kidney phospholipids (Table 9), there is a slight increase in the incorporation of 20:3 (n-6) in PC and PE, when 18:3(n-6) is added to the diet (EPO vs SAF), whereas dietary 18:3 (n-3) increases the formation of 22:6(n-3) (SOY vs SAF). An increase in 20:3(n-6) and 20:4 (n-6) is observed in the CL from rats fed EPO, whereas the incorporation of 18:3(n-6) is negligible. Likewise, the deposition of 18:3 (n-3) in the CL is insignificant, indicating that (n-3) fatty acids cannot substitute (n-6) fatty acids when an ample supply of 18:2(n-6) is given.

The kidney triglycerides (Table 5) contain the same levels of 18:3(n-6) and 18:3(n-3) as the liver triglycerides.

TABLE 5

Fatty Acid Composition of Triglycerides from Liver, Adipose Tissue and Kidney

Dietary group[a]	Liver			Adipose tissue			Kidney		
	EPO	SAF	SOY	EPO	SAF	SOY	EPO	SAF	SOY
Fatty acid[b]	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
12:0	–	–	–	–	–	–	0.37 ± 0.07	0.36 ± 0.11	0.35 ± 0.4
14:0	1.3 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	2.09 ± 0.23	1.80 ± 0.16	1.78 ± 0.08
16:0	29.5 ± 0.7	23.9 ± 2.4	28.7 ± 0.5	18.6 ± 0.9	18.5 ± 0.5	19.3 ± 0.4	29.64 ± 0.78	26.84 ± 0.78	27.70 ± 1.01
16:1	2.2 ± 0.4	2.6 ± 0.3	2.5 ± 0.3	3.4 ± 0.5	3.7 ± 0.3	3.8 ± 0.3	3.74 ± 0.65	3.93 ± 1.11	3.87 ± 0.74
18:0	3.9 ± 0.2	3.7 ± 0.3	2.7 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	3.1 ± 0.1	4.80 ± 0.18	4.74 ± 0.21	4.88 ± 0.50
18:1	15.8 ± 1.7^{A}	20.0 ± 0.7^{B}	25.5 ± 0.2^{C}	19.6 ± 0.6^{A}	21.8 ± 0.8^{B}	31.3 ± 0.6^{C}	20.45 ± 1.38	21.85 ± 2.04	29.70 ± 1.30
18:2	40.3 ± 1.7^{B}	45.7 ± 2.5^{C}	34.6 ± 1.4^{A}	50.3 ± 1.5^{B}	50.8 ± 1.4^{B}	37.0 ± 0.9^{A}	34.62 ± 1.79	37.42 ± 3.12	28.05 ± 0.62
18:3(n-6)	1.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	2.4 ± 0.1^{B}	0.2 ± 0.0^{A}	0.2 ± 0.0^{A}	1.28 ± 0.07	–	–
18:3(n-3)	–	–	2.2 ± 0.1	0.1 ± 0.0^{A}	0.2 ± 0.0^{A}	3.3 ± 0.1^{B}	–	–	1.87 ± 0.09
20:3(n-6)	–	–	–	0.6 ± 0.1^{A}	0.1 ± 0.0^{B}	0.1 ± 0.0^{B}	0.63 ± 0.13	–	–
20:4(n-6)	4.8 ± 0.8^{B}	2.5 ± 0.4^{A}	1.8 ± 0.4^{A}	1.0 ± 0.2^{C}	0.5 ± 0.0^{B}	0.4 ± 0.0^{A}	1.79 ± 0.41	1.54 ± 0.14	0.96 ± 0.04
mg TG/g wet weight	1.64 ± 0.37	2.47 ± 0.67	5.99 ± 2.25	–	–	–	–	–	–

$A < B < C$; $p < 0.05$ by Student's t-test.

[a]Averages of 4 different pools.

[b]Weight percentages determined by GLC.

TABLE 6

Phosphatidylcholines, Phosphatidylethanolamines and Cardiolipins of Rat Heart

Phospholipid	PC			PE			CL		
Dietary group[a]	EPO	SAF	SOY	EPO	SAF	SOY	EPO	SAF	SOY
Fatty acid[b]	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
16 ald	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	5.0 ± 0.4	4.5 ± 0.1	4.7 ± 0.2	–	–	–
16:0	11.9 ± 0.2	12.1 ± 0.4	12.6 ± 0.3	4.8 ± 0.2	4.9 ± 0.1	6.0 ± 0.5	2.2 ± 0.4	0.8 ± 0.1	0.9 ± 0.2
16:1	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	–	–	–	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
18 ald	–	–	–	3.8 ± 0.3	3.5 ± 0.1	3.5 ± 0.2	–	–	–
18:0	31.0 ± 0.5	30.8 ± 0.4	28.8 ± 0.5	26.5 ± 0.2	26.1 ± 0.4	28.2 ± 1.2	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
18:1	5.6 ± 0.2	6.0 ± 0.2	7.2 ± 0.1	4.3 ± 0.2	4.4 ± 0.1	4.9 ± 0.2	5.0 ± 0.3	3.4 ± 0.1	4.5 ± 0.2
18:2	8.6 ± 0.6^{A}	11.3 ± 0.2^{B}	12.5 ± 0.2^{C}	5.8 ± 0.4^{A}	6.8 ± 0.2^{B}	6.7 ± 0.1^{B}	86.3 ± 1.3^{A}	92.1 ± 0.6^{B}	90.4 ± 0.3^{B}
18:3(n-6)	0.2 ± 0.0	–	–	0.1 ± 0.0	–	–	0.3 ± 0.1	–	–
18:3(n-3)	–	0.5 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	–	0.2 ± 0.0	–	–	0.6 ± 0.0
20:2	0.3 ± 0.0	–	0.4 ± 0.1	–	–	–	0.9 ± 0.0	–	0.6 ± 0.0
20:3(n-6)	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	1.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
20:4(n-6)	34.9 ± 0.7^{B}	33.2 ± 0.4^{B}	29.1 ± 0.7^{A}	22.2 ± 0.7^{B}	22.0 ± 0.5^{B}	20.2 ± 0.3^{A}	1.9 ± 0.2	1.7 ± 0.3	1.1 ± 0.1
22:4(n-6)	1.9 ± 0.1^{C}	1.4 ± 0.2^{B}	0.7 ± 0.2^{A}	5.3 ± 0.2^{C}	4.2 ± 0.2^{B}	1.4 ± 0.0^{B}	–	–	–
22:5(n-6)	2.9 ± 0.2^{B}	2.9 ± 0.2^{B}	0.3 ± 0.0^{A}	17.8 ± 0.9	18.1 ± 0.2	1.5 ± 0.1	–	–	–
22:5(n-3)	0.2 ± 0.0	0.1 ± 0.1	1.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	2.1 ± 0.2	–	–	–
22:6(n-3)	0.5 ± 0.1^{A}	0.7 ± 0.1^{A}	4.1 ± 0.3^{B}	2.9 ± 0.2	3.9 ± 0.2	20.2 ± 0.8	–	–	–
μg Pi/g tissue	374.8 ± 7.7	414.2 ± 27.2	398.2 ± 10.8	270.9 ± 7.4^{A}	308.6 ± 11.7^{B}	286.6 ± 11.7	106.6 ± 4.8^{A}	135.3 ± 7.8^{B}	116.7 ± 3.2

$A < B < C$; $p < 0.05$ by Student's t-test.

[a]Averages of 4 different pools.

[b]Weight percentages determined by GLC.

TABLE 7

Fatty Acids in Phosphatidylethanolamines[a] of Rat Brain

Dietary group[b]	EPO	SAF	SOY
Fatty acid	(%)[c]	(%)	(%)
16 ald	5.9 ± 0.5	5.8 ± 0.4	6.3 ± 0.4
16:0	4.4 ± 0.2	4.4 ± 0.4	5.3 ± 0.6
16:1	0.7 ± 0.1	0.7 ± 0.1	0.9 ± 0.4
18:0 ald[d]	11.4 ± 0.6	11.1 ± 0.3	10.8 ± 0.3
18:0	16.0 ± 0.4	16.6 ± 0.5	16.7 ± 0.6
18:1 ald	7.3 ± 0.5	6.5 ± 0.4	7.7 ± 0.9
18:1	11.3 ± 0.8	12.8 ± 0.3	14.2 ± 0.5
18:2(n-6)	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0
18:3(n-6)	0.1 ± 0.0	–	–
18:3(n-3)	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
20:1	2.6 ± 0.1	2.8 ± 0.1	2.9 ± 0.4
20:3(n-6)	0.6 ± 0.0	0.4 ± 0.0	0.3 ± 0.1
20:4(n-6)	12.0 ± 0.2[B]	11.5 ± 0.2	10.7 ± 0.4[A]
22:4(n-6)	6.9 ± 0.1[B]	6.0 ± 0.1[B]	4.8 ± 0.2[A]
22:5(n-6)	4.0 ± 0.1	3.7 ± 0.1	–
22:6(n-3)	15.0 ± 0.5	15.4 ± 0.5	16.6 ± 0.6
μg Pi/g tissue	550 ± 50	510 ± 80	500 ± 50

A < B; $p < 0.05$ by Student's t-test.

[a]Includes plasmalogens.

[b]Averages of 4 pools.

[c]Weight percentages determined by GLC.

[d]Dimethyl acetals from plasmalogens.

TABLE 8

Fatty Acids in Phosphatidylethanolamines[a] and Phosphatidylcholines of Rat Testes

	PE			PC		
Dietary groups[b]	EPO	SAF	SOY	EPO	SAF	SOY
Fatty acid	(%)	(%)	(%)	(%)	(%)	(%)
16 ald	8.4 ± 1.4	9.9 ± 0.4	11.3 ± 0.5	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.2
16:0	18.3 ± 0.4	17.8 ± 0.4	18.3 ± 0.8	31.9 ± 1.2	33.3 ± 1.0	33.5 ± 0.7
18 ald	2.6 ± 0.5	3.2 ± 0.1	3.5 ± 0.1			
18:0	7.9 ± 0.5	7.2 ± 0.2	7.1 ± 0.2	14.4 ± 1.0	13.9 ± 1.9	15.1 ± 2.0
18:1	4.7 ± 0.2	4.5 ± 0.1	4.7 ± 0.1	9.3 ± 0.8	9.5 ± 0.7	9.3 ± 1.2
18:2	1.3 ± 0.1	1.6 ± 0.3	1.8 ± 0.1	1.8 ± 0.3	2.3 ± 0.4	1.9 ± 0.5
18:3(n-6)	–	–	–	–	–	–
18:3(n-3)	–	–	–	–	–	–
20:3(n-6)	0.4 ± 0.0	0.4 ± 0.0	0.6 ± 0.2	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.1
20:4(n-6)	23.0 ± 1.5	20.5 ± 0.5	20.1 ± 0.1	20.3 ± 0.6	18.0 ± 0.5	18.7 ± 1.2
22:4(n-6)	3.9 ± 0.7	3.1 ± 0.1	2.9 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.4 ± 0.2
22:5(n-6)	26.8 ± 1.2	27.6 ± 0.2	25.1 ± 0.4	16.4 ± 1.6	17.3 ± 0.1	15.6 ± 0.7
22:6(n-3)	–	–	1.2 ± 0.0	–	–	0.6 ± 0.0

[a]Includes plasmalogens.

[b]Averages of 4 pools.

[c]Weight percentages determined by GLC.

DISCUSSION

The major essential acid of the diet is linoleic acid. However, it has been established by Thomasson (20) that the desaturation and elongation products of linoleic acid, i.e., 18:3 (n-6), 20:3(n-6) and 20:4(n-6), possess greater EFA activity than does linoleic acid itself.

Marcel et al. (1) and Hassam et al. (2) found that the rate-limiting step in the conversion of linoleic acid into other PUFA is the initial formation of 18:3(n-6) by a Δ6-desaturation of linoleic acid. This process is regulated by product inhibition and by competitive inhibition according to Brenner (21). Since 20:3(n-6) and 20:4(n-6) are precursors for prostaglandins,

TABLE 9

Fatty Acids in Phosphatidylcholines, Phosphatidylethanolamines, Phosphatidylinositols and Cardiolipins of Rat Kidney

Phospholipid	PC			PE		
Dietary group[a]	EPO	SAF	SOY	EPO	SAF	SOY
Fatty acid	(%)[b]	(%)	(%)	(%)	(%)	(%)
14:0	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	4.2 ± 0.2	4.3 ± 0.2	4.8 ± 0.3
16:0	26.5 ± 2.2	25.0 ± 0.4	25.3 ± 0.3	8.9 ± 0.1^{A}	8.8 ± 0.2^{A}	11.0 ± 0.7^{B}
16:1	0.8 ± 0.1	0.7 ± 0.2	0.8 ± 0.0	1.3 ± 0.2	1.4 ± 0.2	1.7 ± 0.3
18:0	21.3 ± 0.8	23.4 ± 1.3	21.5 ± 0.3	23.3 ± 0.3	23.0 ± 0.2	22.4 ± 0.5
18:1	7.0 ± 0.2	7.3 ± 0.2	8.9 ± 0.3	6.5 ± 0.2^{A}	6.7 ± 0.3^{A}	8.5 ± 0.4^{B}
18:2	7.2 ± 0.1^{A}	8.3 ± 0.3^{B}	9.2 ± 0.3^{B}	3.6 ± 0.2	3.8 ± 0.2	4.1 ± 0.4
18:3(n-6)	0.1 ± 0.0	0.1 ± 0.0	–	0.1 ± 0.0^{B}	0.0 ± 0.0^{A}	–
18:3(n-3)	–	–	0.2 ± 0.0	–	–	0.3 ± 0.0
20:2	–	–	–	–	–	–
20:3(n-6)	0.8 ± 0.1^{B}	0.5 ± 0.1^{A}	0.6 ± 0.0	0.4 ± 0.0^{B}	0.1 ± 0.0^{A}	0.1 ± 0.0
20:4(n-6)	33.3 ± 1.5	31.5 ± 0.8	30.3 ± 0.3	45.8 ± 0.4	46.5 ± 0.5	44.0 ± 1.5
22:4(n-6)	0.6 ± 0.2	0.5 ± 0.1^{B}	0.3 ± 0.0^{A}	1.7 ± 0.1^{B}	1.5 ± 0.1^{B}	0.7 ± 0.1^{A}
22:5(n-6)	0.7 ± 0.1	0.7 ± 0.1	–	1.8 ± 0.1	1.8 ± 0.1	–
22:5(n-3)	–	–	0.2 ± 0.1	–	–	–
22:6(n-3)	0.4 ± 0.1^{A}	0.5 ± 0.0^{A}	1.5 ± 0.1^{B}	0.6 ± 0.0^{A}	0.7 ± 0.1^{A}	1.8 ± 0.3^{B}
μg Pi/g tissue	178 ± 10	185 ± 3	200 ± 6	144 ± 3	145 ± 3	168 ± 5

A < B; $p < 0.05$ by Student's t-test.

[a]Averages of 4 pools.

[b]Weight percentages determined by GLC.

TABLE 9 (continued)

Phospholipid	PI			CL		
Dietary group[a]	EPO	SAF	SOY	EPO	SAF	SOY
Fatty acid	(%)	(%)	(%)	(%)	(%)	(%)
14:0	–	–	–	–	–	–
16:0	10.6 ± 1.2	8.7 ± 1.6	10.7 ± 1.0	7.7 ± 0.6	6.9 ± 0.5	6.1 ± 0.5
16:1	0.4 ± 0.1	0.4 ± 0.1	1.0 ± 0.5	2.1 ± 0.2	2.3 ± 0.2	1.9 ± 0.1
18:0	48.6 ± 1.7	45.6 ± 1.8	48.1 ± 2.3	2.1 ± 0.6	2.4 ± 0.3	1.3 ± 0.2
18:1	2.3 ± 0.7	3.2 ± 0.7	3.0 ± 0.6	17.8 ± 1.0	16.8 ± 1.1	17.1 ± 0.6
18:2	2.2 ± 0.3	3.1 ± 0.5	2.1 ± 0.6	58.1 ± 2.1	64.3 ± 2.7	65.6 ± 2.7
18:3(n-6)	–	–	–	0.3 ± 0.1	–	–
18:3(n-3)	–	–	–	–	–	0.5 ± 0.0
20:2	–	–	–	1.6 ± 0.1	1.9 ± 0.2	1.3 ± 0.1
20:3(n-6)	1.2 ± 0.1	0.8 ± 0.1	1.1 ± 0.0	3.5 ± 0.2^{B}	1.9 ± 0.4^{A}	1.8 ± 0.2
20:4(n-6)	32.6 ± 3.2	36.5 ± 4.2	33.8 ± 2.1	5.8 ± 0.3^{B}	4.4 ± 0.3^{A}	3.6 ± 0.3
22:4(n-6)	0.7 ± 0.1	0.6 ± 0.0	–	–	–	–
22:5(n-6)	0.5 ± 0.2	–	–	–	–	–
22:5(n-3)	–	–	tr	–	–	–
22:6(n-3)	–	–	tr	–	–	–
μg Pi/g tissue	26 ± 3	30 ± 4	31 ± 1	39 ± 4	42 ± 3	50 ± 3

A < B; $p < 0.05$ by Student's t-test.

[a]Averages of 4 pools.

[b]Weight percentages determined by GLC.

prostacyclins and thromboxanes, it is of considerable interest to evaluate the possibilities to manipulate the PUFA content by dietary means, for instance by intake of 18:3(n-6) or 20:3(n-6), both of which are unusal fatty acids in the human diet. Previous experiments by Danon et al. (5), Oezl et al. (22) and Stone et al. (23) have established that feeding 20:3 (n-6) to rats, rabbits and humans leads to significant increases of this acid in phospholipids and triglycerides of various tissues, but no detailed examinations of the incorporation into

different phospholipid classes were performed, except for plasma.

The effects of feeding 18:3(n-6) to EFA-deficient rats have been studied in short-term experiments of 5-9 days (5,21-23). Rahm and Holman (24) and Sprecher (25) found incorporation of 18:3(n-6) and 20:3(n-6) in total liver lipids, whereas Hassam et al. (7) and Hassam (26) found 0.48-0.9% 18:3(n-6) and 3.4% 20:3 (n-6) in total liver phospholipids. No attempts were made to differentiate between phospholipid classes. In these experiments, it cannot be excluded that the initial state of EFA deficiency of the experimental animals increased the incorporation of 18:3(n-6). Furthermore, since purified 18:3(n-6) was given together with fat-free diets with or without a supplement of 18:0, no conclusions can be made as to the effect of dietary 18:3(n-6) combined with 18:2(n-6), compared to 18:2(n-6) alone.

In other studies, Hassam et al. (2,4) gave suckling rats a single dose of radiolabeled 18:3(n-6) or 18:2(n-6) and showed that in the liver as well as in the brain most of the 18:2 (n-6) was retained in the phospholipids and triglycerides, whereas 18:3(n-6) was metabolized to 20:3(n-6) and 20:4(n-6). The authors attributed this to the rate-limiting Δ6-desaturation of 18:2(n-6). The data of Hassam et al. (2,4) are not directly comparable with our data, since these authors reported the distribution of labeled fatty acids, thus reflecting the turnover of (n-6)-fatty acids within the total fatty acids of liver and brain, whereas our data give the total fatty acid compositions of individual phospholipids. Hassam et al. (2,4) did not report the fatty acid composition of the adipose tissue of the sucklings. Therefore, the influence of the diets used for the dams cannot be estimated. Since this diet apparently contained soybean oil and linseed oil at a 5:1 ratio (27), it cannot be excluded that the pups, due to the (n-3)-fatty acids from the maternal milk (27) and their own content at birth, had enough (n-3)-fatty acids deposited in liver and brain (17) to exert an inhibition of the Δ6-desaturation as described by Brenner and Peluffo (28, 29). This would reduce the conversion of labeled 18:2(n-6) given orally, but not the conversion of labeled 18:3(n-6).

The present experiments demonstrate that when rats were fed a diet containing sufficient essential fatty acids, a substitution of part of the dietary 18:2(n-6) by an equivalent amount of 18:3(n-6) does not significantly alter the content of C20- and C22-polyenoic fatty acids in PC, PE or CL from rat liver mitochondria. Likewise, the fatty acid compositions of the liver PI and of total liver microsomal lipids are unaffected.

Apparently, there is a difference between results obtained with dietary 18:3(n-6) which does not, in EFA-sufficient animals, lead to elevation of 20:3(n-6) in the liver, and dietary 20:3(n-6) which is incorporated into liver phospholipids. One explanation for this may be that 20:3(n-6) formed in situ enters a substrate pool for the hepatic Δ5-desaturase which has a high activity in the rat, as pointed out by Stone et al. (23) and confirmed by our observations, whereas dietary 20:3(n-6) enters a pool available for formation of phospholipids. This agrees with the observation by Sprecher (30) that 20:3(n-6) is incorporated in vitro into phospholipids.

The lack of any significant incorporation of 18:3(n-6) and 20:3(n-6) into liver microsomes is in agreement with the observation that Δ6- and Δ5-desaturation in vitro is not affected. In the rat fed an EFA-sufficient diet for an extended period of time, it is thus the total amount of (n-6) fatty acids received rather than the distribution between 18:2(n-6) and 18:3 (n-6) that determines the PUFA profile of the membrane phospholipids. The presence of 18:3(n-6) in the liver triglycerides as well as 18:3(n-6) and 20:3(n-6) in the adipose tissue of the EPO group indicates that dietary 18:3(n-6) is taken up in the liver, and that part of the 20:3(n-6) formed in the liver is exported to the adipose tissue, probably in very low density lipoproteins (VLDL) secreted from the liver.

An accumulation of 18:3(n-6) in blood lipids from rats fed 0.5 wt% 18:3(n-6) + 7.7 wt% 18:3(n-3) + 2.3 wt% 18:2(n-6) was found by Larking and Nye (31). These authors, however, did not detect any 20:3(n-6). Recently, Renaud et al. (32) reported that a significant increase in 20:3(n-6) from 2% to 4% of the fatty acids in plasma, platelets and aorta was observed in rabbits, following intake of EPO for 20 weeks. This increase did not affect platelet aggregation and incidence of atherosclerosis.

While the fatty acid compositions of the PC are similar in liver and heart, the profiles of PE and CL significantly more unsaturated in the heart. This may arise from higher activity of elongation systems and Δ4-desaturase in the heart than in the liver, and/or a lower activity of the retroconversion of 22:4(n-6) to 20:4 (n-6) demonstrated by Sprecher (30).

Although the 18:2 residues of cardiolipins have a higher turnover than other fatty acids (33), this does not cause a marked incorporation of 18:3(n-6), emphasizing the significance of 18:2(n-6) in this phospholipid.

When SOY is the dietary fat, giving ca.

5 wt% linoleic acid and 0.7% 18:3(n-3), general decreases in the contents of 20:4(n-6), 22:4 (n-6) and 22:5(n-6) in liver and heart are observed. These decreases are followed by increases in 22:5(n-3) and 22:6(n-3). The contents of 18:2(n-6) in the phospholipids of the SOY group studied are equal to those of the SAF group, with the exception of heart PC. This indicates that the replacement of (n-6) fatty acids by (n-3) is caused by competitive action of (n-3) fatty acids and not by the lower content of 18:2(n-6) in the SOY diet. In the PI, however, a significant increase in the content of 20:3(n-6) of the SOY group is observed.

PUFA in the brain may either be formed in the liver prior to transport to the brain, or they may be formed in the brain from their precursors (34), as the brain possesses the enzyme system for synthesis of PUFA (35).

It appears from our data that diets containing moderate amounts of γ-linolenic acid together with ample supplies of linoleic acid do not influence the fatty acid composition of PE (group EPO vs SAF), the brain phospholipid with the highest content of PUFA. The presence of 22:6(n-3) in the EPO and the SAF groups, which were fed diets with high levels of (n-6) fatty acids and nearly devoid of (n-3) fatty acids for 11 weeks, point at an essentiality of (n-3) fatty acids in the brain.

It has previously been established by Aaes-Jørgensen and Holman (36) that the content of polyenes in testes is subject to considerable changes during the first 18 weeks after weaning. Later it was demonstrated by Kirschman and Coniglio (37) that the content of 22:5(n-6) increases 3-fold from 3 weeks of age to 3 months of age. Furthermore, it has been reported that testes respond readily to alterations in the dietary fatty acids (38). It is, therefore, of particular interest that dietary 18:3(n-6) in the form of EPO does not affect the testicular fatty acid composition. Apparently, the primary factor influencing the testis lipids is the total supply of (n-6) fatty acids and not the distribution between 18:3(n-6) and 18:2(n-6). Only a minor effect on the fatty acid profiles follows from incorporation of 18:3(n-3) in the diet (SOY vs SAF), namely, a small deposition of 22:6(n-3) instead of 22:5(n-6). This is probably due to the ample supply of 18:2(n-6) from the SOY, since it has been shown by Ayala et al. (39) that (n-3) PUFA replace (n-6) PUFA in testes of rats fed fish oils rich in (n-3) fatty acids, but nearly devoid of (n-6) fatty acids.

Tinker and Hanahan (40) have shown that in rabbit renal slices there is an active turnover of all phospholipids. The turnover for PI was larger than for PC and PE. This has also been confirmed in vivo by Soula et al. (41). It is, therefore, not surprising that an incorporation of 20:3(n-6) is found in PI. However, the incorporation of 20:3(n-6) in renal phospholipids following intake of 18:3(n-6) in the form of EPO is much smaller than after intake of 20:3(n-6), as described by Danon et al. (5) for rat kidney total phospholipids and by Knapp et al. (6) for rabbit renal PC, PE or PI+PS. The changes in the fatty acid composition of renal PI indicate that 20:3(n-6) cannot be substituted by a dietary supply of 18:3(n-6).

In kidney triglycerides, the incorporations of 18:3(n-6) and 18:3(n-3) are of the same order of magnitude as in liver triglycerides indicating that these fatty acids have been available for acylation of glycerophosphate. The deposition of 20:3(n-6) following feeding of 18:3 (n-6) is much smaller than reported by Danon et al. (5), following feeding of 20:3(n-6). This supports the hypothesis that 20:3(n-6) formed endogenously is rapidly desaturated into 20:4 (n-6) in contrast to exogenous 20:3(n-6) that is more abundant for synthesis of phospholipids and triglycerides.

The negligible influence of the content of 18:3(n-6) in EPO compared with the 18:2(n-6) in SAF on the composition of various membrane phospholipids from a variety of organs is of particular interest, since it is believed that fatty acids acting as precursors for prostaglandin production in most tissues are liberated from membrane phospholipids by action of phospholipase A_2 (42) or by sequential degradation of PI by the pathway suggested by Bell et al. (43) or Lapetina and Cuatrecasas (44). Manipulations by dietary means of the pools of prostaglandin precursors are possible, as has been demonstrated by Knapp et al. (6), who increased the production of PGE_1 in renal papillae 20-fold by a diet containing 20:3(n-6). Unless there exists a further compartmentalization of the pools of prostaglandin precursors beyond the different phospholipid classes, it thus seems unlikely that inclusion of realistic amounts of 18:3(n-6) in the diet should produce a similar effect on the pools for prostaglandin production in liver, heart, brain, testes or kidney under the conditions used in this experiment.

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Growth of a Sterol Auxotroph Derived from *Saccharomyces cerevisiae* on Chemically Synthesized Derivatives of Cholesterol Possessing Side-Chain Modifications

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ABSTRACT

A number of cholesterol derivatives were analyzed for their ability to satisfy bulk membrane and high-specificity sparking requirements of a yeast sterol auxotroph (RD5-R) (Rodriguez, R. J., Taylor, F. R., and Parks, L. W. [1982], Biochem. Biophys. Res. Commun. 106, 435-441). Substitution of hydrogen by bromine or iodine at C-26 or substitution of C_{26}-methyl by bromine enabled the resulting sterol to satisfy bulk or sparking functions. The presence of a side-chain hydroxyl or keto group at C-25 on a 26-norcholesterol completely abolished the ability of cholesterol to satisfy either sterol requirement. Growth studies revealed that, while the oxygenated cholesterol derivatives were not growth-supportive of RD5-R, they were not growth-inhibitory.
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Recent observation of yeast sterol auxotrophs revealed that sterols are required for 2 separate classes of functions in *Saccharomyces cerevisiae* (1). These classes have been designated bulk membrane and high-specificity sparking functions. The distinction between these 2 classes is illustrated by the fact that sterol auxotrophs were capable of growing on cholestanol only when minute quantities of ergosterol were available. The minute concentration of ergosterol alone was unable to support growth. This phenomenon has been designated the "sparking" of growth (1). These results were not observed in previous experiments (2) because of sterol contamination in commercially available cholestanol and in cells used for inocula (1). Evidence for diverse roles for sterols has been observed in *Dermestes vulpinus* (3) and *Mycoplasma capricolum* (4,5).

Since ergosterol satisfies both sterol functions and cholestanol only fulfills bulk membrane function (1), it is apparent that there are structural specificities associated with the ability of a sterol or stanol to satisfy bulk membrane and/or sparking requirements. This report is concerned with the effect of the sterol side chain on bulk membrane and sparking functions.

It has been reported that in order for sterols to be used as yeast membrane components, they must possess a C-3 hydroxyl, a planar nucleus and an aliphatic side chain (6). Under anaerobic conditions, it appears that the C-28 methyl of ergosterol is required for growth (7). This is not the case with aerobically cultured sterol auxotrophs (8), although changes in the C-28 methyl of ergosterol does cause fluctuations in fatty acid pools (9).

In this paper, we present data on the ability of cholesterol and cholesterol derivatives to satisfy bulk membrane and sparking requirements of a yeast sterol auxotroph. We report that cholesterol, like ergosterol, is able to satisfy both classes of sterol function. Thus, the lack of the C-28 methyl of ergosterol does not seem to affect a sterol's ability to fulfill either class of function. Substitution of a bromide or iodide atom onto the side chain of cholesterol does not alter its ability to satisfy either sterol requirement. However, addition of a hydroxyl or keto group to the cholesterol side chain renders the sterol unsuitable for either bulk membrane or sparking function.

MATERIALS AND METHODS

Yeast Strain and Culture Conditions

S. cerevisiae strain RD5-R was used in all experiments. This strain is auxotrophic for sterol, unsaturated fatty acid, and methionine as a result of *hem*1 and *erg*3 mutations. Cultures were aerated in a Scientific Industries rocking gradient incubator operated isothermally at 28 C. Growth was monitored in a photoelectric colorimeter equipped with a green filter.

To determine whether various sterols and stanols satisfied bulk membrane requirements, inocula were derived from auxotrophic strains

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grown in medium containing 5 μg/ml of ergosterol. The cells were harvested, and washed twice with medium lacking sterol. Pellets were resuspended in the same volume of sterol-less medium, and 0.02 ml transferred to 5 ml fresh medium containing 5 μg/ml of the sterol or stanol of interest. These cells contained enough ergosterol to spark growth when transferred to medium (1:250 dilution) containing cholestanol (1). To assay lipids for their ability to satisfy sparking requirements, inocula cells were depleted of internal sparking levels of sterol as follows: the sterol auxotrophs were cultured to stationary phase; 0.02 ml of cells were then transferred to fresh medium containing cholestanol. The cholestanol culture was grown to stationary phase, harvested by centrifugation (500 x g for 2 min), washed and resuspended to the same volume with medium devoid of sterol. Then, 0.02-ml aliquots were added to 5 ml fresh media containing 5 μg/ml of the sterol or stanol to be analyzed.

Steroidal Substrates

The syntheses of 26-bromo- (10), 26-iodocholesterol (10) and 25-dehydrocholesterol (11) were previously reported from our laboratory. The 26-nor-25-keto cholesterol acetate was prepared from pregnenolone (12).

Reduction of 26-nor-25-keto-cholesterol 3-acetate (sodium borohydride; dioxane/water, 10:1, at 10 C) gave 26-nor-25(RS)-hydroxycholesterol. Treatment of the 26-nor-25-hydroxy-3-acetate with N-bromosuccinimide and triphenylphosphine resulted in 26-nor-25(RS)-bromocholesterol acetate, which was hydrolyzed (5% aq H_2SO_4 in dioxane; 80 C, 1 hr) to yield 26-nor-25(RS)-bromocholesterol (Mason, J. I., Arunachalam, T., and Caspi, E., manuscript submitted). The structure and homogeneity of all compounds were established by thin layer chromatography (TLC), nuclear magnetic resonance (NMR) and mass spectrometry (MS).

Sterol Extraction and Analysis

RD5-R was grown in 10 ml defined medium to stationary phase and sterols were extracted by acid labilization (13) followed by alkaline saponification (14). Samples were extracted with hexane, evaporated, resuspended in 0.1 ml of isopropanol, and then analyzed by high pressure liquid chromatography (HPLC) as previously described (15). Sterols were also analyzed by gas liquid chromatography (GLC) (16).

Materials

All solvents were of reagent grade and redistilled prior to use. Medium supplies were from Difco (Detroit, MI) and HPLC equipment was from Beckman (Palo Alto, CA).

RESULTS

Growth of RD5-R on Cholesterol and Cholesterol Derivatives

The sterols used in this study were analyzed for their ability to satisfy bulk membrane and sparking requirements of RD5-R (1). To determine if the various sterols (Table 1) were able to fulfill the bulk membrane function alone, it was necessary to provide the cells with nanogram quantities of a sterol which would satisfy the sparking requirement. This was accomplished by using cells from a culture grown on medium containing ergosterol. As previously reported (1), ergosterol-grown cells contain enough residual sterol to spark growth and as a result will grow in medium containing cholestanol. As shown in Table 1, all of the sterols tested, with the exception of the oxygenated sterols, supported growth of these cells and therefore satisfied the bulk requirement.

TABLE 1

Growth of RD5-R on Sterols with Different Side-Chain Modifications

Sterol	Ability to satisfy	
	Bulk requirements	Sparking requirements
1. 26-nor-25(RS)-25-Bromocholesterol	+	+
2. 26-Bromocholesterol	+	+
3. 26-nor-25-Hydroxycholesteryl-3-acetate	–	–
4. 26-nor-25-Ketocholesteryl-3-acetate	–	–
5. 26-nor-25(RS)-Hydroxycholesterol	–	–
6. 26-nor-25-Ketocholesterol	–	–
7. Cholesta-5,25-diene-3β-ol	+	+
8. 26-Iodocholesterol	+	+
9. Cholesterol	+	+

To establish if the sterols were able to fulfill the sparking requirements as well as bulk function, it was necessary to prepare inocula cells which lacked sufficient intracellular sterol to spark growth. This was accomplished by cycling ergosterol grown cells on cholestanol. These cells were incapable of growth when transferred to fresh medium containing only cholestanol (1), indicating that they were devoid of sparking levels of ergosterol. It is important to note that, in order for cholestanol cycled cells to grow when transferred to fresh medium containing a sterol of interest (5 μg/ml), the sterol must satisfy both bulk membrane and sparking functions. As shown in Table 1 and Figure 1, the same results were obtained as for bulk membrane analyses. All sterols tested, with the exception of the oxygenated sterols, satisfied both sterol requirements. To ensure that these results were not due to in vivo modification of sterols, stationary phase cells were analyzed by HPLC and GLC for sterol content. In every case, the respective sterols were found unaltered by the cells.

The oxygenated sterols were also shown to be unable to satisfy the sparking requirement alone. This was ascertained by inoculating cholestanol cycled cells into media containing an oxygenated sterol (10 ng/ml) and cholestanol (5 μg/ml). Since cholestanol was able to satisfy bulk membrane function (1), the oxygenated sterols were available for the sparking requirement. As seen in the above experiments, the oxygenated sterols were unable to satisfy the sparking function.

It was of interest to determine why 26-nor-25-keto- and 25-hydroxycholesterols were incapable of satisfying either sterol requirement. It is possible that these oxygenated sterols were growth-inhibitory or were not transported into the cells. Cells grown in medium containing cholesterol (1 μg/ml) with and without 26-nor-25-keto- or 25-hydroxycholesterol (5 μg/ml) were analyzed for growth rate and final cell yield in order to determine if the oxygenated sterols were growth inhibitory. As shown in Table 2, growth rates of RD5-R in the presence of either oxygenated sterol were ca. 15% lower than with cholesterol alone. However, final cell densities were the same in all 3 cultures. To assess transport of the oxygenated sterols, stationary phase cells from the same cultures were analyzed by HPLC and GLC for their sterol content. Cholesterol was detected in all 3 cultures to approximately the same extent. However, in both cultures supplemented with

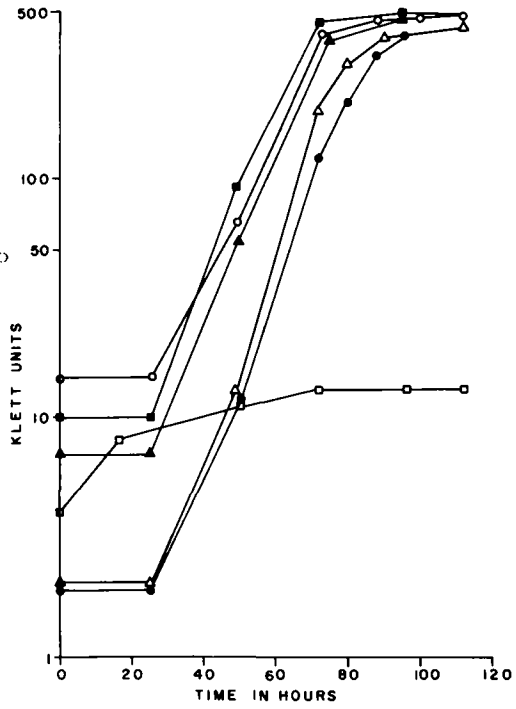

FIG. 1. Growth of RD5-R on cholesterol (▲); 26-bromocholesterol (△); 26-nor-25-bromocholesterol (●); $\Delta^{5,25}$-cholestadienol (■); 26-iodocholesterol (○); and 26-nor-25-hydroxycholesterol or 26-nor-25-ketocholesterol (□). The curve for the 25-keto was virtually identical to that of the 25-hydroxy derivative, differing at some points by only 4 Klett units. Inocula for these growth studies were derived from cells which had been cycled on cholestanol.

TABLE 2

Growth of RD5-R in the Presence and Absence of Oxygenated Sterols

Sterol supplement	Growth rate (doublings/hr)	Final cell yield (Klett units)
Cholesterol	0.20	450[a]
Cholesterol + 26-nor-25-ketocholesterol	0.17	450[b]
Cholesterol + 25-hydroxycholesterol	0.17	450[b]

[a]Culture grew to this density in 51 hr.

[b]Culture grew to this density in 60 hr.

an oxygenated sterol, the respective oxygenated sterol was found to be incorporated to levels greater than those of cholesterol, indicating functional transport.

DISCUSSION

It is apparent that sterols satisfy 2 separate classes of functions in yeast cells (1). One class involves bulk membrane function(s) and the other a high-specificity sparking requirement. Although some sterols can satisfy both functions, others satisfy only the bulk requirement (1) or are unsuitable for either requirement (Table 1). Since cholestanol (Δ^0) does not satisfy the sparking requirement (1) but cholesterol (Δ^5) does, it appears that a C-5,6 unsaturation is critical with regard to the sparking phenomenon. Clearly, the lack of the C-24 methyl group of ergosterol has no effect on the ability of a sterol to fulfill the sparking function since cholesterol will spark RD5-R.

The ability of a Δ^5-sterol to satisfy sparking requirements can be abolished by certain modifications of the sterol side chain. This was observed when an oxygen atom in the form of a keto or hydroxy moiety was substituted for a methyl group at the C-25 position. Both oxygenated derivatives of cholesterol were ineffectual in satisfying sterol requirements. However, addition of a bromide or iodide atom, or a C-25 (26) unsaturation did not affect the ability of cholesterol to satisfy either sterol function.

The slight decrease in growth rates observed in the presence of the oxygenated sterols and cholesterol is not sufficient to explain the inability of the oxygenated sterols to satisfy either sterol function. In addition, this inability cannot be ascribed to a nonfunctional transport for the oxygenated sterols. It is possible that the oxygenated sterols, although transported into the cells, are not utilized as membrane components. One way cells can ensure that unsuitable sterols are not incorporated into membranes is to esterify them to long-chain fatty acids and deposit them in lipid droplets (17). Although the oxygenated sterols are found in the steryl ester fraction, the situation appears to be more complicated since they are also present, to a lesser degree, in the free sterol pool (data not shown). More experimentation is needed to understand this phenomenon better. It is possible that the inhibitory component is a degradative product of the oxygenated analogs.

RD5-R exhibits selectivity with regard to sterols capable of satisfying sterol requirements. This appears to be a relatively specific process but either nuclear (saturation of a C-5(6) double bond) (1) or side-chain alterations (substitution of a keto or hydroxy moiety for C-26 on the side chain) significantly alter the ability of a sterol to satisfy bulk membrane and/or sparking functions.

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Fatty Acid Composition of Triglycerides from Adipose Tissue Transplanted between Obese and Lean Mice

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ABSTRACT

The subcutaneous adipose tissue of genetically obese mice (ob/ob) differs from that of lean littermates not only by virtue of its larger cells but also in its fatty acid composition; it contains a higher proportion of palmitoleic acid and a lower proportion of linoleic acid. To determine whether these differences in fatty acid composition were inherent in fat cells, subcutaneous adipose tissue from obese and lean mice was transplanted under the kidney capsules of lean and obese host mice and the fatty acid composition of the neutral lipids of the graft and of the host perirenal and subcutaneous fat was determined 1 or 2 months later. The fatty acid composition of grafts from lean donors in obese mice resembled that of the perirenal adipose tissue of the obese hosts after 1 month, with a lower proportion of linoleic acid and a higher proportion of palmitoleic acid than in lean mice. Grafts from obese mice in lean mice had fatty acid compositions which were either unchanged, partially changed or which completely resembled that of the host. The use of grafts prelabeled by feeding the donor margaric acid indicated that total lack of fatty acid turnover, rather than selective metabolic processes, was responsible for the failure of some grafts from obese mice in lean mice to acquire the fatty acid composition of the perirenal adipose tissue of the host.
Lipids 18:776-780, 1983.

INTRODUCTION

Adipose tissue from obese-hyperglycaemic mice is characterized by a fatty acid composition with a higher proportion of palmitoleic acid and a lower proportion of linoleic acid than that of adipose tissue from lean mice (1-3). These differences arise from increased rates of fatty acid synthesis in the liver and adipose tissue of obese mice (4-6), causing a greater dilution of the dietary linoleic acid, and also from increased synthesis of palmitoleic acid by the raised activity of acyl-CoA Δ9 desaturase (7). The lipogenic enzymes, acetyl-CoA carboxylase (8), fatty acid synthetase (8,9) and acyl-CoA desaturase (10), are insulin-dependent. However, it is not clearly established whether their increased activities in obese mice are dependent on the prevailing hyperinsulinaemia, although procedures which decrease the concentration of plasma insulin may lower their activities (3,11). The aim of this study was to determine whether normalization of the physiological environment would return the fatty acid composition of adipose tissue of obese mice to normal. The transplantation of adipose tissue from obese into lean mice provides a way of rapidly transforming the physiological environment. As previous studies (12-14) show such grafts to be viable, this technique was employed to determine whether normalization of environment results in normalization of the fatty acid composition.

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METHODS AND MATERIALS

Animals

Male, C57Bl/6J-ob mice were grafted at 6-8 weeks old. In the first experiment, obese mice (ob/ob) and their lean littermates (ob/+ or +/+) were from Olac Ltd., Bicester, UK and, in the second experiment, obese mice (ob/ob) and closely related lean mice (+/+), obtained by selecting out the ob gene, were from the colony at the Meat Research Institute, derived from C57Bl/6J-ob stock of the Jackson Laboratory, Bar Harbor, MA. Mice were given water and a commercial pelleted diet (Spratts Laboratory Diet No. 1, containing 22% protein, 3.7% lipid and 48.0% available carbohydrate, from Spillers Ltd.) ad libitum and housed singly after the transplant procedure.

Transplantation

Adipose tissue from donor lean or obese mice was transplanted under one kidney capsule of obese and lean mice as described previously (12). Donor adipose tissue was obtained from the subcutaneous depot lying over the lateral aspect of the posterior part of the cutaneous maximus muscle, just anterior to the muscles of the upper hind limb. In the first experiment, all mice were killed by cervical dislocation after 1 month. The grafts were carefully removed, rinsed in saline and placed in solvent for lipid extraction. In the second experiment, the adipose tissue of the donor

obese mouse was labeled with additional margaric acid by feeding methyl margarate, 0.2 ml/day, by stomach tube for 5 days, ending 3 days before grafting. Grafted lean hosts were killed 1 or 2 months later. Samples, 20-100 mg, of perirenal adipose tissue adjacent to the transplant and subcutaneous adipose tissue from the same site as the donor were taken from all mice at slaughter.

Lipid Extraction and Analysis

Grafts and samples of perirenal and subcutaneous adipose tissue were washed in isotonic saline, blotted and placed in 5 ml of chloroform/methanol (2:1, v/v) containing 2,6-di-*tert*-butyl-*p*-cresol (BHT, 100 mg/l) as antioxidant. After homogenization, the extract was filtered, evaporated to dryness at 60 C and taken up in 2.0 ml heptane. Phospholipids were removed by adding 50 mg of silicic acid (Mallinckrodt, 100 mesh), followed, immediately after mixing, by 2.0 ml of chloroform. The solution of neutral lipids was decanted and the fatty acids prepared after saponification in the usual manner (7). These were methylated with diazomethane and the methyl esters analyzed isothermally at 180 C on a column (7 ft × 1/8 in.) containing 12% polyethylene glycol adipate on 100-120 mesh Gas-Chrom Q. Peaks were identified by comparison with standards and were quantified with an Infotronics 304-50 computing integrator (LDC Division, Milton Roy Inc.).

Materials

Solvents were of analytical grade where available and were distilled before use. Fatty acid standards (Applied Science Division, Milton Roy Inc.) were obtained from Pierce Warriner (UK) Ltd., and methyl margarate, ca. 95%, was from Sigma Chemical Co. (St. Louis, MO).

Statistics

Results were analyzed by Student's t-test and differences between means where $P<0.05$ were taken to be significant.

RESULTS

In the first experiment, 10 lean mice and 10 obese mice received adipose tissue grafts. Five of the lean mice and 5 of the obese mice received a graft of fat from a lean donor mouse and 5 mice of each genotype received a graft of fat from an obese donor mouse. Two lean and 2 obese donors were used. Only 2 obese host mice did not survive transplantation. Grafts were recovered 1 month later from all except

TABLE 1

Fatty Acid Composition of Adipose Tissue from Lean and Obese Donor and Host Mice

	Fatty acids (% by weight)[a]							
	14:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2
Lean donors(2)[b] Expt. 1 subcutaneous	4.7 ± 0.5	28.9 ± 1.9	7.7 ± 0.6	0.20 ± 0.02	0.25 ± 0.00	3.2 ± 0.4	28.7 ± 0.4	19.0 ± 0.7
Obese donors (2) Expt. 1 subcutaneous	4.8 ± 2.2	27.3 ± 0.2	10.8 ± 0.2	0.16 ± 0.01	0.25 ± 0.02	2.3 ± 0.2	32.2 ± 3.7	13.3 ± 0.1
Obese donor (1) Expt. 2 subcutaneous	5.2	27.2	12.5	1.73	1.55	2.2	28.3	14.1
Lean hosts (10) Expt. 1,2 subcutaneous	1.7 ± 0.1	26.2 ± 0.5	9.0 ± 0.3	0.18 ± 0.01	0.19 ± 0.03	3.4 ± 0.4	33.8 ± 0.3	20.7 ± 0.7
	***c	NS	***	***	NS	**	***	***
Obese hosts (7) Expt 1 subcutaneous	2.3 ± 0.2	25.2 ± 0.4	12.7 ± 0.2	0.13 ± 0.01	0.24 ± 0.03	2.0 ± 0.1	38.7 ± 0.8	14.2 ± 0.1
Lean hosts (10) Expt 1,2 perirenal	2.1 ± 0.2	26.8 ± 0.9	8.8 ± 0.4	0.17 ± 0.01	0.20 ± 0.04	4.3 ± 0.5	33.3 ± 0.9	18.5 ± 1.1
	NS	NS	***	***	NS	**	*	***
Obese hosts (7) Expt. 1 perirenal	1.9 ± 0.1	28.0 ± 0.5	13.4 ± 0.3	0.09 ± 0.01	0.23 ± 0.01	2.2 ± 0.2	36.4 ± 0.6	12.7 ± 0.5

[a]% by weight of total fatty acids. Minor components to 100%.
[b]Results are means ± SEM for the number of mice in parenthesis. Donor samples were analyzed when tissue was taken for grafting. Host samples were taken at the time grafts were removed.
[c]Significance of difference between obese and lean mice: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, not significantly different, $P>0.05$, by Student's t-test.

1 obese mouse transplanted with tissue from an obese donor, and 1 lean mouse, transplanted with tissue from a lean donor, in which no graft could be seen. The 9 host lean mice grew from 18.6 ± 0.5 g to 26.8 ± 0.8 g and the 7 obese hosts from 20.9 ± 0.6 g to 38.6 ± 0.5 g. The obese donors had a lower percentage of linoleic acid (18:2) and a higher percentage of palmitoleic acid (16:1) than the tissue from the donor lean mice (Table 1). These differences were also present in the subcutaneous and perirenal adipose tissue of lean and obese host mice 1 month after grafting (Table 1).

Transplants between mice of the same type had similar proportions of 16:1 and 18:2 to the host perirenal fat (Table 2). Grafts from lean mice placed under the kidney capsule of obese mice also took on the fatty acid composition of the host's perirenal fat with no significant difference ($P>0.05$) in the proportions of palmitoleic acid and linoleic acid. These effects are amplified by considering the changes in the ratio of 18:2 to 16:1 (L:P ratio). The mean L:P ratio of subcutaneous fat from lean mice was 2.4 and this fell only slightly to 2.2 when it was transplanted into lean mice. However, the L:P ratio fell to 1.0 when lean fat was transplanted into obese hosts. The L:P ratio of obese donor fat also decreased slightly when grafted into obese mice, from 1.2 to 0.8. However, grafts from obese donors in lean hosts displayed varied fatty acid compositions. Two of the grafts had similar proportions of linoleic acid and palmitoleic acid to those in the donor subcutaneous adipose tissue at the time of grafting, whereas 2 other grafts had changed their fatty acid composition to resemble that of the host adipose tissue (results not shown). Those grafts in which the fatty acid composition was unchanged could either have retained the metabolic characteristics of the obese donor or, more likely, may have lost the ability to turn over their fatty acids.

A second experiment was, therefore, set up to test these possibilities. An obese donor mouse was fed methyl margarate to increase the concentration of margaric acid in its adipose tissue. At the time of grafting, the subcutaneous tissue of this mouse contained 1.7% margaric acid compared with the usual amounts which do not exceed 0.3% (Table 1). Thus, any turnover of the fatty acids in the grafted tissue would result in a decrease in the proportion of margaric acid, since it could not be replaced by the low concentrations present in the host tissue, and as the cells of the graft would be decreasing in size, falls in the proportion of margaric acid could not be due to dilution by deposited fatty acids.

The obese donor, aged 34 days, weighed 15 g at the time of transplantation and reached 48 g 2 months later. Subcutaneous adipose tissue from this mouse was grafted under the kidney capsule of 8 lean mice 6 weeks of age. Four lean hosts were killed 1 month after transplantation and the other 4 were killed after 2 months. The group of lean hosts, killed 1 month after transplantation, had increased

TABLE 2

Proportion of Palmitoleic Acid and Linoleic Acid in Donor, Host and Graft Adipose Tissue

		Fatty acid (% by wt) Adipose tissue sample			
Sample	Fatty acid	Donor subcutaneous	Host subcutaneous	Host perirenal	Graft
"Lean" graft in lean host	Palmitoleic	7.8 ± 0.6(2)[a]	9.6 ± 0.2(4)	8.6 ± 0.7(4)	9.2 ± 0.8(4)[b]
	Linoleic	19.0 ± 0.7	21.6 ± 0.6	20.5 ± 1.4	20.4 ± 0.8
	L:P ratio	2.4 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	2.2 ± 0.2
"Lean" graft in obese host	Palmitoleic	7.8 ± 0.6(2)	13.0 ± 0.3(3)	13.6 ± 0.1(3)	13.2 ± 0.5(3)
	Linoleic	19.0 ± 0.7	14.1 ± 0.3	13.5 ± 0.5	13.1 ± 0.3
	L:P ratio	2.4 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
"Obese" graft in obese host	Palmitoleic	10.8 ± 0.2(2)	12.4 ± 0.4(4)	13.2 ± 0.6 (4)	14.0 ± 1.5(4)
	Linoleic	13.4 ± 0.2	14.2 ± 0.1	12.3 ± 0.7	11.2 ± 0.8
	L:P ratio	1.2 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
"Obese" graft in lean host	Palmitoleic	12.5(1)	8.4 ± 0.4 (6)	8.9 ± 0.4(6)	9.6 ± 1.1(6)
	Linoleic	14.2	20.0 ± 0.1	17.1 ± 1.3	20.6 ± 1.2
	L:P ratio	1.1	2.4 ± 0.1	1.9 ± 0.2	2.2 ± 0.3

[a]Results are expressed as means ± SEM with number of animals in parenthesis.
[b]There were no significant differences ($P>0.05$) between host perirenal fats and grafts in the proportions of palmitoleic acid, linoleic acid or the L:P ratio in any treatment group.

in weight from 16.8 ± 1.0 g to 23.4 ± 0.5 g and those killed after 2 months had grown from 16.3 ± 1.5 g to 25.1 ± 1.5 g. Of the 4 grafts recovered after 1 month, 3 had acquired similar proportions of linoleic acid to the host tissues and 1 retained the donor levels of linoleic acid together with 1.3% margaric acid. A similar pattern was observed in 4 grafts removed after 2 months. One graft had a very low proportion of linoleic acid and 1.7% margaric acid, as in the initial graft. The other 3 grafts had similar proportions of linoleic acid and palmitoleic acid to the hosts. The results for the 6 mice with concentrations of margaric acid below 1.0% have been combined in Table 2. The L:P ratio had increased from 1.1 in the donor tissue to a mean of 2.2 when the grafts were removed similar to the 1.9 in host perirenal fat. The relationship between the proportions of margaric acid and linoleic acid in the lipids of the grafts of experiment 2 is shown in Figure 1. The regression shows a significant ($P<0.01$) negative correlation between the proportions of these 2 fatty acids.

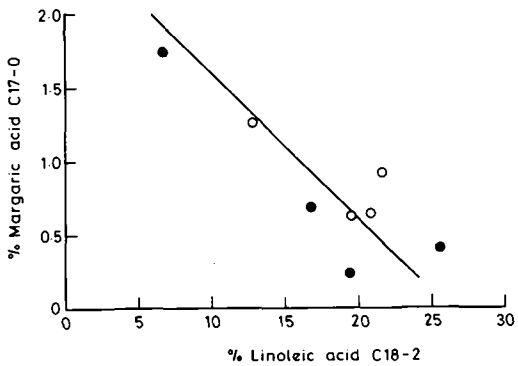

FIG. 1. Relationship between the percentages of margaric acid and linoleic acid in the grafts from an obese donor mouse under the perirenal capsule of lean host mice. (○) Grafts removed after 1 month; (●) grafts removed after 2 months.

DISCUSSION

These results clearly demonstrate that the differences in the fatty acid composition of adipose tissue neutral lipids between obese and lean mice depends on the physiological characteristics of the animal and not on genetic differences in the adipose tissue. This is similar to the results of studies which have demonstrated that transplanted fat cells assume the size characteristics of the host adipose tissue (12-14). The decreased proportion of linoleic acid in the adipose tissue triglycerides of obese mice results from a dilution of the dietary linoleic acid by excessive fatty acid synthesis, de novo, associated with hyperphagia. Similar changes in fatty acid composition have been reported as a result of hyperphagia induced by gold-thioglucose treatment of rats (15). If the high rates of fatty acid synthesis were an inherent property of adipocytes from obese mice, a dilution of linoleic acid might have been expected in adipocytes from obese mice grafted into lean hosts, even if cell shrinkage were taking place. Equally, retention of a high acyl-CoA Δ9 desaturase activity would have been expected to maintain higher than normal proportions of palmitoleic acid. There was no evidence for either effect: linoleic acid increased and palmitoleic acid decreased. While lipogenesis depends on the supply of glucose as a substrate, it is possible that the reported lower sensitivity of adipocytes from obese mice to the stimulation of glucose uptake by insulin (16) might limit lipogenesis. However, it seems likely that the insulin insensitivity is more apparent than real and that in the obese mouse the tissue is in a permanently stimulated state (17). The changes in the fatty acid composition of the adipocytes from lean mice when transplanted into obese mice may also be interpreted in favor of normal intrinsic properties of cells in the obese mice since "lean" cells in the obese situation did not over- or under-respond compared with "obese" cells.

The fatty acid composition of adipose tissue of obese mice does not change when the animals are forced to lose weight through restriction of their food intake (1,15). However, such a result probably stems from the retention of abnormally high rates of fatty acid synthesis in obese mice under these conditions (18) and one would expect turnover to continue under such conditions. There is, in fact, a slight decrease in the linoleic acid to palmitoleic acid (L:P) ratio under those conditions, suggesting de novo synthesis of fatty acids is less affected than the supply of dietary linoleic acid. The small falls observed in the L:P ratios in the present study when lean grafts are placed in lean hosts, or obese grafts are placed in obese hosts probably arises from increases in the quantity of fat synthesized as the animals age and caloric requirements for growth of other body tissues are decreased.

The grafts from obese donors into lean mice which retained their "obese" fatty acid composition apparently did so because they were moribund and showed virtually no fatty acid turnover judged by the margaric acid levels. Since the grafted tissue was recognizable, and previous studies have consistently demonstrated that "obese" cells shrink in lean mice (12),

it suggests that turnover and net lipolysis have been dissociated. It is possible that the relatively low hormone and substrate concentrations in the lean host result in net loss of fatty acids and that turnover may be limited by metabolic deficiency or lack of effective vascularization or innervation. However, the main conclusion is that the environment in which the fat cell finds itself is the major determinant of its content of linoleic acid and palmitoleic acid and no genetic differences in fatty acid metabolism of adipocytes from obese mice are apparent when the cells are transplanted into lean mice.

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Effect of Dietary Fatty Acids on Δ5 Desaturase Activity and Biosynthesis of Arachidonic Acid in Rat Liver Microsomes

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ABSTRACT

The effect of different fatty acids supplemented to a fat-free diet on the activity of Δ5 desaturase was studied. Fat-free diet produces a reduction in the conversion of eicosa-8,11,14-trienoic acid to arachidonic acid. The addition of the *cis*-ω6 acids, linoleic, γ-linolenic or arachidonic to the diet produces an increase of eicosatrienoic acid desaturation, shifting Δ5 desaturase activity towards the controls on a balanced diet. This reactivation is apparently produced by induction of enzyme biosynthesis since linoleate effect was suppressed by simultaneous cycloheximide injection. On the contrary, no changes in Δ5 desaturation activity were found when the diet was supplemented with palmitic or 9-*trans*,12-*trans*-linoleic acid. The changes on the activity of Δ5 desaturase were compared with the fatty acid composition of plasma and liver microsomes.
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The fatty acid composition of the dietary fat produces marked effects on the biosynthesis of unsaturated fatty acids in the liver. A fat-free or a hydrogenated fat-supplemented diet generally increases Δ9 and Δ6 desaturation activity (1-7). The addition of linoleic or arachidonic acid to this diet reduces linoleic acid desaturation to normal values (7). Diets rich in linoleic acid also modify Δ6 desaturation of linoleic acid (8). The control of unsaturated fatty acid biosynthesis is considered to reside mainly in the regulation of Δ6 and Δ9 microsomal desaturase activity, and has been extensively studied (1-9). The regulatory functions of the Δ5 desaturase enzyme that catalyzes the second desaturation step in the biosynthesis of polyunsaturated acids has been studied less (9). Similar to the Δ6 desaturase, Δ5 desaturase is modified by hormones (10-12) and a very low protein diet also reduces the activity of this enzyme, leading to an impairment of arachidonic acid synthesis (13). However, the effect of diets on the behavior of this desaturase differs from that of the Δ6 desaturase. Essential fatty acid deficiency produces a reduction in Δ5 desaturation activity, an effect that is reversed on supplementing the diet with ethyl linoleate (6.9). Similar results were found by Jeffcoat and James after feeding corn oil to rats for 14 days (8).

The differential behavior of Δ6 and Δ5 desaturases poses interesting questions about the concerted effect of both enzymes upon arachidonic acid biosynthesis.

Therefore, more precise information was needed on the very early effects of linoleic, γ-linolenic and arachidonic acids on Δ5 desaturase activity. For this reason, the present study was conducted to show the influence of the short-term intake of linoleic, γ-linolenic, arachidonic and linoelaidic acid esters on liver microsomal Δ5 desaturase activity of rats maintained on a fat-free diet. The fatty acid pattern of plasma and hepatic microsomes was also analyzed to give a broad picture of the role of dietary fatty acids on unsaturated fatty acid biosynthesis. Cycloheximide effect on the reactivation of Δ5 desaturase by dietary linoleic acid was also investigated.

MATERIALS AND METHODS

Chemicals

[1-^{14}C] Eicosa-8,11,14-trienoic acid (57.5 mCi/mmol, 98% radiochemical purity) was purchased from New England Nuclear Corp., Boston, MA. NADH, ATP, CoA, linoelaidic acid ethyl ester and cofactors were provided by Sigma Chemical Company, St. Louis, MO. Ethyl γ-linolenate (99% purity) was purchased from Nu-Chek Prep. Inc., Elysian, MN, and ethyl arachidonate (98% pure) was a gift of Hoffman-La Roche and Co., Basle, Switzerland. Methyl linoleate was prepared from sunflower seed oil following the procedure described by Keppler et al. (14). This preparation was 99% pure.

Animals and Treatment of Animals

The experiments were carried out with male weanling rats of the Wistar strain. Animals

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were fed ad libitum for 1 month on either a balanced diet consisting of: (in cal) 55% starch, 20% casein and 25% sunflower seed oil, or on a fat-free diet comprising 73.4% starch and 26.6% defatted casein. The fatty acid content of the basal 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 acid. Both diets were supplemented with minerals and a mixture of vitamins (15). Water was given ad libitum. After a month, the rats on the fat-free diet were divided in different groups of 6 animals each. One group was maintained on the same diet and in the other groups the diet was supplemented with 1.5 g % of either ethyl esters of γ-linolenic and linoelaidic acids or methyl linoleate. The rats were killed at 24 and 48 hr from the time of supplementation of the diet. Control groups of rats fed a balanced diet were sacrificed at the same time. In another experiment, the influence of the addition of ethyl arachidonate and ethyl palmitate to the fat-free diet was tested. Animals were treated as above, but they were sacrificed 12 and 48 hr after the supplementation of the diet. Four animals per group were used in this experiment. In a third experiment, groups of 6 animals each, maintained an a control diet, fat-free diet or fat-free diet supplemented with methyl linoleate for 24 hr were simultaneously treated with cycloheximide. The cycloheximide was administered intraperitoneally in single dose of 250 μg per 100 g body weight in saline solution 24 hr before killing the rats. Control groups without cycloheximide were sacrificed simultaneously.

The rats were killed by decapitation. The blood was allowed to drain and was collected for the determination of the fatty acid composition. Livers were rapidly excised and immediately placed in ice-cold homogenizing medium (1). After the homogenization, microsomes were separated by differential centrifugation at 100,000 × g as described previously (1) and immediately used for the desaturation assays.

Analytical Procedure

Desaturation of fatty acids by liver microsomes was measured by estimation of the percentage conversion of [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid. Five nmol of the labeled acid and 95 nmol of unlabeled acid were incubated with 5 mg of microsomal protein in the 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 $MgCl_2$, 2.25 μmol glutathione, 62.5 μmol NaF, 0.5 μmol nicotinamide and 62.5 μmol phosphate butter (pH 7). After 10 min, the reaction was stopped by addition of 2 ml of 10% KOH in methanol. 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 3 M HCl (3 hr at 68 C) and the distribution of the radioactivity between substrate and product was measured by gas liquid radiochromatography in an apparatus equipped with a Packard proportional counter. Percentage conversion was calculated from the distribution of radioactivity between substrate and product measured directly on the radiochromatogram (16).

Lipids of liver microsomes were extracted with chloroform/methanol (2:1, v/v) by the procedure of Folch et al. (17). The fatty acids of the lipids were converted to methyl esters by the procedure already described and analyzed by gas liquid chromatography in a Packard apparatus equipped with a flame detector. The column was packed with 15% EGSS-X coated on Chromosorb WHP (80-100 mesh), Supelco Inc., Bellefonte, PA. The acids were identified by comparison with standards and the composition was calculated by measuring the area under the peaks.

Protein content of the ractions was determined by the biuret method of Gornall et al. (18).

RESULTS

Δ5 Desaturation Activity

The effect of dietary regimen on the desaturation of eicosa-8,11,14-trienoic acid to arachidonic acid by rat liver microsomes is shown in Figure 1. The maintenance of rats on a fat-free diet resulted in a significant reduction in Δ5 desaturation activity when compared to animals fed a balanced diet. The addition of linoleic or γ-linolenic acid to the fat-free diet produced an increase in the conversion of eicosatrienoic acid to arachidonic acid. This effect was more remarkable when the rats were supplemented with γ-linolenic acid. In this case, after 48 hr of refeeding, the Δ5 desaturation activity reached the control values. The supplementation of the diet with 9-*trans*,12-*trans*-linoleic acid produced no changes in this enzymatic activity comparing to that observed in liver microsomes of rats fed a fat-free diet.

The effect of supplementation of the fat-free diet with either arachidonic acid or palmitic acid on the percentage change of Δ5 desatura-

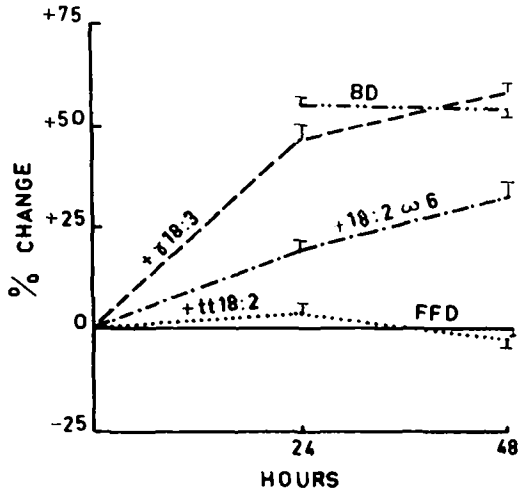

FIG. 1. Effect of different dietary fatty acids supplemented to rats maintained on a fat-free diet on the change of eicosa-8,11,14-trienoic acid conversion to arachidonic acid. Zero corresponds to the percentage conversion (average 48.0 ± 2.5) of animals fed a fat-free diet (FFD). Balanced diet (BD) (-·-·-·-·-), FFD supplemented with: linoleic acid (– · – · –), γ-linolenic acid (– – – – –), and 9-*trans*,12-*trans*-linoleic acid (.....). Each point represent the average of 6 animal ± SEM.

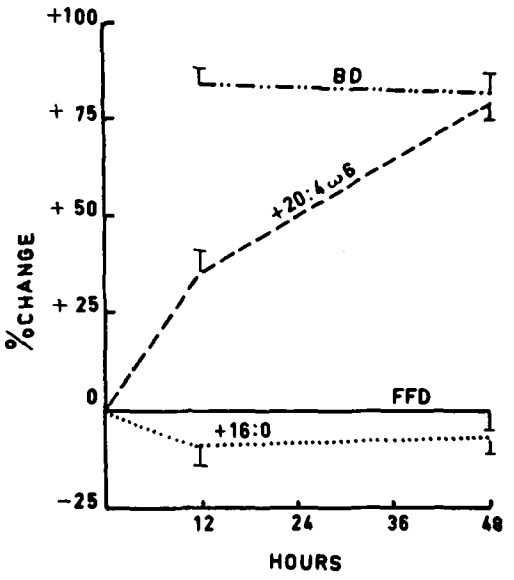

FIG. 2. Effect of the addition of palmitic and arachidonic acids in the diet of rats maintained on fat deprivation, on the change of liver microsomal conversion of eicosa-8,11,14-trienoic acid to arachidonic acid. Zero corresponds to the percentage conversion (average 38.2 ± 2.8) of animals fed a fat-free diet (FFD). Balanced diet (BD) (-·-·-·-·-), FFD supplemented with: palmitic acid (.....), and arachidonic acid (- – – – – – –). Each point represents the average of 4 rats ± SEM.

tion activity is shown in Figure 2. The addition of arachidonic acid produced an increase in the conversion of eicosa-8,11,14-trienoic acid to arachidonic acid that reached the control values after 48 hr of treatment. The administration of palmitic acid produced no changes on Δ5 desaturation activity. In this case, the data were similar to those observed under the fat-free diet. Therefore, it is clear that the ingestion of *cis* polyunsaturated fatty acids of the linoleic acid series results in an increase of Δ5 desaturase activity. Figure 3 shows the effect of linoleic acid refeeding on Δ5 microsomal desaturation activity of rats maintained on a fat-free diet under cycloheximide treatment. The percentage conversion of eicosa-8,11,14-trienoic acid to arachidonic acid decreased in the rats maintained on a fat-free diet and returned to the control values after refeeding the animals with linoleic acid. Cycloheximide treatment produced a reduction in Δ5 desaturation activity in the control rats and in the fat-free rats, and prevented the recovery observed after linoleic acid refeeding.

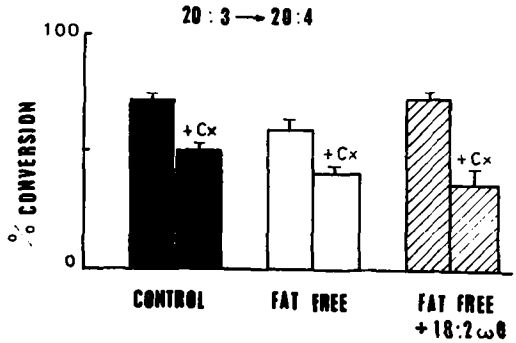

FIG. 3. Effect of cycloheximide treatment on the percentage conversion of eicosa-8,11,14-trienoic acid to arachidonic acid of liver microsomes of rats fed a complete diet (■), a fat-free diet (□), or a fat-free diet supplemented with linoleic acid (▨). Values are the mean of 6 animals ± SEM.

Fatty Acid Composition

The fatty acid composition of plasma lipids is given in Table 1. The percentage distribution of the plasmatic fatty acid of the rats fed a fat-free diet reflects the essential fatty acid (EFA) status of the animal: increased amount of monoenoic and 20:3ω9 fatty acids and a low level of 18:2ω6 and 20:4ω6 acids. Fatty acid supplementation with linoleic and γ-linolenic acids produced significant differences in the plasmatic fatty acids. The levels of palmitic, palmitoleic and eicosatrienoic acids de-

TABLE 1

Fatty Acid Composition of Plasma

Fatty acids[a]	Complete diet	Fat-free diet	Fat-free diet supplemented with: Linoleic acid 24 hr	Linoleic acid 48 hr	γ-Linolenic acid 24 hr	γ-Linolenic acid 48 hr	Linoelaidic acid 24 hr	Linoelaidic acid 48 hr
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
16:0	18.1[b] ± 0.3	27.8 ± 0.7	22.8 ± 1.6	21.3 ± 0.6	19.6 ± 0.6	20.7 ± 0.9	28.9 ± 1.1	28.6 ± 2.6
16:1	1.9 ± 0.2	6.6 ± 0.6	6.1 ± 0.4	4.3 ± 0.9	3.9 ± 0.3	3.7 ± 0.3	7.5 ± 0.2	6.5 ± 0.3
18:0	10.5 ± 0.3	12.4 ± 0.6	12.0 ± 0.8	12.2 ± 0.6	11.5 ± 0.4	11.5 ± 0.2	10.7 ± 1.0	11.2 ± 0.9
18:1	15.5 ± 0.6	21.9 ± 1.0	22.0 ± 2.3	21.9 ± 1.4	13.8 ± 0.9	13.8 ± 1.3	19.1 ± 0.6	20.2 ± 1.4
18:2 *tt*	–	–	–	–	–	–	5.9 ± 0.5	6.6 ± 0.6
18:2ω6	27.8 ± 0.8	7.0 ± 0.5	13.0 ± 1.1	10.4 ± 1.9	4.3 ± 0.2	3.7 ± 0.2	9.0 ± 0.6	9.8 ± 0.8
γ-18:3	tr	tr	tr	tr	6.9 ± 0.3	6.4 ± 0.6	tr	tr
20:3ω9	1.2 ± 0.1	5.8 ± 0.6	3.7 ± 0.7	3.8 ± 0.6	3.5 ± 0.3	2.0 ± 0.2	6.5 ± 0.4	5.2 ± 0.5
20:4ω6	20.9 ± 0.7	12.2 ± 1.2	19.1 ± 2.1	23.7 ± 1.8	29.0 ± 1.1	30.2 ± 1.5	10.2 ± 0.7	9.7 ± 0.6

For details, see Materials and Methods.
[a]Only the main fatty acids were computed.
[b]Results are the mean of 6 animals ± SEM.

creased significantly compared to the rats fed a fat-free diet. However, γ-linolenic acid was more effective than linoleic acid in decreasing oleic acid. This is supported by a greater effect of γ-linolenic acid than linoleic acid on hepatic Δ9 desaturase (manuscript in preparation). As expected, the levels of both linoleic and arachidonic acids were higher then linoleic acid was added to the fat-free diet, indicating a rapid conversion to 20:4ω6. The groups fed γ-linolenic acid accumulated this acid and arachidonic acid, and decreased the percentage of linoleic acid. The concentration of arachidonic acid reached even higher percentages than in control animals. The addition of linoelaidic acid to the diet produced an accumulation of this acid in the lipids but no important changes were found on the other fatty acids compared to those observed in the rats fed a fat-free diet. However, a slight increase of linoleic acid was noted.

Table 2 shows the effect of the different diets on the relative percentage of fatty acids of liver microsomes. As was observed in plasma lipids, the fatty acid composition of liver microsomes of the rats maintained on a fat-free diet is typical of essential fatty acid deficiency. Monoenoic fatty acids were enhanced significantly in EFA-deficient rats, expressing an increase in Δ9 desaturation activity already shown under this kind of diet (3,7,8). When *cis*-ω6 acids were added to the diet of rats raised on a fat-free diet, significant change were found in the fatty acid composition of the microsomes. Linoleic acid was incorporated as such, and converted to arachidonic acid whereas γ-linolenic acid was very little incorporated but was highly converted to arachiconic acid and ω6 acids of 22:4 and 22:5. All the *cis* acids reduced the levels of 20:3ω9, oleic and palmitoleic acids. However, γ-linolenate was more efficient than the other fatty acids. Linoelaidic acid was incorporated in the liver microsomes. Its supplementation to the diet enhanced the content of *cis*-linoleate and decreased the arachidonic acid proportions in nonstatistically significant figures. It did not modify the composition of the other fatty acids.

The effect of supplementation of the fat-free diet with arachidonic acid on the fatty acid composition of liver microsomes is shown in Table 3. Similarly to the other *cis*-ω6 fatty acids, the addition of 20:4ω6 to the diet significantly decreased monoenoic fatty acids and 20:3ω9 acid and significantly increased the relative percentage of arachidonic acid.

The triene/tetraene ratio (20:3ω9/20:4ω6) of liver lipids (Tables 2 and 3) clearly shows the essential fatty acid deficiency. This ratio was not significantly increased when supplementing the diet with linoelaidic acid, but fell when linoleic, γ-linolenic or arachidonic acids were added to the diet. γ-Linolenic acid returned the triene/tetraene ratio to normal values.

DISCUSSION

The administration of a fat-free diet to weanling rats evokes changes in the fatty acid composition that are characteristic of EFA deficiency (19,20). These changes are shown very early in rat liver microsomes (6). Arachidonic acid decays as a consequence of a decreased biosynthesis due to a progressive linoleic acid depletion. The organism apparently reacts under this change, trying to compensate this depletion by mobilizing linoleic acid from

TABLE 2

Fatty Acid Composition of Liver Microsomal Lipids of Rats Fed a Fat-Free Diet Supplemented with Different Fatty Acids

Fatty acids	Balanced diet	Fat-free diet	Fat-free diet supplemented with: Linoleic acid 24 hr	Linoleic acid 48 hr	γ-Linolenic acid 24 hr	γ-Linolenic acid 48 hr	9-*t*,12-*t*-Linoleic acid 24 hr	9-*t*,12-*t*-Linoleic acid 48 hr
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
14:0	0.3[a] ± 0.03	0.4 ± 0.02	0.4 ± 0.03	0.5 ± 0.02	0.5 ± 0.03	0.4 ± 0.04	0.5 ± 0.02	0.4 ± 0.02
16:0	16.5 ± 0.3	21.1 ± 0.5	20.9 ± 0.5	21.4 ± 0.4	20.6 ± 0.4	19.8 ± 0.3	20.6 ± 0.4	20.2 ± 0.6
16:1	1.4 ± 0.1	4.8 ± 0.3	3.0[b] ± 0.2	2.8[b] ± 0.2	2.6[b] ± 0.2	1.8[b] ± 0.1	3.7 ± 0.3	3.7 ± 0.2
18:0	19.6 ± 0.3	16.8 ± 0.5	17.6 ± 0.5	18.7 ± 0.5	18.3 ± 0.3	19.2[c] ± 0.4	15.1 ± 0.7	15.2 ± 0.8
18:1	10.4 ± 0.4	19.7 ± 0.9	16.4 ± 0.9	13.5[b] ± 0.7	13.2[b] ± 0.6	9.8[b] ± 0.3	16.5 ± 0.8	18.1 ± 0.5
18:2 *tt*	–	–	–	–	–	–	6.5[b] ± 0.4	7.7[b] ± 0.5
18:2ω6	13.8 ± 0.3	6.7 ± 0.4	9.8[b] ± 0.3	10.3[b] ± 0.2	2.3[b] ± 0.2	1.9[b] ± 0.1	7.0 ± 0.2	8.5[b] ± 0.2
γ-18:3	0.4 ± 0.05	0.5 ± 0.1	0.5 ± 0.03	0.5 ± 0.02	1.0[c] ± 0.08	0.9[c] ± 0.06	0.3 ± 0.1	tr
20:3ω9	1.0 ± 0.2	6.1 ± 0.3	5.1 ± 0.2	3.4[b] ± 0.3	2.4[b] ± 0.2	0.9 ± 0.06	6.3 ± 0.4	5.7 ± 0.3
20:3ω6	0.8 ± 0.1	2.1 ± 0.1	2.3 ± 0.3	2.1 ± 0.1	1.7 ± 0.1	1.1 ± 0.04	1.8 ± 0.1	1.6 ± 0.05
20:4ω6	26.6 ± 0.4	14.1 ± 0.8	16.7 ± 0.6	20.1[b] ± 0.7	25.7[b] ± 0.4	28.6[b] ± 0.3	12.7 ± 0.7	12.3 ± 0.4
22:4ω6	2.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.06	1.0 ± 0.04	2.3[b] ± 0.1	3.3[b] ± 0.1	0.8 ± 0.3	0.5 ± 0.09
22:5ω6	4.0 ± 0.3	2.8 ± 0.2	2.6 ± 0.05	2.8 ± 0.1	6.4[b] ± 0.5	8.6[b] ± 0.4	3.0 ± 0.3	2.4 ± 0.2
22:5ω3	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.04	0.8 ± 0.02	0.4 ± 0.03	0.5 ± 0.04	1.2 ± 0.4	0.5 ± 0.1
22:6ω3	2.4 ± 0.2	3.2 ± 0.3	3.1 ± 0.07	3.2 ± 0.2	2.9 ± 0.1	3.0 ± 0.1	4.2 ± 0.4	3.3 ± 0.4
20:3ω9/20:4ω6	0.04	0.43	0.31	0.17	0.09	0.03	0.50	0.46

For details, see Materials and Methods.
[a]Results are the mean of 6 animals ± SEM.
[b]$P<0.001$ compared to the fat-free diet.
[c]$P<0.01$ compared to the fat-free diet.

TABLE 3

Fatty Acid Composition of Liver Microsomes; Influence of the Addition of Arachidonic Acid to the Diet

Fatty acids[a]	Complete diet	Fat-free diet	Fat-free diet supplemented with Arachidonic acid 12 hr	48 hr
	(%)	(%)	(%)	(%)
16:0	17.6[b] ± 1.0	20.7 ± 0.6	19.6 ± 1.5	17.9 ±0.4
16:1	2.0 ± 0.3	5.1 ± 0.4	2.8[d] ± 0.2	2.0[c] ±0.2
18:0	23.5 ± 1.0	17.8 ± 1.0	18.8 ± 0.7	21.9 ±0.8
18:1	11.1 ± 0.6	25.5 ± 1.8	20.4 ± 1.1	14.5[d] ±1.3
18:2ω6	12.8 ± 0.3	7.1 ± 0.6	7.6 ± 1.2	6.6 ±0.8
20:3ω9	1.0 ± 0.3	5.3 ± 0.3	3.7[d] ± 0.3	3.2[d] ±0.4
20:4ω6	27.5 ± 1.9	13.8 ± 0.7	24.7[c] ± 0.3	29.7[c] ±1.3
20:3ω9/20:4ω6	0.04	0.38	0.15	0.11

For details, see Materials and Methods.
[a]Only the main fatty acids were computed.
[b]Results are the mean of 4 animals ± SEM.
[c]$P<0.001$ compared with the fat-free diet.
[d]$P<0.01$ compared with the fat-free diet.

fat depots and increasing Δ6 desaturase activity (6). Δ6 Desaturase is the first desaturating enzyme of the ω6 essential fatty acid family and is considered to control the biosynthesis of the series (2). However, it is remarkable that EFA deficiency evokes an early decrease of the activity of the Δ5 desaturase that is the second enzyme of the series (6). In the present work, it is shown (Figs. 1 and 2) that, after 30 days on a fat-free diet, the Δ5 desaturase activity is still low, whereas it was found (7,9) that Δ9 and Δ6 desaturations are increased at the same time. These results agree with the work by Jeffcoat and James (8) in which different types of diets were administered to rats. Therefore, Δ6 and Δ5 desaturases modify their activities in opposite way under the dietary changes already mentioned.

The administration of *cis* unsaturated acids of the linoleic acid family during only a few hours (Figs. 1 and 2) evokes a reactivation of the Δ5 desaturase, whereas neither palmitic acid nor all *trans*-linoleic acid evoke such a change. These results indicate that the activation is produced by *cis* unsaturated acids. However, the efficiency of dietary linoleic, γ-linolenic and arachidonic acids to evoke the reactivation is different, being the highest for γ-linolenic and arachidonic. In less than 48 hr, both γ-linolenic and arachidonic acids reactivate Δ5 desaturase to the levels found in control animals.

The reactivation is apparently produced by Δ5 desaturase biosynthesis since the linoleate effect was nullified by simultaneous cycloheximide injection, and it is known that cycloheximide inhibits protein synthesis in mammalian cells.

Although the above described experiments show that the administration of fatty acids of linoleic acid family to fat-deficient rats evokes an increase of the Δ5 desaturase activity, it has been found in similar experiments that the same acids decrease the Δ6 desaturase (7,9). Therefore, considering that the two desaturases, Δ6 and Δ5, are involved in arachidonic acid biosynthesis and that Δ5 desaturation activity increases after the addition of either linoleic, γ-linolenic or arachidonic acids to the diet, the Δ6 desaturation of linoleic to γ-linolenic acid in vivo would be a rate-limiting step in the conversion of linoleic to arachidonic acid. Indirect support of this concept was given by Hassam and coworkers (21) comparing the incorporation of radioactivity from 18:2 [^{3}H] and γ-18:3[^{14}C] into the liver, brain and plasma lipids of the rat. All these results are in accordance with previous findings (2,9), and show evidently that the supplementation of the fat-free diet with fatty acids of linoleic acid family produces an activation of eicosatrienoic acid desaturation to arachidonic acid. This enzymatic behavior could be, in the case of linoleate and γ-linolenate administration, a physiological mechanism for coping with the increased amount of eicosa-8,11,14-trienoic acid produced by the cell in spite of decreased Δ6 desaturase activity when those acids were ingested. That is, the cell would enhance the amount of Δ5 desaturase to be able to transform the increased level of substrate. At first sight, this hypothesis could not be so easily

used to explain the effect of arachidonic acid administration. However, the cellular level of arachidonic acid is related to the linoleate level. Therefore, it would be necessary for arachidonic acid to produce a similar effect to that of linoleate or γ-linolenate in order to make Δ5 desaturase activity adequate to cope with increased levels of substrate. Moreover, if arachidonate had an antagonistic effect to linoleate or γ-linolenate, it would disorganize the regulatory mechanism.

From all these experiments, it is apparent that the mechanisms whereby individual desaturases are regulated by dietary fat may be unique to each enzyme, although the ultimate consequence leads to a coordinate change in the activities of these enzymes. This coordinate change would be relevant in determining the relative amounts of 20:3ω6 and 20:4ω6 that produce different eicosanoids.

The 4 acids, 18:2ω6, γ-18:3, *t,t*-18:2ω6 and arachidonic, were incorporated into the microsomal lipids (Tables 2 and 3), but only *cis*-linoleic and γ-linolenic acids were converted to arachidonic acid. Linoelaidic acid not only did not evoke an increase of Δ5 desaturase activity, but also was not converted into arachidonic acid. This last result agrees with an old work demonstrating that it is not a substrate in vitro for the rat Δ6 desaturase (22). γ-Linolenic acid was less incorporated in the microsomal lipids, probably because this acid is rapidly removed by chain elongation (23) and subsequent Δ5 desaturation. The striking difference in the relative increases of the percentage of arachidonic acid in microsomal lipids and the Δ5 desaturase activity when linoleic acid administration was replaced by γ-linolenic acid (Table 2) would corroborate this hypothesis. Moreover, only γ-linolenic and arachidonic acids evoked a recovery of arachidonic acid to control rat level in 48 hr. Therefore, it might be possible that the mechanism of linoleate or γ-linolenate activation of Δ5 desaturase biosynthesis is produced through their conversion to arachidonic acid. However, this suggestion needs further investigation. The fatty acid composition of the microsomal lipids of the animals supplemented with γ-linolenic acid shows a content of 22:4ω6 and 22:5ω6 acids, higher than the linoleic-treated group. These results indicate that γ-linolenate is more efficient than linoleate in the conversion to polyunsaturated acids of 20 and 22 carbons, very probably because the Δ6 desaturase step has been avoided. The relative percentages of linoleic acid in γ-18:3 treated rats were significantly lower than those of the fat-deficient animals, probably expressing an enhancement of linoleic acid metabolism.

It has been well demonstrated that dietary *trans* fatty acids are absorbed and incorporated into animal and human tissues (24,25) and, in this experiment, it is shown that there is a rapid incorporation of 9-*trans*,12-*trans*-18:2 either in plasma or in liver lipids (Tables 1 and 2). Besides, it is known that linoelaidic acid is not converted in vitro in γ-linolenic acid (22) and in vivo into an eicosatrienoic acid (26,27). However, the inclusion of the *trans* supplement in the fat-free diet (Tables 1 and 2) increased the 18:2ω6 concentration in plasma and liver microsomes, which indicates that the metabolism of linoleic acid was at least partially impaired. In agreement with this observation, De Schrijver and Privett (28) found that the conversion of linoleic to γ-linolenic acid was suppressed by the dietary *trans* fatty acid. Earlier in vitro experiments carried out by Brenner and Peluffo (22) also demonstrated that linoelaidic inhibits the conversion of linoleic acid to γ-linolenic acid. Besides, dietary *trans*-18:2 decreased the levels of arachidonic acid in the rat liver even when appropriate amounts of linoleic acid were fed (29). Moreover, it has been well documented that the *trans,trans*-isomer of linoleic acid possesses no EFA activity in the sense of curing or preventing classical EFA symptoms (30-32). Moreover, linoelaidic aggravates EFA deficiency when fed to animals as a sole dietary fat (31,33). The results obtained in the present experiment show that this aggravation of essential fatty acid deficiency may lie in the Δ6 desaturase inhibition since the activity of Δ5 desaturase does not differ from that obtained in the animals fed a fat-free diet.

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Effect of Bile Acid Feeding on Hepatic Steroid 12 α-Hydroxylase Activity in Hamsters[1]

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ABSTRACT

The effects of feeding cholic acid, chenodeoxycholic acid and ursodeoxycholic acid on the activity of the hepatic steroid 12α-hydroxylase, gallbladder bile acid composition, fecal neutral sterol output, cholesterol synthesis and bile acid synthesis were determined in female hamsters. The 12α-hydroxylase activity was inhibited to 56% by cholic acid, to 62% by chenodeoxycholic acid, and to 78% by ursodeoxycholic acid compared with the control. Bile acid composition was altered by feeding of cholic acid and chenodeoxycholic acid to be rich in the given bile acids. Fecal neutral sterol output increased about twice by feeding chenodeoxycholic acid and ursodeoxycholic acid, whereas cholic acid had no significant effect. Body cholesterol synthesis increased to 217% by chenodeoxycholic acid and to 274% by ursodeoxycholic acid, whereas effect of cholic acid was not significant. Bile acid synthesis was suppressed to 48% of control only by chenodeoxycholic acid. A positive correlation between the 12α-hydroxylase activity and the bile acid synthesis was observed in the control, chenodeoxycholate-fed and ursodeoxycholate-fed animals. In conclusion, ursodeoxycholic acid might have less inhibitory effect on the steroid 12α-hydroxylase and the bile acid synthesis than chenodeoxycholic acid.
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Chenodeoxycholic acid and ursodeoxycholic acid have been extensively applied as therapeutic agents in the treatment of cholesterol gallstones (1,2). The administration of these bile acids reduces biliary cholesterol secretion (3,4), decreases cholesterol saturation in bile (5,6), and induces gallstone dissolution (1,2). These bile acids have also been reported to decrease intestinal absorption of cholesterol (7,8) and to inhibit hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (9,10), resulting in decreased hepatic cholesterol synthesis. Despite these studies, however, information about the effects of chenodeoxycholic acid and ursodeoxycholic acid on the hepatic steroid 12α-hydroxylase remains limited. 12α-Hydroxylase is a microsomal mixed function oxidase which catalyzes the conversion of 7α-hydroxycholest-4-en-3-one to 7α,12α-dihydroxycholest-4-en-3-one and is one of the key enzymes involved in the metabolism of cholesterol to the bile acids in the liver (11). Together with the mitochondrial 26-hydroxylase which catalyzes the initial step of the side-chain degradation, the microsomal 12α-hydroxylase may regulate the relative amounts of cholic acid and chenodeoxycholic acid synthesized in the liver (11, 12). Danielsson showed that the activity of the 12α-hydroxylase in rats is inhibited by taurochenodeoxycholic acid (13). However, rats are known to carry out 6β-hydroxylation of chenodeoxycholic acid to make α-muricholic acid; this, in turn, is converted into its 7β-epimer, β-muricholic acid (14), which can also be made from ursodeoxycholic acid (15). It is, therefore, possible that the observed suppression of the 12α-hydroxylase activity was not a direct effect of chenodeoxycholic acid itself, but rather was mediated by α- and β-muricholic acids. Recently, Ahlberg et al. reported that the 12α-hydroxylase activity in human liver microsome was inhibited ca. 50% by chronic oral administration of chenodeoxycholic acid (16). However, for further investigation of the effects of bile acid feeding on the enzyme, there exist ethical problems in obtaining liver specimens from subjects whose hepatobiliary systems are not affected. It was, therefore, considered of importance to get a suitable experimental animal whose bile acid metabolism resembles that of humans. To investigate the effect of chenodeoxycholic acid and ursodeoxycholic acid on the hepatic 12α-hydroxylase, the hamster appears to be a more suitable animal model than the rat, since the hamster does not have the hepatic 6β-hydroxylase and the individual bile acid composition is similar to that of humans (17). Thus, we have now studied effects of cholic acid, chenodeoxycholic acid and ursodeoxycholic acid on the hepatic steroid 12α-hydroxylase in hamsters. We also report effects of these bile acids on the gallbladder bile

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acid composition, fecal neutral sterol output, cholesterol synthesis and bile acid synthesis.

EXPERIMENTAL PROCEDURES

Materials

7α-Hydroxycholest-4-en-3-one, 5β-cholestane-3α,7α,12α-triol, 7α,12α-dihydroxy-5β-cholestan-3-one, 7α,12α-dihydroxycholest-4-en-3-one, and tritium-labeled 7α-hydroxycholest-4-en-3-one (specific radioactivity 49 μCi/mg) were synthesized as previously reported (17). 7α,12α-Dihydroxy-5β-cholanoic acid was synthesized as previously described (18). Cholic acid (Sigma Chemical Co., St. Louis, MO) was recrystallized 3 times from methanol. Chenodeoxycholic acid and ursodeoxycholic acid of better than 99% purity were generously supplied by Tokyo Tanabe Pharmaceutical Co. (Tokyo, Japan) and used without further purifications. Piperidinohydroxypropyl Sephadex LH-20 was prepared as described by Goto et al. (19). All other chemicals were reagent grade. Chow containing 0.2% of cholic acid, chenodeoxycholic acid or ursodeoxycholic acid was prepared by mixing 2.0 g of each bile acid in 300 ml of ethanol with 1.0 kg of Standard Powder Chow FM of Oriental Yeast Co. Ltd. (Tokyo, Japan).

Animals

Twenty female Golden Syrian hamsters with a mean body weight of 92 g were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan) and were randomly divided into 4 groups after 2 weeks of acclimation period. Each group was fed one of the standard chow, 0.2% cholic acid chow, 0.2% chenodeoxycholic acid chow and 0.2% ursodeoxycholic acid chow for 2 weeks. Lighting was between 6 a.m. and 6 p.m. and room temperature was controlled at 25 C. Chows and water were given ad libitum. During this period, one animal from the control group and 2 from the ursodeoxycholic acid group died of unknown etiology. Food intake was weighed daily. Feces of the last 7 days were collected, lyophilized and stored in desiccator until analyzed.

Sacrifice was performed between 9 and 10 a.m. Under ether anesthesia, the abdomen and the chest were opened; blood was aspirated from the heart. The gallbladder was removed and the bile was deproteinized with ethanol. The liver was then excised, weighed, and used for the incubation.

Measurement of Microsomal Steroid 12α-Hydroxylase Activity

Two g portion of the liver was used for measuring the activity of hepatic microsomal steroid 12α-hydroxylase. The methods for the preparation of the microsomes and for the determination of the rate of the conversion of 7α-hydroxycholest-4-en-3-one to 7α,12α-dihydroxycholest-4-en-3-one have been described in detail (17).

Analysis of Gallbladder Bile Acids

The determination of individual bile acid composition of the gallbladder bile was carried out by gas liquid chromatography as methyl ester-dimethylethylsilyl ether derivatives, as described previously (17).

Analysis of Fecal Bile Acids and Neutral Sterols

Seven days' pool of feces was ground down and mixed thoroughly. Five mg of 7α,12α-dihydroxy-5β-cholanoic acid and 1 mg of 5α-cholestane were added as internal standards to 2 g portion of feces. The feces were mixed with 20 ml of 2 N KOH in a Teflon beaker and heated at 130 C for 3 hr. The hydrolysate was filtered and the residue was extracted with hot ethanol for 24 hr. The ethanolic extract was condensed by evaporation of ethanol under a reduced pressure, combined with the filtrate, diluted with water, acidified with d-HCl, and extracted twice with ether and twice with ethyl acetate. The extracts were combined, washed with water to neutrality, dried over anhydrous Na_2SO_4, filtrated and evaporated under reduced pressure. The residue was dissolved in 5 ml of 90% ethanol and chromatographed on a column (1.6 × 10 cm) of piperidinohydroxypropyl Sephadex LH-20. Neutral sterols were eluted with 60 ml of 90% ethanol and bile acids were eluted with 200 ml of 0.1 M acetate in 90% ethanol. Each fraction was evaporated under reduced pressure to dryness. The neutral sterols were converted to trimethylsilyl ether derivatives and the bile acids were converted to methyl ester-trimethylsilyl ether derivatives. Analyses of both the neutral sterols and the bile acids were carried out by gas liquid chromatography using glass column packed with 2% Poly-I-110 on 100-120 mesh of Chromosorb W. Column temperature was 250 C. Suitable corrections were made using the internal standards. Cholesterol and bile acid syntheses were calculated using the chromatographic balance method (20,21).

Statistical Methods

Results were expressed as mean ±SD. Group means were compared by paired Student's t-test. Bivariate regression analyses were performed by the method of least squares.

RESULTS

Under the conditions of the present study, the hamsters receiving bile acid diets ingested an average of 17 mg/day of each bile acid. The animals on these diets showed no significant differences from the control animals, in their weight gain and food intake for the entire experimental period.

Steroid 12α-Hydroxylase Activity

The mean activity of hepatic microsomal 12α-hydroxylase of each group is shown in Figure 1. The activity was 104.8 ± 6.8 pmol/mg protein/min for the control group, 58.8 ± 5.5 pmol/mg protein/min for the cholic acid group, 64.2 ± 17.3 pmol/mg protein/min for the chenodeoxycholic acid group, and 81.7 ± 5.2 pmol/mg protein/min for the ursodeoxycholic acid group. Statistical differences between the control and each bile acid group were $P < 0.0005$ (cholic acid), $P < 0.0025$ (chenodeoxycholic acid) and $P < 0.0025$ (ursodeoxycholic acid). The difference between the cholic acid group and the ursodeoxycholic acid group was also statistically significant ($P < 0.001$).

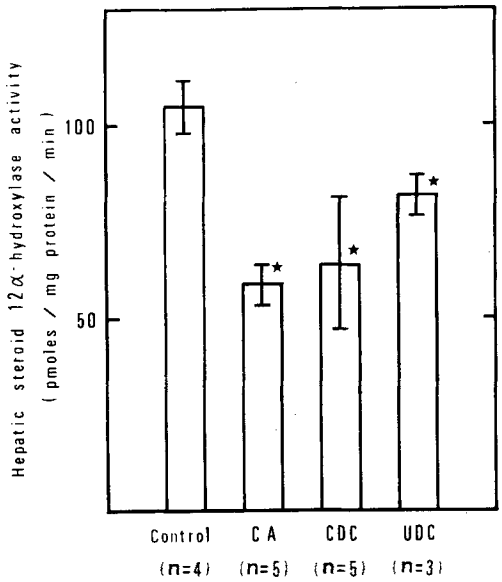

FIG. 1. Effect of bile acid feeding on the activity of the hepatic microsomal steroid 12α-hydroxylase in female hamsters. The values are the means ±SD. The activity is pmol of 7α,12α-dihydroxycholest-4-en-3-one formed from 7α-hydroxycholest-4-en-3-one/mg of microsomal protein/min. CA, CDC and UDC mean cholic acid, chenodeoxycholic acid and ursodeoxycholic acid, respectively. ★: Significantly different from control values ($P < 0.005$ or better by paired t-test).

Bile Acid Composition in Gallbladder Bile

The bile acid composition in each group is shown in Table 1. Bile of the control group contained ca. 80% of cholic acid plus its metabolites (i.e., deoxycholic acid and 7-ketodeoxycholic acid). Cholic acid administration resulted in increased percentage of deoxycholic acid and conspicuous disappearance of chenodeoxycholic acid. Administration of both chenodeoxycholic acid and ursodeoxycholic acid decreased the percentages of cholic and deoxycholic acids and increased those of chenodeoxycholic and lithocholic acids. Ursodeoxycholic acid occupied ca. 20% of total bile acid in the ursodeoxycholic acid group.

Fecal Bile Acids and Neutral Sterols

The fecal steroid excretion during the last week is shown in Table 2. Fecal neutral sterol output increased about twice by feeding of

TABLE 1

Effects of Bile Acid Feeding on Gallbladder Bile Acid Composition in Female Hamsters (mean ±SD)

Bile acid	Control (n = 4)	Cholic acid (n = 5)	Chenodeoxycholic acid (n = 5)	Ursodeoxycholic acid (n = 3)
		(%)		
Cholic acid	70.4 ± 9.3	67.9 ± 3.5	9.4 ± 4.1[b]	12.1 ± 0.6[b]
Chenodeoxycholic acid	17.6 ± 3.9	ND[a,b]	81.5 ± 6.5[b]	58.8 ± 5.3[b]
Deoxycholic acid	8.2 ± 10.9	31.0 ± 3.7[b]	1.1 ± 0.7[b]	0.6 ± 0.3[b]
Lithocholic acid	1.4 ± 0.7	0.2 ± 0.1	7.9 ± 2.5[b]	7.7 ± 1.0[b]
7-Ketodeoxycholic acid	2.4 ± 1.3	1.0 ± 0.3	ND	ND
Ursodeoxycholic acid	ND	ND	ND	20.8 ± 3.4[b]

[a]Not detected.

[b]Significantly different from control values ($P < 0.05$ or better by paired t-test).

TABLE 2

Effects of Bile Acid Feeding on Fecal Neutral Sterol Output, Cholesterol Synthesis, and Bile Acid Synthesis in Female Hamsters (mean ±SD)

	Control (n = 4)	Cholic acid (n = 5)	Chenodeoxy-cholic acid (n = 5)	Ursodeoxy-cholic acid (n = 3)
			(mg/day)	
Cholesterol intake	5.84 ± 0.97	4.95 ± 0.37	5.06 ± 0.24[a]	6.22 ± 0.48
Fecal neutral sterol output	2.98 ± 0.83	3.30 ± 0.81	6.56 ± 1.15[a]	6.72 ± 1.14[a]
Cholesterol synthesis[b]	1.69 ± 1.16	2.16 ± 1.38	3.66 ± 0.88[a]	4.63 ± 1.67[a]
Bile acid synthesis[c]	4.52 ± 1.17	3.81 ± 0.78	2.16 ± 0.26[a]	4.12 ± 0.75

[a]Significantly different from control values ($P < 0.05$ or better by paired t-test).

[b]Total fecal steroid excretion (fecal neutral sterol output + fecal bile acid output) – total dietary steroids (cholesterol intake + bile acid intake).

[c]Fecal bile acid output – bile acid intake.

chenodeoxycholic acid and ursodeoxycholic acid compared with the control. There was no significant change between the control and the cholic acid group. Administration of chenodeoxycholic and ursodeoxycholic acids increased cholesterol synthesis ca. 2-3 times, whereas cholic acid administration showed no significant effect. Bile acid synthesis was suppressed markedly by chenodeoxycholic acid administration ($P < 0.0005$). Bile acid synthesis in cholate-fed and ursodeoxycholate-fed animals was slightly decreased by 16% and 9% of the control, respectively; however, the differences between means were insignificant.

As shown in Figure 2, the bile acid synthesis was correlated well ($r = 0.823$, $P < 0.001$, $n = 12$) with the activity of the steroid 12α-hydroxylase in the control, chenodeoxycholate-fed and ursodeoxycholate-fed hamsters.

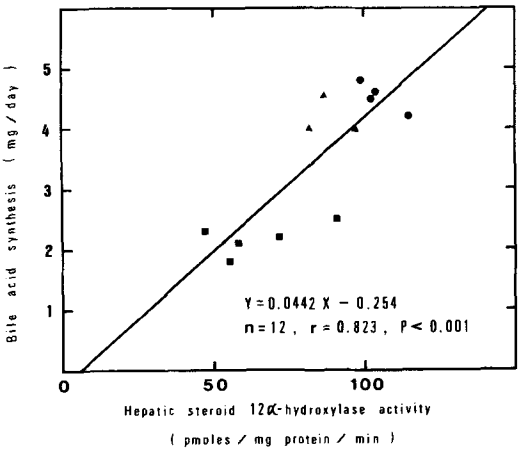

FIG. 2. Correlation between the activity of the hepatic microsomal steroid 12α-hydroxylase and the bile acid synthesis in control, chenodeoxycholate-fed and ursodeoxycholate-fed female hamsters. The best-fit linear function was determined by the method of least squares. The bile acid synthesis was calculated using the chromatographic balance method (fecal bile acid output minus bile acid intake). The symbols indicate the different treatment groups: control (●), chenodeoxycholate-fed (■), and ursodeoxycholate-fed (▲).

DISCUSSION

The results of the present study show that the steroid 12α-hydroxylase activity in hamsters was influenced by feeding of various bile acids and that the activity responded differently to different bile acids. Our data show that the activity of the 12α-hydroxylase was suppressed markedly when cholic acid or chenodeoxycholic acid had been fed. The same effect on the enzyme has been reported by Danielsson, who observed decreased 12α-hydroxylase activity by feeding of taurocholic acid and taurochenodeoxycholic acid to rats (13). However, the previous report on the inhibitory effect of taurochenodeoxycholic acid was inconclusive, since the rats possess a 6β-hydroxylase that converts chenodeoxycholic acid to α- and β-muricholic acids (14). Our data provide direct evidence for the inhibitory effect of chenodeoxycholic acid itself on the 12α-hydroxylase in experimental animals. More recently, Ahlberg et al. showed the inhibitory effect of chenodeoxycholic acid on the 12α-hydroxylase activity in patients with cholesterol gallstone disease (16). Our data in hamsters are consistent with theirs and the extents of inhibition are almost the same levels (39% in hamsters and 46% in man).

Effect of ursodeoxycholic acid on the 12α-hydroxylase activity has not been studied directly. However, Thistle et al. compared the effects of feeding chenodeoxycholic acid and

ursodeoxycholic acid in man and found that administered ursodeoxycholic acid does not suppress endogenous cholic acid synthesis to the same extent as does chenodeoxycholic acid (22). The results suggest that the inhibition of the 12α-hydroxylase by ursodeoxycholic acid is not as effective as that of chenodeoxycholic acid. In accord with the suggestion, our data show that the 12α-hydroxylase activity in the ursodeoxycholate-fed hamsters was higher than that in the chenodeoxycholate-fed animals, although the difference between both groups was not statistically significant. It must be mentioned that the effect of ursodeoxycholic acid on biliary bile acid composition in the hamsters differed from that in man. When ursodeoxycholic acid was administered to man, it became the predominant biliary bile acid with a reciprocal decrease of chenodeoxycholic acid and, to a lesser extent, of cholic acid (6). In the ursodeoxycholate-fed hamsters, however, the administered bile acid accounted for 21% of total biliary bile acids, while chenodeoxycholic acid became the major bile acid (59%) in the bile. The reasons for this difference are not known. A possibility is a much greater conversion of ursodeoxycholic acid to chenodeoxycholic acid in hamsters than in man. It has been reported that in the intestinal tract a major fraction of unabsorbed ursodeoxycholic acid is 7-dehydroxylated to form lithocholic acid by the action of microorganisms (23), and that this secondary bile acid, if absorbed, is largely converted to chenodeoxycholic acid during hepatic passage in hamsters (24). Another possible pathway for the formation of chenodeoxycholic acid from ursodeoxycholic acid involves the bacterial 7-dehydrogenation of ursodeoxycholic acid to form 7-ketolithocholic acid, which is then reduced to chenodeoxycholic acid by intestinal bacteria or the liver (25,26).

Anyhow, because the major biliary bile acid in the ursodeoxycholate-fed hamsters was not ursodeoxycholic acid but rather chenodeoxycholic acid, it is reasonable to assume that the suppression of the 12α-hydroxylase activity in these animals was not an effect of ursodeoxycholic acid alone but rather combined effects of ursodeoxycholic and chenodeoxycholic acids. Clearly, additional studies are needed to elucidate the effect of ursodeoxycholic acid alone on the 12α-hydroxylase activity. Nevertheless, we interpret our data to indicate that ursodeoxycholic acid has a lesser inhibitory effect on the 12α-hydroxylase than chenodeoxycholic acid, because the sum (80%) of the contents of ursodeoxycholic and chenodeoxycholic acids in the bile of the ursodeoxycholate-fed hamsters was the same as the content (81%) of chenodeoxycholic acid in the chenodeoxycholate-fed animals, and the only difference in the bile acid composition between both groups was the presence or absence of relatively small amounts of ursodeoxycholic acid.

The present study shows that daily fecal neutral sterol output increased about twice by feeding of chenodeoxycholic acid and ursodeoxycholic acid compared with the control and cholate-fed hamsters. Previous studies on the effect of bile acid feeding on cholesterol absorption from the gastrointestinal tract in man and experimental animals have shown that cholesterol absorption was depressed after feeding diets of chenodeoxycholic acid (7) and ursodeoxycholic acid (8,27), while the administration of cholic acid (27,28) was associated with an increase of cholesterol absorption. We believe that the increased output of fecal neutral sterols during chenodeoxycholic acid and ursodeoxycholic acid feeding is the reflection of the decreased absorption of cholesterol.

The effect of dietary bile acid on endogenous cholesterol synthesis is still controversial. Some of the available data suggest that bile acids directly inhibit the hepatic HMG-CoA reductase, the rate-controlling enzyme of cholesterol synthesis, resulting in a decrease of cholesterogenesis (9,10,29). Other studies seem to suggest that, during ursodeoxycholic acid feeding, an increased synthesis of cholesterol is mediated through the decreased cholesterol absorption (30,31). Our finding that chenodeoxycholic acid and ursodeoxycholic acid feeding increased endogenous cholesterol synthesis, while cholic acid feeding caused no significant effect on cholesterogenesis, seems to support this latter mechanism. In addition, there is a possible explanation of the abovementioned discrepancy; i.e., the HMG-CoA reductase of the intestinal mucosa participates considerably in the cholesterol synthesis and has a different character from that of the liver, so a reduced activity of the hepatic HMG-CoA reductase does not always prove a decreased cholesterol synthesis of whole body (32).

The bile acid synthesis was inhibited by chenodeoxycholic acid feeding, whereas ursodeoxycholic acid had little effect (Table 2). This result is consistent with that of Thistle et al. who suggested that administered ursodeoxycholic acid suppressed endogenous bile acid synthesis much less than chenodeoxycholic acid (22). It has been reported that cholic acid administration suppressed the hepatic cholesterol 7α-hydroxylase activity, the rate-determining enzyme of bile acid synthesis (13,29), and consequently bile acid synthesis in rats (28). In the present study, the depression of the

bile acid synthesis during cholic acid feeding was also observed, although the difference between means was not statistically significant.

It is worth mentioning that there is also a positive correlation between the activity of the steroid 12α-hydroxylase and the bile acid synthesis in the control, chenodeoxycholate-fed and ursodeoxycholate-fed animals (Fig. 2). The bile acid synthesis may reflect the activity of cholesterol 7α-hydroxylase, so the above-mentioned correlation suggests that cholesterol 7α-hydroxylase and steroid 12α-hydroxylase might be inhibited in a similar fashion by these 3,7-dihydroxylated bile acid feedings.

In conclusion, the steroid 12α-hydroxylase is inhibited intensely by cholic and chenodeoxycholic acids and slightly by ursodeoxycholic acid. The bile acid synthesis is decreased markedly by chenodeoxycholic acid and slightly by cholic and ursodeoxycholic acids.

ACKNOWLEDGMENTS

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Comparison of Ultracentrifugation and Gel Filtration for the Isolation of Bovine Lipoproteins

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ABSTRACT

Lipoproteins from the plasma of three nonlactating Holstein cows were isolated using either preparative ultracentrifugation or gel filtration chromatography. Lipoprotein classes obtained by ultracentrifugation were very low density plus chylomicra, <1.006 g/ml; low density, 1.007-1.039 g/ml; high density$_1$, 1.040-1.063 g/ml; and high density, 1.064-1.22 g/ml. These lipoprotein classes were individually applied to an agarose gel column to determine at what volume they eluted in comparison to lipoproteins that were separated after applying total bovine lipoproteins to the column. Three major peaks corresponding to very low density lipoproteins plus chylomicra, low density, and high density lipoproteins resulted after gel filtration of total lipoproteins. Very low density lipoproteins plus chylomicra, obtained by ultracentrifugation, eluted as a single peak, as did low density and high density lipoproteins. However, high density$_1$ lipoproteins eluted as two peaks. The first peak eluted at the same volume as low density lipoproteins, and the second peak eluted at a volume similar to that of the ascending slope of the high density lipoprotein peak. Results from disc polyacrylamide gel electrophoresis, immunoelectrophoresis and double immunodiffusion of lipoprotein fractions, and SDS polyacrylamide gel electrophoresis of their apoproteins, similarly indicated that the lipoproteins present in the 1.040-1.063 g/ml density interval are a mixture of low and high density lipoproteins rather than a unique class of lipoproteins.
Lipids 18:795-802, 1983.

INTRODUCTION

Analytical and preparative ultracentrifugation, electrophoresis, precipitation by sulfated polysaccharides, and gel filtration are techniques which have been used to isolate and characterize bovine lipoproteins (1-6). Preparative ultracentrifugation has been used extensively to isolate bovine lipoproteins on the basis of their hydrated densities; however, this method has several disadvantages. Centrifugation time required to obtain one fraction ranges from 18 to 24 hr, hence the isolation of several fractions from a single serum or plasma sample is very time-consuming. Secondly, lipoproteins obtained using this technique are often contaminated by other blood constituents having similar densities. Additionally, because bovine very low density lipoproteins may change density if not maintained at physiological temperatures (7), isolation must proceed at ca. 37 C. Ferreri and Gleockler (2) fractionated bovine lipoproteins according to molecular size using gel filtration chromatography and suggested it as an alternative isolation technique because it avoided many problems associated with ultracentrifugation. The objective of this study was to make a direct comparison between preparative ultracentrifugation and gel filtration chromatography as methods for separating bovine lipoproteins.

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MATERIALS AND METHODS

A flowchart of the experimental procedure is shown in Figure 1. Eight hundred ml of blood were obtained from the internal iliac artery of each of 3 nonlactating Holstein cows. The vessel in which the blood was collected contained amounts of disodium ethylenediaminetetraacetate (Na_2•EDTA), sodium azide (NaN_3), and gentamycin sulfate to provide concentrations in blood of 0.1%, 0.05%, and 0.005%, respectively. To process the samples as quickly as possible, 2 ultracentrifuges were used (Beckman Models L2-65B and L5-50B). Total lipoproteins were isolated from 150 ml of plasma (collected by centrifuging whole blood at 9,000 × g for 20 min at 20 C) by adjusting the background density to 1.22 g/ml with solid potassium bromide and centrifuging at 30,000 rpm (Beckman 30 rotor) for 40 hr in the L2-65B centrifuge operating at 20 C. The lipoproteins were collected by aspiration and concentrated to a volume of ca. 8 ml by ultrafiltration (50 ml Amicon stirred cell Model 8050). All steps used in the sequential isolation of lipoproteins, including centrifugation of blood to obtain plasma, were carried out at 37 C. A Beckman 50.2 Ti rotor and L5-50B ultracentrifuge were used for isolation of lipoproteins from 198 ml of plasma. Very low density lipoproteins and chylomicra (VLDL plus CM; <1.006 g/ml) were obtained by aspiration following ultracentrifugation of plasma for

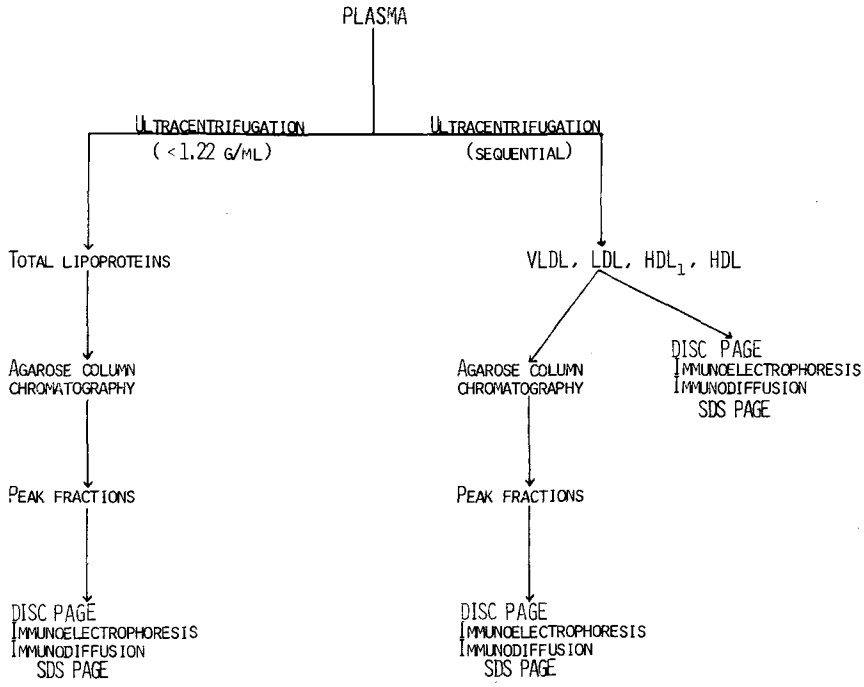

FIG. 1. Flowchart of procedures used in isolation and characterization of bovine lipoproteins.

18 hr at 35,000 rpm. Salt solutions prepared according to Lindgren (8) were used for density manipulations during sequential isolation of low density (LDL; 1.007-1.039 g/ml), high density$_1$ (HDL_1; 1.040-1.063 g/ml), and high density (HDL; 1.064-1.22 g/ml) lipoproteins. LDL and HDL_1 were removed by aspiration following centrifugation for 20 hr at 35,000 rpm, and HDL were similarly obtained after centrifugation for 24 hr at 35,000 rpm. Isolated lipoprotein fractions were concentrated to 6-8 ml by ultrafiltration. Salt solutions used for density adjustment contained Na_2 • EDTA (0.04%), NaN_3 (0.05%) and gentamycin sulfate (0.005%). Solution densities were measured using an American Optical Model 10423 refractometer.

A 0.5-ml aliquot of each concentrated lipoprotein sample was frozen for analysis by disc polyacrylamide gel electrophoresis (PAGE), immunoelectrophoresis, double immunodiffusion and SDS PAGE of their lipoproteins. The remainder of the lipoprotein sample was subjected to gel filtration chromatography (9). Bio-Gel A5-M, 200-400 mesh (Bio/Rad Laboratories) was packed to a bed height of 92 cm in a K 26/100 column (Pharmacia). Samples were applied to the column and eluted at 20 C with 1 M sodium chloride (NaCl) containing 1.2% Tris Base (Sigma) and 0.2% NaN_3, adjusted to pH 8.0. The rate of elution was ca. 17 ml/hr and 90 5-ml fractions were collected for each lipoprotein sample. The relative concentration of lipoprotein in each fraction was measured by absorption of light at 280 nm. Several fractions from each peak were composited and concentrated to ca. 1-3 ml to be used for disc and SDS PAGE. The fraction from each peak with the greatest absorbance at 280 nm was concentrated by ultrafiltration (10 ml Amicon Stirred Cell Model 12) to a volume of ca. 0.5 ml to be used for immunoelectrophoresis and double immunodiffusion analyses.

Disc PAGE was conducted using the procedures of Naito et al. (10) for sample and spacer gels and Narayan (11) for the separating gel. A 3.75% acrylamide separating gel was necessary for adequate separation of VLDL and LDL.

Electrophoresis was conducted by applying a constant current of 7 mA/tube for 40 min. SDS PAGE of lipoprotein apoprotein constituents was performed according to Edelstein and Scanu (12). Fifty μg of protein from each fraction was used.

Immunoelectrophoresis and double immunodiffusion techniques employed were as described by Ouchterlony and Nilsson (13). Antisera were obtained by injecting 2 New Zealand white rabbits with 0.5 ml whole

bovine sera emulsified with an equal volume of Freund's complete adjuvant. Injections were made subcutaneously at 4 different sites on the rabbits' backs. Booster injections were made 3 weeks later with 0.25 ml whole bovine sera emulsified with an equal volume of Freund's incomplete adjuvant. Rabbits were bled 4 weeks later and serum from each animal was pooled. Double immunodiffusion was performed on 45 x 95 mm plates (Miles Laboratories) coated with 10 ml gel media consisting of 1.0% agarose in 0.04 M barbital buffer, pH 8.6. Sample wells were cut in the gel media to allow application of 25 μl antigen, saline or antisera. Forty-eight hr were allowed for the formation of immunoprecipitates. Slides for immunoelectrophoresis were prepared by coating 76 x 25 mm glass slides with 2 ml gel media. Side wells were made to contain 5 μl antigen and the center trough to contain 65 μl of antisera. After electrophoresis of antigens by applying a potential difference of 10 V/cm with a current of 5 mA per plate, antiserum was added to the center trough and immunoprecipitates were allowed to form for 48 hr.

RESULTS

The elution profiles of lipoproteins obtained from the plasma of 3 nonlactating Holstein cows are shown in Figure 2. Profiles were similar for the 3 animals and agree with those reported by Ferreri and Gleockler (2). Three major peaks were observed and correspond to (I) VLDL plus CM, (II) LDL, and (III) HDL. A small peak eluted at ca. 450 ml. Ferreri and Gleockler (2) reported the presence of an extra peak which eluted after the HDL which they suggested to be albumin. The small peak we observed was shown not to be albumin since the latter eluted much earlier. Although CM have been separated from VLDL by gel filtration (14), the agarose gel used in this study did not facilitate the separation of lipoproteins in the size range of the VLDL and CM. The quantities of LDL were constant among cows; however, the amounts of HDL and VLDL varied considerably. Adequate separation of VLDL plus CM from LDL was obtained, but complete separation between LDL and HDL peaks was not achieved. A similar elution pattern was obtained by Ferreri and Gleockler (2) and they speculated that the reason for the lack of complete resolution of LDL and HDL was due to the presence of HDL_1, a class of lipoprotein previously isolated by ultracentrifugation and characterized by Puppione et al. (4) and Raphael et al. (15). Therefore, it was a major objective of this study to determine where the sub-

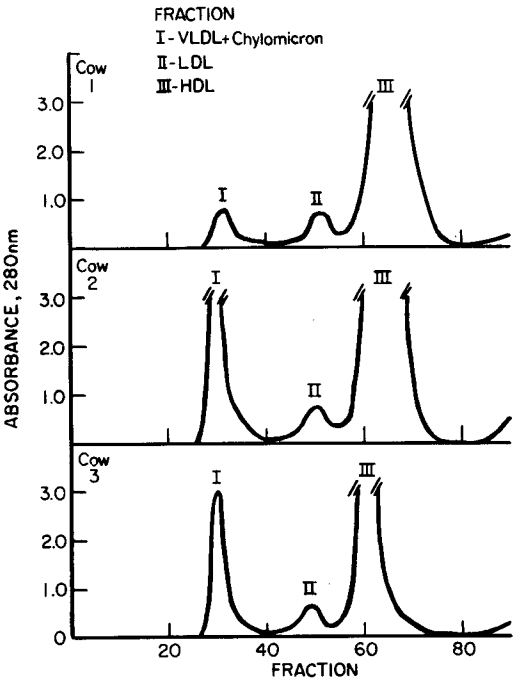

FIG. 2. Elution profiles of lipoproteins from 3 nonlactating Holstein cows. Total lipoproteins isolated by ultracentrifugation were separated using gel filtration chromatography.

class of lipoprotein, HDL_1, isolated by ultracentrifugation, would elute in comparison to VLDL, LDL and HDL also isolated by ultracentrifugation. Figure 3 shows the elution profiles of VLDL plus CM, LDL, HDL_1 and HDL obtained by ultracentrifugation from 1 cow and is representative of all 3 animals sampled. VLDL plus CM, LDL and HDL isolated by ultracentrifugation appeared relatively free of other lipoproteins although slight contamination was occasionally encountered. However, HDL_1 from each animal formed a double peak. The first peak eluted at the same volume as the LDL peak, whereas the second peak always eluted at a volume corresponding to the ascending portion of the HDL peak. These results indicate that the HDL_1 class is not a separate entity but rather a combination of LDL and HDL.

To test this hypothesis further, disc PAGE, immunoelectrophoresis and double immunodiffusion were employed to characterize the isolated lipoprotein fractions. Results from disc PAGE, which separates particles on the basis of size and electrical charge, were supportive of those obtained from examination of gel filtration elution profiles. Figure 4 shows acrylamide gels of lipoproteins isolated by ultracentrifugation (a) before and (b) after column chromatog-

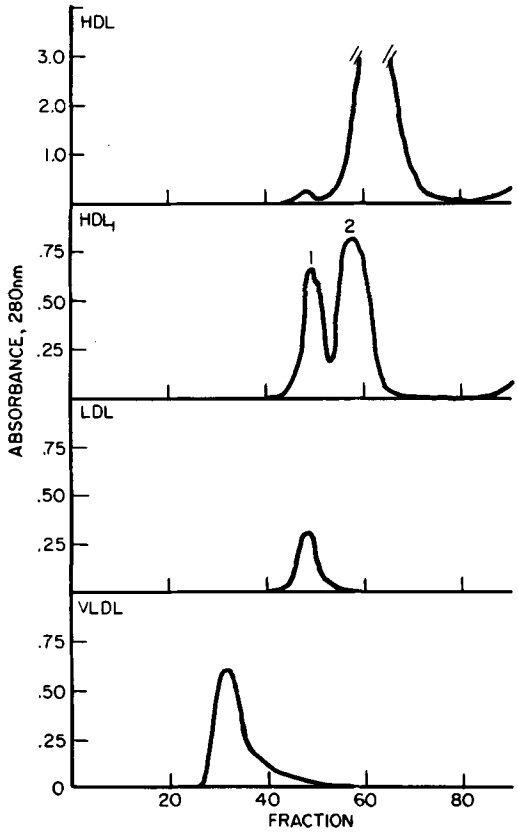

FIG. 3. Elution profiles of lipoproteins previously separated by preparative ultracentrifugation. Density intervals used for separation were: VLDL plus chylomicron, <1.006 g/ml; LDL, 1.007-1.039 g/ml; HDL_1, 1.040-1.063 g/ml and HDL, 1.064-1.22 g/ml.

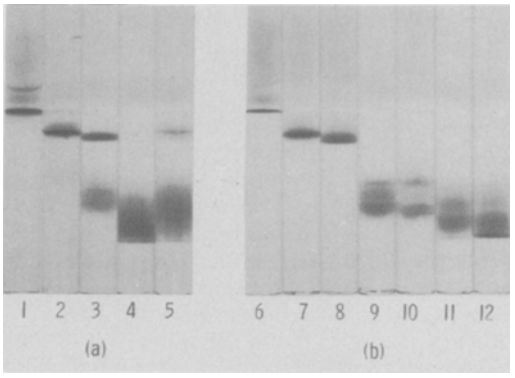

FIG. 4. Disc polyacrylamide gel electrophoresis of bovine lipoproteins isolated by preparative ultracentrifugation (a) before and (b) after gel filtration chromatography. (a): 1-VLDL, <1.006 g/ml; 2-LDL, 1.007-1.039 g/ml; 3-HDL_1, 1.040-1.063 g/ml; HDL, 1.064-1.22 g/ml; 5-total lipoproteins. (b): 6-VLDL, fractions 30-38; 7-LDL, fractions 48-50; 8-HDL_1, peak 1-fractions 46-50; 9-HDL_1, peak 2-fractions 54-60; 10-HDL, fractions 55-58; 11-HDL, fractions 59-65; 12-HDL, fractions 67-72.

raphy. VLDL plus CM isolated by ultracentrifugation (Fig. 4a) separated into 2 bands. A sharp band just penetrated the separating gel while a faint band remained in the spacer gel. Ferreri and Gleockler (2) speculated that the faint band may consist of CM. Electrophoresis of HDL_1 before passing through the agarose column revealed that it comprised both α and β migrating lipoproteins. The β migrating band is clearly equivalent to the β migrating band obtained from electrophoresis of LDL, whereas the α band was quite diffuse and migrated to a region similar to a portion of the very heterogeneous HDL fraction. Figure 4b shows the electrophoretic mobility of lipoproteins from peaks obtained after subjecting lipoproteins isolated by ultracentrifugation to column chromatography. The lipoproteins eluting in the first HDL_1 peak exhibited β migration almost identical to that of LDL, and the lipoproteins from the second peak showed similar migration as lipoproteins obtained from the ascending fractions of the HDL peak. The acrylamide gel (3.75%) was too porous for sharp resolution of HDL bands; however, the presence of multiple broad diffuse bands may be indicative of heterogeneity within this lipoprotein class.

Immunoelectrophoresis was used to characterize the lipoprotein fractions further. The ability of lipoproteins to migrate through 1% agarose gel depends mainly on their electrical charge, and the extent of migration is visualized by development of an antigen-antibody precipitate. Immunoelectrophoresis of VLDL plus CM resulted in a distinct single line of precipitation (data not shown). The absence of a doublet may have resulted because chylomicra were not present at a high enough concentration to facilitate a visual antibody-antigen precipitate. The lipoproteins contained in the most concentrated fraction of HDL_1 peaks 1 and 2 obtained by chromatography are compared to those in the most concentrated fraction from LDL and HDL peaks (Fig. 5). Although not identical, the precipitation line of LDL (fraction 47) was similar to that of HDL_1 peak 1 (fraction 48). The slight difference in pattern may be due to heterogeneity of LDL and slight variations in electrical charge of lipoproteins within this class. Patterns obtained from immunoelectrophoresis of HDL_1 peak 2 (fraction 56) and HDL (fraction 60) were similar showing the presence of 2 lines of precipitation. Two rather distinct protein-containing particles apparently elute together. The precipitate line closest to the sample well resembles LDL; however, it is difficult to imagine that LDL would still be eluting at this volume. It is also unlikely that the pattern reflects heterogeneity of HDL because of the striking difference between the

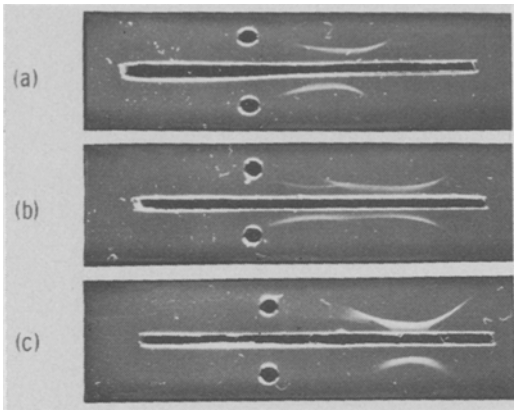

FIG. 5. Immunoelectrophoresis of bovine lipoproteins obtained by gel filtration chromatography and bovine serum albumin. (a) LDL, fraction 47 (top); HDL_1, fraction 48 (bottom). (b) HDL_1, fraction 56 (top); HDL, fraction 60 (bottom). (c) Bovine serum albumin, 1 mg/ml (top); bovine serum albumin, 0.1 mg/ml (bottom). Center trough contained antibovine sera.

two lines. The possibility exists that one of the lines, probably that closest to the well, corresponds to a nonlipoprotein protein that is of similar size as HDL yet has a different electrical charge. Because the line farthest from the well appeared at a location similar to that of albumin, the pattern obtained from HDL was compared to that of albumin (Fig. 5d). A bow-shaped precipitate characteristic of albumin was not observed in the precipitate line farthest from the well, which suggests that this line is probably HDL and not albumin contamination.

Double immunodiffusion was utilized to determine the immunological relation between lipoprotein fractions. Of particular interest was the relationship between LDL and HDL_1 from peak 1, since the precipitate patterns from immunoelectrophoresis of these lipoproteins was similar but not identical. Precipitation lines from LDL (fraction 47) and HDL_1 from peak 1 (fraction 48) fuse and form an arc (Fig. 6a). This type of reaction indicates the presence of identical antigenic determinants in the samples compared. A similar reaction occurs when HDL_1 from peak 2 (fraction 56) is compared to HDL (fraction 60; Fig. 6c). Therefore, it appears that LDL and HDL_1 eluting in peak 1 are identical in regard to their immunological properties, as are HDL and HDL_1 from peak 2. If HDL_1 from peak 1 (fraction 48) and peak 2 (fraction 56) are compared (Fig. 6b), the precipitation pattern shows fusion and the presence of a spur which is indicative of a partial identity between the 2 antigens. Since lipids are weak antigens, these results indicate that LDL and HDL_1 from peak 1 contain the same complement of apoproteins as does HDL and HDL_1 from peak 2. The partial relationship between HDL_1 from peaks 1 and 2, i.e., between LDL and HDL, is evidence that these 2 lipoprotein classes contain some but not all similar apoproteins. These results are contradictory to those of Stead and Welch (5) who indicated that HDL and LDL (LDL_1 in their terminology) were immunologically identical and LDL and HDL_1 (LDL_2 in their terminology) were immunologically distinct.

Because of these results and the fact that monospecific antisera was not used, apoprotein content of various lipoprotein fractions was determined using SDS PAGE (Fig. 7). Apoprotein content of LDL, HDL_1 and HDL isolated by ultracentrifugation was similar, with the amount of each apoprotein varying among fractions. Although not adequately shown in Figure 7, LDL contained the most protein which was unable to enter the 10% acrylamide gels used in this analysis. This protein may

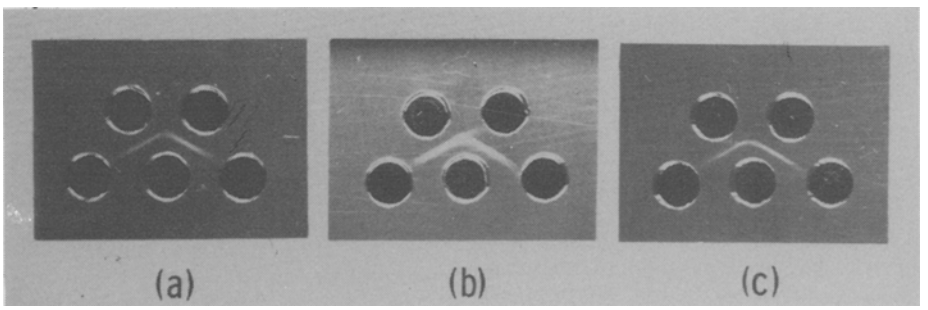

FIG. 6. Double immunodiffusion of bovine lipoproteins obtained by gel filtration chromatography. (a) Bottom wells: left and right–0.9% saline, center–antibovine sera; top wells: left–LDL, fraction 47, right–HDL_1, fraction 48. (b) Bottom wells: same as (a); top wells: left–HDL_1, fraction 48, right–HDL_1, fraction 56. (c) Bottom wells: same as (a); top wells: left–HDL, fraction 60, right–HDL_1, fraction 56.

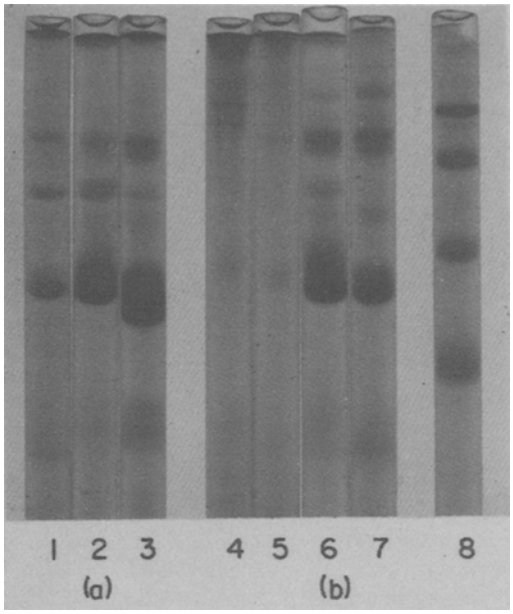

FIG. 7. SDS polyacrylamide gel electrophoresis of bovine apoproteins from lipoproteins isolated by preparative ultracentrifugation (a) before and (b) after gel filtration chromatography. (a): 1-LDL, 1.007-1.039 g/ml; 2-HDL_1, 1.040-1.063 g/ml; 3-HDL, 1.064-1.22 g/ml. (b): 4-LDL, fractions 48-50; 5-HDL_1, peak 1–fractions 46-50; 6-HDL_1, peak 2-fractions 54-60; 7-HDL, fractions 59-65; 8-protein standards–bovine serum albumin, egg albumin, trypsinogen, cytochrome c.

represent apoB which in other species has a molecular weight too large to migrate into 10% acrylamide gels (16). HDL had the least protein remaining atop the gel, while HDL_1 was intermediate in this respect. The protein contained within the bold prominent band seen in the HDL fraction had a molecular weight of ca. 22,000 and may represent bovine apoA-I. However, Jonas (17) and Puppione et al. (18) have reported a molecular weight of ca. 27-28,000 for bovine apoA-I. LDL contained the least amount of this protein and HDL_1 an intermediate quantity. Two heavier proteins having molecular weights of ca. 40,000 and 55,000 were also present in LDL, HDL_1 and HDL fractions. The identity of these proteins is unknown; however, their presence in all 3 fractions from each animal suggests that they may be apoproteins rather than contaminating protein. The low molecular weight proteins (8,200-9,600) observed in the HDL fractions may represent C apoproteins.

SDS PAGE of LDL, HDL_1 and HDL protein after gel filtration indicated that LDL and HDL_1 from peak 1 contained almost exclusively protein which did not enter the acrylamide gels. If it is assumed that this protein is apoB, then bovine LDL is similar to that of humans (16) in that it contains predominantly apoB. The relative absence of other apoproteins may be indicative of a greater purity of lipoproteins isolated by gel filtration as compared to ultracentrifugation. HDL_1 from peak 2 and HDL had a very similar apoprotein content with the 22,000 molecular weight protein being the predominant band observed. Results from apoprotein analysis, therefore, are supportive of other analyses conducted in suggesting that LDL and HDL_1 from peak 1 are similar, as are HDL_1 from peak 2 and HDL.

DISCUSSION

In agreement with Ferreri and Gleockler (2), results from this study indicate that gel filtration is an excellent method for separating bovine lipoproteins. Use of Bio Gel A5-M facilitates the separation of bovine lipoproteins into 3 major groups, undoubtedly corresponding to VLDL plus CM, LDL, and a very heterogeneous class of HDL. Chylomicra have been separated from VLDL using gel filtration (14) and we are currently attempting to resolve HDL using this method. However, due to the extreme range in size of bovine lipoproteins, no single gel can provide for separation of all the lipoprotein classes and subclasses simultaneously.

A comparison of ultracentrifugation and gel filtration for isolation of lipoproteins raises considerable doubt as to the validity of using the density interval of 1.040-1.063 g/ml to obtain a distinct class of bovine lipoproteins. Considerable confusion exists in the literature in regard to the make-up of lipoproteins isolated within the 1.006-1.063 g/ml density range. Stead and Welch (5) have subfractionated bovine LDL by ultracentrifugation and have referred to the 2 subfractions as LDL_1 (1.019-1.039 g/ml) and LDL_2 (1.039-1.060 g/ml). LDL_1 exhibited α migration and LDL_2 β migration when subjected to disc PAGE. This confirmed their earlier work (1) in which LDL_1 and LDL_2 corresponded to density intervals of 1.019-1.039 g/ml and 1.039-1.050 g/ml. Dryden et al. (19) also observed that bovine LDL consisted of 2 components and subsequently subfractionated the LDL class into 2 subclasses: LDL_1 (1.007-1.044 g/ml) and LDL_2 (1.044-1.063 g/ml). Results from analysis by analytical ultracentrifugation indicated that LDL_1, LDL_2 and HDL had densities of 1.030, 1.057 and 1.077 g/ml, respectively.

Raphael et al. (15) subfractionated bovine

LDL by ultracentrifugation since they had also observed the presence of α migrating lipoprotein in the 1.006-1.063 g/ml density range. However, in contrast to Stead and Welch (5), lipoproteins within the 1.006-1.039 g/ml density interval, referred to as LDL, exhibited predominantly β migration, whereas those isolated in the 1.039-1.063 g/ml density interval showed mainly α migration. Because of the predominance of α migrating lipoproteins and the similarity in composition to HDL, they termed this fraction HDL_1. The amount of overlap of α and β migrating lipoproteins within these subfractions appeared dependent on the physiological state of the cow (e.g., lactating vs nonlactating). Ferreri and Gleockler (2) used gel filtration to isolate bovine lipoproteins and reported that the lipoproteins eluting between the LDL and HDL peak showed increasing α migration with decreasing lipoprotein size. They concluded that this heterogeneous group of lipoproteins may be equivalent to the HDL_1 isolated in the 1.039-1.063 g/ml density interval reported by Raphael et al. (15).

Results from this study differ from those of Stead and Welch (5) but are similar to those reported by Raphael et al. (15) and Ferreri and Gleockler (2). What does differ between our findings and those above is interpretation of data. The lipoproteins eluting from the column between the LDL and HDL peaks are present in the 1.039-1.063 g/ml density interval. In addition, a significant portion of the LDL and HDL peak fall within this density range. HDL_1 isolated by ultracentrifugation and applied to a gel filtration column eluted in two peaks, one corresponding to the LDL peak, the other to the ascending edge of the HDL peak. This observation was confirmed using PAGE, immunoelectrophoresis and double immunodiffusion techniques. These methods enable separation and/or identification of bovine lipoproteins according to molecular size, electrical charge and immunological properties. Based on these physical and chemical characteristics, there is no basis to believe that in the bovine, a unique lipoprotein class exists that has a density within the range of 1.039-1.063 g/ml. Therefore, the justification for isolating a lipoprotein class within this density interval by ultracentrifugation seems questionable. However, more work in this area would be beneficial to ensure that discrepancies between this study and others (1,5) are not due to animal variability since small animal numbers have typically been used. Lactating cows were not sampled in this experiment. There is evidence to suggest that the level of lipoproteins in the 1.039-1.063 g/ml density interval increases during lactation, in particular late lactation (2,15). In further work, we have examined elution profiles of lipoproteins obtained from lactating cows and evidence indicates that, indeed, this occurs. However, this increase appears to be due to greater HDL levels and greater heterogeneity of lipoproteins within this class.

Gel filtration provides an alternative method to ultracentrifugation for the separation of bovine lipoproteins; however, the agarose gel column used in this study is not suitable for the separation of CM from VLDL or for resolution of subclasses of HDL. Multiple columns in series, each containing gels with varying exclusion limits, could be used to accomplish a more complete separation of bovine lipoproteins; however, this would be very time-consuming and cumbersome. The development and use of techniques employing high performance liquid chromatography may alleviate some of these problems in the future. Density intervals appropriate for isolation of bovine lipoproteins by ultracentrifugation which result in the least amount of cross-contamination need to be identified. Zonal centrifugation and isopycnic density gradient centrifugation may prove useful for establishment of suitable density intervals for the separation of bovine lipoproteins.

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Oleic Acid Transfer from Microsomes to Egg Lecithin Liposomes: Participation of Fatty Acid Binding Protein

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ABSTRACT

Oleic acid transfer from microsomes or mitochondria to egg lecithin liposomes was stimulated by fatty acid binding protein. By gel filtration, it could be demonstrated that this protein incorporates oleic acid into liposomes. Fatty acid binding protein transfer activity was higher using microsomes rather than mitochondria, which suggests a selective interaction with different kinds of membranes. Transfer of oleic acid by this soluble protein is greater than that of stearic acid. The results indicate that fatty acid binding protein may participate in the intracellular transport of fatty acids.
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The transfer of hydrophobic molecules between membranes is a process of biological significance. Thus, the function and activity of phospholipids, fatty acids, cholesterol, steroid hormones, fat-soluble vitamins and lipophilic drugs in cells and in whole organisms require their transfer between numerous bilayers and micellar lipid structures.

During the last 10 years, several phospholipid exchange or transport proteins have been isolated and characterized from different sources (1). Reports of steroid hormone binding protein (2) and of cholesterol binding protein (3), which are believed to be involved in the intracellular transport of these lipids in a specific and controlled fashion, suggested there might be a protein which could function in a similar way in the intracellular transport of fatty acids.

A soluble fatty acid binding protein (FABP), MW 12,000, present in cytosol obtained from various tissues (4) seems to be a good candidate for fatty acid transport between membranes. However, there is no direct evidence for the participation of FABP in a transport process between well characterized membranes. The present study was thus undertaken to check whether such protein is involved in fatty acid transport between membranes.

MATERIALS AND METHODS

Labeled fatty acids were purchased from The Radiochemical Centre, Amersham, England. Sephadex G75 and Sepharose 2B were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, NJ. Unlabeled oleic and stearic acids were generously provided by NuChek Prep., Elysian, MN.

Gel filtration of supernatants was performed as described (5). FABP was partially purified (as the 12,000 MW fraction) by filtration through Sephadex G75 (bed volume, 250 ml) and concentrated in an Amicon ultrafiltration cell (Amicon Corp., Lexington, MA) by using a UM-2 membrane. Mitochondria and microsomes were isolated as previously described (5). Egg lecithin was prepared by the method of Singleton et al. (6) dissolved in chloroform and stored at −20 C under nitrogen. Egg lecithin liposomes were prepared according to Rogers and Strittmatter (7).

Phospholipids (8) and protein (9) were determined as already described.

Incorporation of [1-^{14}C] Oleic Acid into Microsomes

[1-^{14}C] Oleic acid (0.5 μCi) specific activity 57.4 mCi/mmol, and 2.2 μmol of unlabeled oleic acid were dispersed with the aid of 100 μl propylene glycol and incubated in the presence of 1.5 ml of microsomal suspension containing 50 mg protein. After incubation at 37 C for 10 min, the suspension was diluted with 5 ml 0.25 M sucrose and centrifuged at 105,000 × g for 30 min. The final pellet was resuspended in 0.25 M sucrose in its original volume (1.5 ml). The amount of fatty acid bound to the microsomes was determined by extraction of an aliquot of the final suspension with chloroform/methanol (2:1).

Fatty Acid Transfer System

Transfer activity was determined by measuring the transfer of labeled oleic acid from microsomes to egg yolk lecithin liposomes. [1-^{14}C] Oleic acid labeled microsomes (2.5 mg protein, 34 nmol [1-^{14}C] oleic acid) were in-

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cubated with liposomes (0.16 μmol phospholipid-P) and cytosolic fractions with affinity for oleic acid or cytosol in a total volume of 1.5 ml 0.25 M sucrose, 0.001 M EDTA, 0.01 M Tris-HCl (pH 7.4). Incubation was carried out for 20 min at 25 C. At the end of the incubation, the pH of the medium was quickly adjusted to 5.1 by addition of 0.5 ml 0.2 M sodium acetate-acetic acid (pH 5.0) in 0.25 M sucrose. Microsomes were quantitatively sedimented at 10,000 × g for 10 min. Aliquots of the supernatant were counted in toluene counting fluid containing 30% Triton X100.

The pellet was dissolved in 0.5 ml sodium deoxycholate 2.5% and radioactivity was determined as described above. The supernatant containing the liposomes and soluble protein was analyzed by gel filtration.

Separation of Liposomes and FABP by Gel Filtration

Supernatant (1 ml) obtained in fatty acid transfer system, containing liposomes and FABP, was applied to a Sepharose 2B column (1 × 15 cm) and eluted with 0.01 M Tris-HCl (pH 7.4). Routinely, 1-ml fractions were collected. Liposomes emerged in the void volume, whereas FABP eluted in fractions 14-18 (not shown). Radioactivity, phospholipids and protein were determined in each fraction.

RESULTS

Sephadex G75 chromatography of rat liver cytosol incubated with [1-^{14}C] oleic acid showed association of radioactivity with a low molecular weight protein which has been designated "fatty acid binding protein" (fraction II). Radioactivity was also associated with components in the excluded (void) volume (fraction I) (Fig. 1).

To study transfer of fatty acids between membranes, microsomes containing [1-^{14}C] oleic acid were incubated with egg lecithin liposomes in the presence of cytosol or cytosolic fractions showing affinity for oleic acid. The transfer of oleic acid was not significantly affected by cytosol or fraction I. Appreciable transfer was observed in the presence of fraction II (Fig. 2).

The transfer of oleic acid from microsomes to liposomes was dependent on the incubation time (Fig. 3). The maximum transfer rate attained was 0.65 nmol of [1-^{14}C] oleic acid/min/mg of fraction II. In the absence of liposomes, more than 98% of the radioactivity was recovered associated to microsomes. Appreciable spontaneous transfer of oleic acid (10% in 20 min of incubation at 25 C) was observed in the absence of fraction II.

For determination of the amount of oleic acid incorporated in acceptor vesicles as a function of fraction II protein concentration, supernatants obtained in fatty acid transfer system after centrifugation were analyzed by Sepharose 2B gel filtration. In these experiments, the column eluates were analyzed chem-

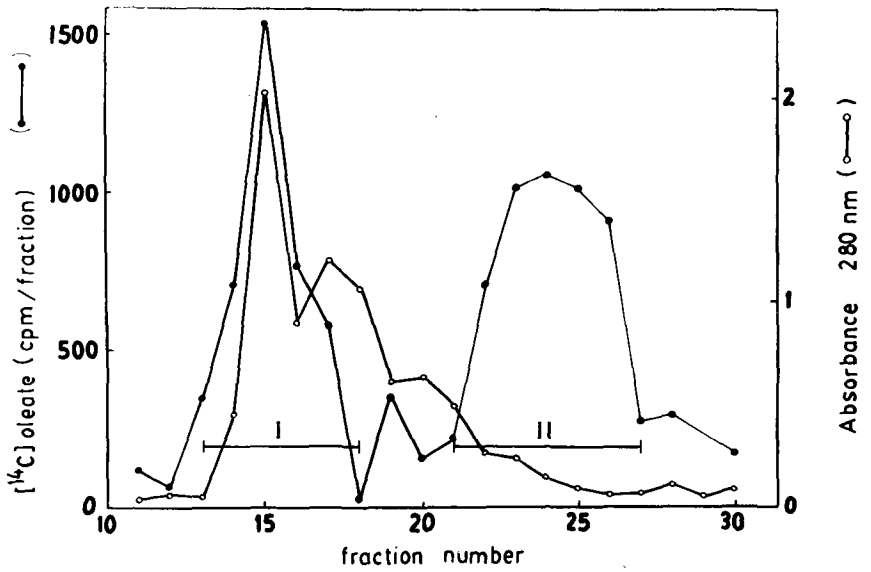

FIG. 1. Gel filtration chromatography of rat liver cytosol in the presence of [1-^{14}C] oleic acid. 25 nmol of [1-^{14}C] oleic acid (in 50 μl of propylene glycol) were added to 10 mg of cytosolic protein in 0.5 ml of 0.01 M Tris-HCl (pH 7.4) and chromatographed in Sephadex G75 (1 × 43 cm, 12 ml/hr, 1 ml fractions, elution buffer 0.01 M Tris-HCl, pH 7.4).

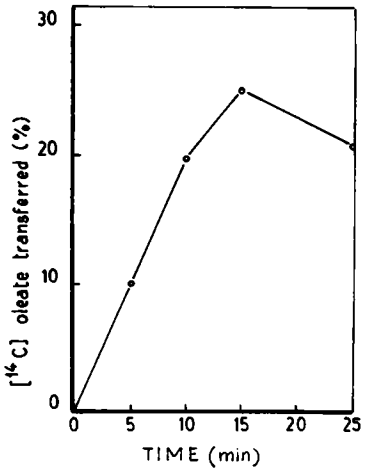

FIG. 2. Transfer of oleic acid during incubation of rat liver labeled microsomes with egg lecithin liposomes in the presence of cytosol or cytosolic fractions. The incubation was at 25 C for 20 min. The incubation mixture contained 2.5 mg microsomal protein (34 nmol [1-^{14}C] oleic acid) and liposomes (0.16 μmol phospholipid-P) in a total volume of 1.5 ml 0.25 M sucrose, 0.001 M EDTA, 0.01 M Tris-HCl (pH 7.4). Fraction II (○), fraction I (▲), cytosol (●).

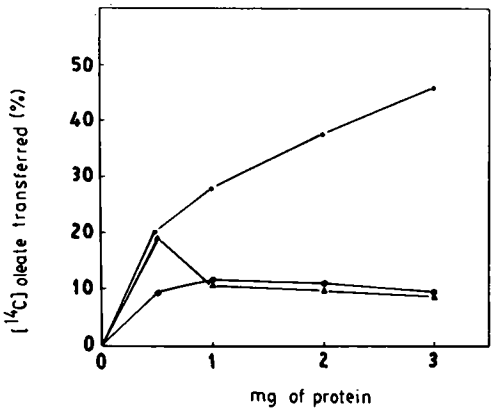

FIG. 3. Time course of oleic acid transfer reaction accelerated by fraction II (1 mg protein). Oleic acid transfer activity was assayed under the standard conditions described in Material and Methods. Incubation was at 25 C for the indicated time.

ically both for phospholipids and protein in addition to determining the distribution of radioactivity.

Our results indicate that oleic acid was effectively transferred from microsomes to liposomes. As can be seen (Fig. 4), specific radioactivity of the liposomal phospholipids was increased considerably, whereas oleate incorporated per mg of protein (fraction II) decreased as a function of transfer protein con-

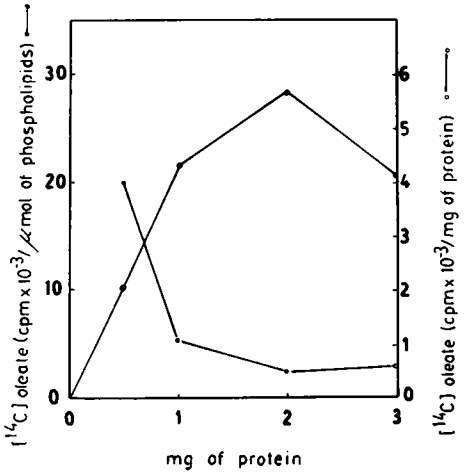

FIG. 4. Oleic acid distribution in egg lecithin liposomes and fraction II as a function of transfer protein concentration. Supernatant prepared as described in fatty acid transfer system was subjected to Sepharose 2B column (see Materials and Methods). Radioactivity, phospholipids and protein were determined in each fraction.

centration. The increase in the specific radioactivity of liposomal phospholipids and the fact that no changes in the supernatant phospholipid content could be detected, suggest the influence of FABP in the movement of oleic acid from microsomes to liposomes rather than a partition of the fatty acid among the 3 components of the transfer assay system. Thus, it indicates that oleic acid transfer was from microsomes to fatty acid binding protein to egg lecithin liposomes.

The transfer activity obtained when mitochondria containing [1-^{14}C] oleic acid was incubated with egg lecithin liposomes at 25 C in the presence of fraction II was lower than that observed using labeled microsomes as donors of oleic acid (Fig. 5). It must be noted that the amount of oleic acid present in mitochondrial membranes was in the same range as that present in microsomal membranes. As in the case of oleic acid labeled microsomes, fraction I showed no effect in the transfer of oleic acid from mitochondria to egg lecithin liposomes.

Next, the transfer of labeled oleic acid was compared to the transfer of labelled stearic acid. Microsomes containing either [1-^{14}C] oleic acid or [1-^{14}C] stearic acid were prepared and incubated with liposomes in the presence of fraction II (3 mg protein). Stearic acid was transferred at a rate 7.6% of that for oleic acid (results not shown).

The amount of fatty acids disappearing from microsomal or mitochondrial membranes

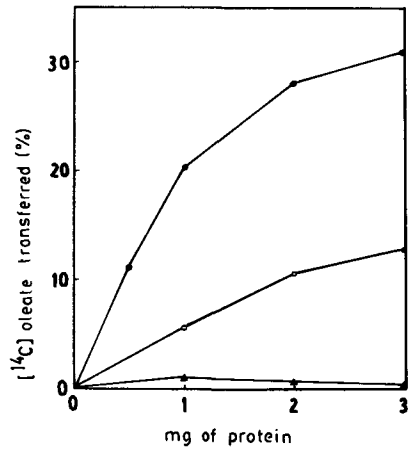

FIG. 5. Transfer of oleic acid from microsomes or mitochondria to liposomes. Microsomes (2.5 mg protein) containing [1-^{14}C] oleic acid were incubated in the presence of fraction II (●). Mitochondria (2.5 mg protein) containing oleic acid were incubated in the presence of fraction II (○) or fraction I (▲).

agreed well with the amount recovered in the acceptor vesicles. The material showing transfer activity present in the second Sephadex peak could be stored at −20 C without significant loss of activity for one month.

DISCUSSION

Fatty acid uptake by hepatic cells is a passive process, fatty acids appear to interact with the plasma membrane, and no specific receptors have been demonstrated (10,11). It has been postulated that fatty acid binding protein facilitates the desorption of fatty acids from the inner membrane, and that the fatty acid-FABP complex diffuses to the endoplasmic reticulum where the fatty acid is activated to its acyl-CoA derivative. We have previously shown (5) evidence in the transfer of palmitic acid from fatty acid binding protein to microsomes, mitochondria and lipid vesicles, suggesting the possibility that fatty acids may be transferred from microsomes or mitochondria to lipid vesicles in the presence of FABP. The present studies confirm the concept that the 12,000 MW protein fraction of rat liver 105,000 × g supernatant is involved in the transfer of fatty acids between membranes.

The data presented clearly show that there is a functional interaction between hepatic endoplasmic reticulum or mitochondria with FABP, since the partial purified protein (fraction II) is capable of transferring oleic acid from microsomes or mitochondria to egg lecithin liposomes.

The ability of FABP to transfer [1-^{14}C] oleic acid incorporated in microsomes at a higher rate than that observed using the fatty acid incorporated in mitochondria indicates that FABP interacts selectively with different kinds of membranes and suggests that such an interaction may be of physiological significance in vivo during efflux or uptake of free fatty acids by the liver. In this regard, biological membranes are thought to consist of a phospholipid bilayer interspersed with proteins. The fact that phospholipid composition of the outer mitochondrial membrane is similar to that of microsomal membranes (12) suggests the possibility that the observed differences in transfer of [1-^{14}C] oleic acid from microsomes or mitochondria to lipid vesicles should be the result of a selective kind of interaction between FABP and membrane proteins.

Our findings are in agreement with results obtained using isolated hepatocytes (13) which indicate that FABP specifically directs the metabolism of long-chain fatty acids towards microsomal esterification.

It has also been reported that stearic acid bound to FABP is transferred to the mitochondrial β-oxidative system in the heart (14), suggesting a role as transcytoplasmic fatty acid carrier for this protein.

Mishkin et al. (15), in studies of FABP isolated from rat liver supernatant, showed that binding of oleic acid exceed that of stearic acid, which could explain the differences observed in the transfer of these fatty acids in our assay system. Oleic acid was transferred at a rate 13 times higher than stearic acid from microsomes to egg phosphatidylcholine liposomes.

Other possible explanations on the differences observed in the transfer of stearic and oleic acids could be due to differences in the association of the fatty acid with either specific binding microsomal proteins or bulk membrane phospholipids.

Under these conditions, it appears that fatty acid transfer involves a sequence of binding to FABP and subsequent insertion of the acyl chain into the hydrocarbon region of the liposomal bilayer.

Recently, it has been demonstrated that FABP is an entity of 3 similar components with respect to amino acid analysis, electrophoretic mobility, and immune crossreactivity (16). Differences between them may be secondary to preparatory methods and ligand load.

Additional studies in this area using the transfer assay system described in this paper may be expected to clarify the physiological function of fatty acid binding protein and its participation in fatty acid uptake and utilization.

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Metabolism of Fatty Acid, Glycerol and a Monoglyceride Analogue by Rat Cardiac Myocytes and Perfused Hearts

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ABSTRACT

Studies have been conducted on the uptake and metabolism of unesterified fatty acid, free glycerol and 1-hexadecyl glyceryl ether by rat cardiac myocytes, and of fatty acid, intact triglyceride and the glyceryl ether by perfused rat hearts. Cardiac myocytes efficiently extracted, oxidized and esterified oleic acid, but demonstrated little ability to utilize free glycerol. Although the glyceryl ether was efficiently extracted by myocytes, it was neither hydrolyzed or esterified. The perfused heart also extracted and metabolized unesterified fatty acid, and the fatty acid released during lipolysis of circulating lipoprotein triglyceride. The glyceride glycerol, however, was largely recovered (90%) in the perfusate suggesting inefficient myocardial utilization of either free glycerol or partial glycerides. Myocardial extraction of glyceryl monoether was demonstrated, but the monoglyceride analogue was also unmetabolized by intact heart tissue. The results suggest that if monoglycerides are produced by the action of lipoprotein lipase on circulating triglycerides, reutilization of intact monoglycerides for higher glyceride synthesis is not a major fate of these products.
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INTRODUCTION

The intact heart can efficiently utilize the triglycerides of circulating chylomicrons and very low density lipoprotein (VLDL) (1-4). Available evidence suggests that these glycerides are partially or completely hydrolyzed at the capillary endothelial surface by the action of functional (membrane-supported) lipoprotein lipase (5,6). The released products are rapidly utilized by the tissue, since these do not appear to accumulate extracellularly during organ perfusion studies in vitro (6,7).

Free glycerol, resulting from the action of lipoprotein lipase, is not efficiently reutilized for glycerolipid resynthesis in heart muscle due to low levels of glycerokinase activity (8). Thus, synthesis of phospholipids and glycerides in heart muscle requires either the availability of α-glycerophosphate during glycolytic production of dihydroxyacetone phosphate, or reutilization of monoglyceride, as in the case of intestinal tissue. There are suggestive data (9) that, under certain conditions, the heart may extract intact glycerides. However, the fate of the extracted glyceride has not been determined. Thus, it has been difficult to assess whether monoglyceride formation and utilization in heart muscle may represent an important aspect of lipoprotein lipase activity in situ.

As an approach to assessing the possibility that intact glycerides may be utilized by heart tissue, the uptake and metabolism of 1-[1-^{14}C]hexadecyl glyceryl ether by the perfused rat heart and by rat cardiac myocytes have been compared to that of unesterified oleic acid, free glycerol and lipoprotein-associated triglyceride. These monoglyceride analogues have been effectively employed as model substrates for elucidating specific aspects of glycerolipid resynthesis in intestinal epithelial tissue. Since glyceryl monoethers are not hydrolyzed by lipases (10), their incorporation into di- and trialkoxy ether derivatives has been taken as a measure of monoglyceride metabolism in tissue (10, 11).

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MATERIALS AND METHODS

Materials

Trypsin (180 units/mg), collagenase (type 2, 136 units/mg) and lima bean trypsin inhibitor were obtained from Worthington Biochemicals (Freehold, NJ). Bovine serum albumin (fraction 5, fatty acid-poor) was purchased from Sigma Chemical Co., St. Louis, MO. Lipids (>99% purity) were from Supelco Corp., Bellefonte, PA, and labeled compounds were from Amersham-Searle Corp., Arlington Heights, IL. All other chemicals were of highest purity and purchased from Fisher Chemical Co., La Jolla, CA. Adult male Wistar rats were obtained from Charles River Laboratories (Wilmington, MA). The 1-[1-^{14}C]hexadecyl glyceryl monoether was kindly provided by Dr. R. Wykle,

Department of Biochemistry, Bowman Gray School of Medicine.

Lipoprotein Isolation and Labeling

Adult male rats (200-300 g) were allowed standard laboratory chow ad libitum, and were subjected to cannulation of the left thoracic lymphatic duct (12). Lymph was collected on ice for periods up to 24 hr during which animals were not provided food, but received a continuous intraduodenal infusion of 0.9% NaCl to maintain lymph flow. Large chylomicrons ($d<1.006$ g/ml) were removed by preparative ultracentrifugation of 3×10^6 g-avg min. The very low density lipoproteins (VLDL or small chylomicrons) were obtained at $d<1.006$ g/ml NaCl, dialyzed, and characterized by analysis of apolipoprotein composition using SDS-polyacrylamide disc electrophoresis (13) and analysis of lipid compositions (14).

The VLDL fraction was labeled with triglyceride in vitro by a modification of the method of Brenneman and Spector (15). This involved addition of glyceryl tri[1-^{14}C]oleate (30 μCi) and [2-^{3}H]glyceryl trioleate (150 μCi) to 30 g Celite and evaporation of solvents under nitrogen. Six ml of the VLDL preparation (0.5 mg protein/ml) were added to the Celite, the mixture was incubated for 2 hr at room temperature and the suspension was passed through 1.2-μm millipore filter. The filtered lipoprotein was washed with 1 mM albumin in saline to remove unesterified fatty acids resulting from the procedure and the resulting lipoprotein preparation contained 85-90% of the [^{14}C] and [^{3}H] labels in the core triglyceride fractions.

Heart Perfusions

Hearts from adult male rats were perfused by a modified Langandroff procedure in a closed recirculating apparatus, as described earlier (16). The perfusion media (20 ml) consisted of a modified Krebs bicarbonate buffer containing 0.5% albumin to which lipids were added as described for individual studies. The media was circulated in the apparatus for 5 min prior to insertion of heart. Aliquots of the perfusate were obtained at 0 and 45 min perfusion for analysis of $^{14}CO_2$ and lipids as described earlier (14,16,17). After perfusion, the coronaries were flushed with 5 ml of unlabeled media and hearts were blotted, weighed and homogenized in buffer for lipid extraction (18). For individual studies, perfusion media were prepared to contain: 400 μM unesterified fatty acid complexed to bovine serum albumin; freshly prepared lymph VLDL (400 μM fatty acid equivalent in triglycerides), containing [2-^{3}H]glyceryl tri[1-^{14}C]oleate; or 1-[1-^{14}C]hexadecyl glyceryl ether (60 μM) added in acetone to the albumin-containing media.

Preparation and Incubation of Heart Cells

The procedure for enzymatic dissociation of rat ventricular tissue was a modification of the described earlier (19). The buffered media for both tissue dissociation and resuspension of cells consisted of (g/l): NaCl, 6.8; KCL, 0.4; NaH_2PO, 0.21; Na_2HPO_4, 0.06; EDTA, 0.01; and glucose, 0.9. The pH was adjusted to 7.4 and osmolarity to 300 mOsm/l. Left ventricular tissue from adult rat hearts was minced and subjected to successive 20 min incubations in 2 ml 0.6% trypsin (2 x), 2 ml 0.3% trypsin inhibitor (1 x), and 2 ml 0.3% collagenase (4-5 x). Supernatants from each collagenase incubation were collected and maintained on ice; these were subsequently combined and filtered through 350-μm nylon mesh. The harvested cells were washed 2-3 times with buffer allowing cells to settle after each washing. These were resuspended in fresh buffer for cell counts and determination of viability (19).

For studies on lipid uptake and metabolism, the myocytes were diluted in 10 ml buffer containing 0.5% albumin to a final cell count 0.5×10^5 cells/ml in individual studies, incubation media were prepared to contain the following: 4 μmol [1-^{14}C]oleic acid (10 μCi) complexed to bovine serum albumin; 1.2 μmol [2-^{14}C]glycerol (50 μCi); 1.2 μmol 1-[1-^{14}C]-hexadecyl glyceryl ether (1 μCi) added in 5 μl acetone to the albumin-containing media;

Incubations were for 45 min at 37 C. Aliquots of the cell suspensions were obtained at 1 min and at 15-min intervals for determinations of $^{14}CO_2$ (20). Aliquots (1.5 ml) were also subjected to centrifugation for 4 min at 2 C to sediment cells, for direct isotopic analysis of cells and supernatant, or for lipid extraction and analysis of lipid fractions.

Analyses

$^{14}CO_2$ in the heart perfusion media and CO_2 trap (16), and in the myocyte incubation media, was assessed by the method of Cuppy and Crevasse (20). Lipids in heart perfusion and cell incubation media, and in the tissue preparations were extracted by the method of Folch et al. (18) using 20 vol of chloroform/methanol (2:1, v/v). After evaporation of the chloroform extract under N_2 and reextraction of lipids in hexane, aliquots of the hexane extract were subjected to thin layer silicic acid chromatography in either hexane/diethyl ether/acetic acid (80:16:2, v/v) or hexane/acetone/acetic

acid (89:11:3, v/v). The latter system has been effectively employed to separate alkoxy derivatives of glycerolipids (11). Lipid classes were identified by comigration with authentic standards. Individual silicic acid areas corresponding to cholesteryl esters, triglycerides (or alkoxy diglycerides), unesterified fatty acids, mono- and diglycerides (or alkoxy monoglycerides and unmetabolized glyceryl ether) and phospholipid were scraped into vials for subsequent isotope analysis by liquid scintillation spectrometry (11).

RESULTS

Heart Perfusion Studies

The comparative myocardial extraction and metabolism of free and esterified fatty acid, glyceride glycerol and the glyceryl monoether is summarized in Table 1. During the 45-min recirculating perfusion of 8 μmol (0.4 mM) albumin-bound oleic acid, 2.5 μmol, or 31.2 ± 4.0%, was extracted by the intact heart. Of this, 0.6 μmol, or 24.1 ± 2.8%, was completely oxidized to $^{14}CO_2$ and the remainder was accounted for as tissue lipid (33.1 ± 1.5%) and water-soluble metabolites (42.8 ± 4.0%). Ca. 91% of the lipid radioactivity from [1-^{14}C]-oleate was recovered as esterified lipids and primarily as triglyceride. These levels of tissue extraction, oxidation and distribution of unesterified fatty acids are comparable to those reported earlier (19,21) using an identical approach.

We have recently reported on studies using VLDL labeled in the core lipids with [2-^{3}H]-glyceryl tri[1-^{14}C]oleate (22). It was shown that the correlation coefficient of disappearance of triglyceride mass and label from VLDL during heart perfusion is 0.96 ± 0.04, demonstrating the utility of this in vitro labeled model for studies on triglyceride lipolysis.

In the present studies, perfusion of hearts with VLDL for 45 min resulted in lipolysis of 32.5 ± 1.6% of the available triglyceride fatty acid. Of this (2.6 μmol), 2.4 μmol, or 92.3%, was extracted by the heart and the remainder was recovered as unesterified fatty acid in the perfusing media. Ca. one-third (29.8%, 0.72 μmol) of the extracted oleic acid was oxidized to $^{14}CO_2$, and only 7.6% was recovered in tissue lipids (Table 1). The remainder was accounted for as water-soluble metabolites, presumably derived from oxidation of oleic acid. The distribution of the extracted oleate among tissue lipids was comparable to that observed during perfusion of unesterified oleic acid.

During lipolysis of VLDL triglycerides by

TABLE 1

Extraction and Metabolism of Oleic Acid, VLDL Triglyceride and Glyceryl Monoether by Perfused Rat Hearts

Substrate[a]	Lipolysis (μmol/g)	Tissue uptake (μmol/g)	Oxidation to CO_2 (μmol/g)	Distribution of tissue lipid radioactivity				
				UFA[b] (%)	TG (%)	MG-DG (%)	PL (%)	Other (%)
[1-^{14}C]Oleic acid (8 μmol)	–	2.500 ±0.32	0.60 ± 0.07	7 ±2	80 ±2	7 ±1	4 ±0.3	–
Glyceryl tri[1-^{14}C]oleate (8 μmol)	2.60 ± 0.13	2.420 ±0.13	0.72 ± 0.14	12 ±1	71 ±1	11 ±1	6 ±2	–
[2-^{3}H]Glyceryl trioleate (2.4 μmol)	0.89 ± 0.09	0.083 ±0.010	0	0 ±0	70 ±4	22 ±2	7 ±3	
1-[1-^{14}C]Hexadecyl glyceryl ether (1.2 μmol)	–	0.700 0.16	0	0 ±0	2 ± 0.5	4 ±0.1	0 ±0	94[c] ± 1

[a]Recirculating perfusion of intact rat heart was conducted for 45 min in a closed system (16), using the substrates indicated, in 20 ml Krebs buffer, pH 7.4, containing 0.5% bovine serum albumin. Preparation of the fatty acid and glyceryl monoether substrates are described under Methods. Labeled glyceryl trioleate was incorporated into VLDL core lipids as described under Methods. Data represent means from 4-6 studies ± SEM.

[b]Abbreviations: UFA, unesterified fatty acid; TG, triglyceride; MG-DG, mono- and diglycerides; PL, phospholipids.

[c]Recovered as authentic 1-hexadecyl glyceryl monoether.

the perfused heart, 0.89 μmol of [2-^{3}H] glycerol (37%) was hydrolyzed from [2-^{3}H] glyceryl trioleate. Of this, 90.3 ± 15.1% was recovered in the perfusate (Table 1) and only 5.3 ± 0.2% was accounted for in tissue lipids (Table 1). The remainder (4.0 ± 1.1%) was recovered as nonlipid glycerol radioactivity. Analysis of the distribution of lipid glycerol (Table 1) indicated that ca. 92% was as partial glycerides and triglyceride and the remainder as phospholipid glycerol. No radioactivity was recovered as unesterified fatty acid. Thus, overall, the ratio of VLDL triglyceride fatty acid release to glycerol release was 2.92, whereas the ratio of tissue extraction of fatty acid and glycerol was 28.9, or almost 10-fold. These data are also compatible with those reported earlier (21).

Recirculating perfusion of [1-^{14}C] hexadecylglyceryl-1-monoether for 45 min resulted in a extensive tissue extraction representing 58% of the available glyceride analogue. Based on analysis of the perfusates and tissues, there was no lipolysis of the ether (e.g., no unesterified hexadecyl alcohol or fatty acid) and there was no oxidation of the labeled hexadecyl moiety. Thus, the extracted label was completely recovered in tissue lipids, and of this, 94% was recovered as the unmetabolized ether. The remainder was distributed in chromatographic fractions corresponding to partial glycerides (4%) and triglycerides (2%).

Cell Studies

Studies with cardiac ventricular myocytes were conducted only for qualitative comparisons of tissue lipid extraction and metabolism. Since we have previously demonstrated that these cells are unable to metabolize VLDL triglycerides (21), comparative studies were conducted using unesterified oleic acid, glycerol and the glyceryl monoether. During 45-min incubations of cells (5 × 10^5 cells/ml) with [1-^{14}C] oleic acid, total cellular extraction of the fatty acid (Table 2) was 18.3 ± 2.2 nmol/mg protein (24.8 ± 3.1% of the available fatty acid). Of this uptake, 2.85 ± 0.40 nmol/mg protein, or ca. 16%, was oxidized to $^{14}CO_2$ by the end of incubation. As shown in Figure 1, the extracted fatty acid was rapidly esterified, primarily to triglycerides, during the course of incubation, and by 45 min, only 12% was recovered as unesterified fatty acid (Table 2).

When myocytes were incubated with [2-^{14}C] glycerol, only 0.4 ± 0.08% was extracted by the cells and none of this appeared as $^{14}CO_2$ (Table 2). Analysis of the cellular radioactivity after the 45-min incubation showed that 90% of the extracted glycerol was recovered as

TABLE 2

Uptake and Metabolism of Oleic Acid, Glycerol and Glyceryl Ether by Rat Cardiac Myocytes

Substrate[a]	Cellular uptake (nmol/mg protein)	Oxidation to Co_2 (nmol/mg protein)	Distribution of cellular lipid radioactivity				
			UFA[b] (%)	TG (%)	MG-DG (%)	PL (%)	Other (%)
[1-^{14}C] Oleic acid (4 μmol)	18.3 ±2.2	2.85 ± 0.40	12 ± 2	78 ±4	4 ± 0.3	8 ± 2	–
[2-^{14}C] Glycerol (1.2 μmol)	0.089 ±0.018	0	0 ± 0	42 ± 2	39 ± 2	19 ± 2	–
[1-^{14}C] Hexadecyl glyceryl ether (1.2 μmol)	3.45 ±0.02	0	1 ± 0.5	1 ± 0.2	0 ± 0	0 ± 0	98[c] ± 1

[a]Cardiac myocytes (5 × 10^5 cells; 53.9 mg protein; 10 ml) were incubated for 45 min at 37 C with 400 μM oleic acid albumin bound, 120 μM glycerol or 120 μM glyceryl monoether. Incubations were conducted in sealed vessels (95% O_2, 5% CO_2) containing hyamine hydroxide in center wells for analysis of $^{14}CO_2$ (20). Cells and media were individually analyzed for lipids or glycerol as described under Methods. Figures represent mean of 3-4 studies ± SEM.

[b]Abbreviations: UFA, unesterified fatty acid; TG, triglycerides; MG-DG, mono- and diglycerides; PL, phospholipids.

[c]Recovered as unmetabolized 1-hexadecyl glyceryl ether.

nonlipid glycerol. The 10% associated with tissue lipids (0.009 μmol/mg protein) was entirely as esterified lipids, of which ca. 80% was as partial glycerides and triglycerides (Table 2). The time course of esterification of these small amounts of glycerol into glycerides and phospholipid is shown in Figure 2.

Incubation of myocytes with the glyceryl monoether resulted in cellular extraction of 15.5 ± 0.1% (3.45 ± 0.02 nmol/mg protein) of the available glyceride analogue. Of this uptake, none was recovered as $^{14}CO_2$ during the 45-min incubation. As in the case of the perfused organ, the extracted radioactivity was completely recovered as tissue lipid, and of this, 98% was associated with the chromatographic area corresponding to the authentic glyceryl monoether.

DISCUSSION

Previous studies have demonstrated the low levels of heart glycerokinase, and the inefficient metabolism of free glycerol by myocardial tissue (8). This is also clearly demonstrated in the present studies using both the intact organ model and isolated cardiac myocytes. With cardiac myocytes, utilization of free glycerol was less than 0.5% of the available substrate (1.2 μmol). In contrast, incubations with unesterified oleic acid, at a level approximating a 3:1 ratio to glycerol, was efficiently extracted (24.8%) and rapidly incorporated into esterified lipids. Thus, the relative utilization of fatty acid to glycerol was 200:1, despite an initial concentration ratio of 3:1.

During the perfusion of hearts with VLDL containing [2-^{3}H]glyceryl trioleate, there was essentially no accumulation of [^{3}H] partial glycerides in the perfusion medium, and by 45 min, over 90% of the label appeared in the perfusate as water-soluble tritium. From these studies, it could not be determined whether total hydrolysis of triglyceride occurred by the action of membrane-associated LPL, or that partial glycerides were produced, extracted by the heart and subsequently hydrolyzed by tissue lipases. In either case, only 5.3% of the triglyceride glycerol released during perfusion was recovered in tissue lipids, and this was largely as partial glycerides and triglycerides.

In contrast to the inefficient utilization of glyceride glycerol by the intact heart, the released triglyceride fatty acid was efficiently extracted (92%) and utilized by perfused heart. Thus, at best, utilization of the triglyceride fatty acid was almost 29-fold that of the glycerol moiety. These data collectively suggest that either partial glycerides are not a major

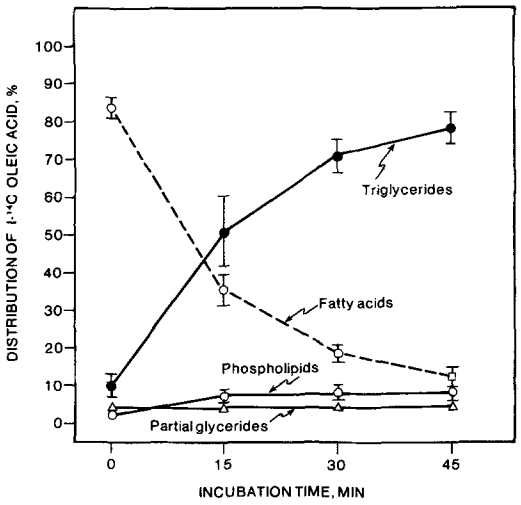

FIG. 1. Esterification and distribution of [1-^{14}C]-oleic acid during incubations with adult rat cardiac myocytes. Cells (5 X 10^5 cells/10 ml) were incubated with 4 μmol oleic acid complexed to bovine serum albumin for 45 min at 37 C in air. At the times indicated, aliquots were removed for reisolation of cells, lipid extraction and separation, and analysis of recovery and distribution of radioactivity.

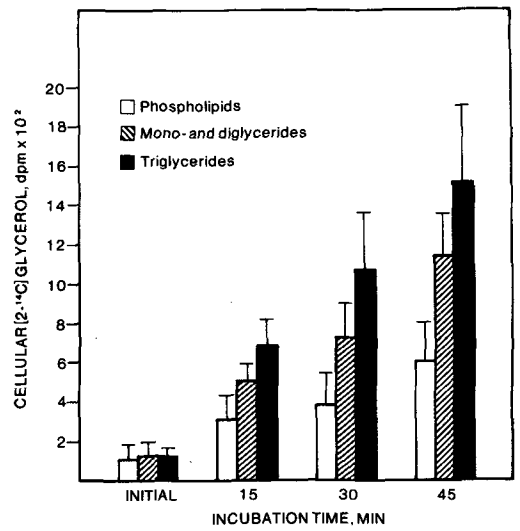

FIG. 2. Esterification and distribution of [2-^{14}C]-glycerol during incubations with rat cardiac myocytes. Cells (5 X 10^5 cells/10 ml) were incubated with 1.2 μmol glycerol for 45 min at 37 C in air. At the times indicated, aliquots were removed for reisolation of cells and lipid extraction, separation and distribution of radioactivity.

end product of LPL action in situ, or that, if they are produced and extracted by the heart, the glycerides are subsequently hydrolyzed rather than utilized by a monoacylglycerol

pathway.

Studies with the glyceryl monoether were predicated on their physicochemical similarity to monoglycerides and on their resistance to lipolysis in other model systems (10). Similar analogues have been effectively empolyed to elucidate specific aspects of the monoglyceride pathway in intestine (10,11). In the present studies, both the isolated cell model and the perfused heart were able to extract the glyceryl monoether from the respective media. With both tissue preparations, 94-98% of the tissue associated with radioactivity was recovered as unmetabolized ether, and at best, only 6% was incorporated into higher glyceride analogues. However, there is no direct evidence that the monoether was quantitatively internalized by cardiac cells or by the perfused heart.

Although the quantitative data from the tissue models empolyed in these studies should not be compared directly, the overall results suggest that cardiac tissue does not efficiently utilize either free glycerol or monoglycerides for subsequent formation of higher glycerides.

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1-*O*-Alkyl-Linked Phosphoglycerides of Human Platelets: Distribution of Arachidonate and Other Acyl Residues in the Ether-Linked and Diacyl Species

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ABSTRACT

In this study, the 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine content of human platelets was determined. The distribution of arachidonate among the 1,2-diacyl, 1-*O*-alkyl-2-acyl, and 1-*O*-alk-1′-enyl-2-acyl classes of choline- and ethanolamine-containing phosphoglycerides was also assessed. The major platelet phospholipids were choline-containing phosphoglycerides (38%), ethanolamine-containing phosphoglycerides (25%) and sphingomyelin (18%), with smaller amounts of phosphatidylserine (11%) and phosphatidylinositol (4%). In addition to the diacyl class, the choline-linked fraction was found to contain both 1-*O*-alkyl-2-acyl (10%) and 1-*O*-alk-1′-enyl-2-acyl (9%) species. The ethanolamine-linked fraction, on the other hand, had an elevated level of the 1-*O*-alk-1′-enyl-2-acyl (60%) species and a small amount of the 1-*O*-alkyl-2-acyl component (4%). The major fatty acyl residues found in all classes of the choline and ethanolamine phospholipids were 16:0, 18:0, 18:1(Δ^9), 18:2(n-6) and 20:4(n-6). The 1-*O*-alkyl and 1-*O*-alk-1′-enyl fraction of the ethanolamine-linked phospholipids also contained substantial amounts of 22:4(n-6), 22:5(n-3) and 22:6(n-3) acyl chains. Arachidonate comprised 44% of the acyl residues in the *sn*-2 position of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine. Corresponding values for the diacyl and 1-*O*-alk-1′-enyl-2-acyl species were 23% and 25%, respectively, based on all 20:4 (n-6) being linked to the *sn*-2 position of all classes. In the ethanolamine-linked phosphoglycerides, arachidonate constituted 60%, 20% and 68% of the acyl groups in the *sn*-2 position of the 1,2-diacyl, 1-*O*-alkyl-2-acyl and 1-*O*-alk-1′-enyl-2-acyl classes, respectively. The content of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine appears sufficient to support the synthesis of platelet activating factor by a deacylation-reacylation pathway in platelets. Our findings also demonstrate that human platelets contain a significant amount of 1-*O*-alkyl-2-arachidonyl-*sn*-glycero-3-phosphocholine that could possibly serve as a precursor of both platelet activating factor and bioactive arachidonate metabolites.

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INTRODUCTION

PAF, in addition to other biological activities, is a potent stimulator of the aggregation and degranulation of both platelets and neutrophils (1,2). It has now been identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (1-*O*-alkyl-2-acetyl-GPC) (3,4). Originally, PAF was found to be secreted from antigen-stimulated IgE-sensitized basophils and has since been shown to be produced by other cells including platelets (5-7).

Evidence for the synthesis of 1-*O*-alkyl-2-acetyl-GPC by a deacylation-reacylation pathway has been obtained in microsomal preparations of rat spleen and several other tissues (8-10). This mode of synthesis, which involves the transfer of acetate from acetyl-CoA to 1-*O*-alkyl-2-lyso-GPC by an acetyltransferase, is dependent on the presence of intracellular 1-*O*-alkyl-2-acyl-GPC containing a long-chain residue in the *sn*-2 position. On stimulation of the cell, a phospholipase A_2 reaction (11) would make available the necessary substrate for acetylation. There is now evidence that this pathway is operative in platelets (11,12), and in other cells including neutrophils (13,14) and macrophages (9,15).

There are also numerous accounts in the literature documenting that platelets can convert endogenous arachidonate into various cyclooxygenase products including prostaglandins and thromboxanes, and to the lipoxygenase product, 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). Arachidonate esterified to the *sn*-2 position of membrane phosphoglycerides, including phosphatidylcholine, has been shown to be the source for these metabolites (16,17).

Since platelets are involved in the synthesis of both PAF and arachidonate metabolites, this study was carried out with a 2-fold purpose in mind. First, we wanted to assess the levels of 1-*O*-alkyl-2-acyl-GPC in the cell, which would serve as a precursor for PAF in the deacylation-reacylation scheme. Secondly, we wanted to determine the degree of association of arachidonate with ether lipid species in the cell, since this information might yield a clue to the interrelationship of PAF and arachidonate metabolism.

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Abbreviations: PAF, platelet activating factor; GPC, *sn*-glycero-3-phosphocholine; TLC, thin layer chromatography; PRP, platelet-rich plasma; PC, choline-linked phosphoglycerides; PE, ethanolamine-linked phosphoglycerides; GPE, *sn*-glycero-3-phosphoethanolamine; GLC, gas liquid chromatography, HPLC, high performance liquid chromatography.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade or better. Phospholipase C (*Bacillus cereus*), primulin dye and Silica Gel H were purchased from Sigma (St. Louis, MO). Silica Gel G-coated TLC plates were obtained from Analtech, Inc. (Wilmington, DE). Vitride ($NaAlH_2[OCH_2CH_2OCH_3]_2$) was purchased from Alfa products (Beverly, MA). Phospholipid standards were purchased from Supelco (Bellefonte, PA), 1-*O*-alkylglycerol standards from Serdary Research Laboratories (London, Ontario, Canada) and fatty alcohol acetate standards from Nu-Chek Prep (Elysian, MN). Fatty acid methyl ester standards were obtained from Supelco and Nu-Chek Prep.

Preparation of Cells

Human platelets were prepared as described earlier (18). Briefly, 450 ml of venous blood was collected into 50 ml of 110 mM sodium citrate. PRP was prepared by centrifugation at 190 × g and 22 C for 20 min. The supernatant PRP was removed and centrifuged twice more at 190 × g and 22 C for 20 min to remove contaminating leukocytes. EDTA was then added to the supernatant to give a final concentration of 5 mM, and the PRP was put on ice for 20 min. The platelets were pelleted by centrifugation at 3000 × g and 4 C for 20 min; resuspended in 50 ml of 10 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 11 mM glucose and 1 mM EDTA; and put on ice for 20 min. The platelets were then pelleted again by centrifugation at 3000 × g and 4 C for 20 min, and resuspended in 10 ml of the Tris buffer solution. Examination by oil immersion microscopy demonstrated a pure platelet preparation.

TLC Solvent Systems

Several solvent systems were used in the purification of the cellular lipids and their derivatives: system I, chloroform/methanol/glacial acetic acid/H_2O (50:25:8:4, v/v); system II, hexane/ethyl ether (70:30, v/v); system III, ethyl ether/hexane/conc ammonium hydroxide (60:40:1, v/v); system IV, ethyl ether/hexane (60:40, v/v).

Extraction and Fractionation of Lipids

Cellular lipids were extracted by the method of Bligh and Dyer (19) immediately after cell isolation. The extracts from 11 units of blood were combined and stored at −20 C, and aliquots were taken from this pooled extract for analysis. The choline-linked (PC) and ethanolamine-linked (PE) phosphoglycerides were purified by TLC on Silica Gel H using solvent system I. After visualization with a primulin spray reagent (20), the purified PC and PE fractions were eluted from the gel by Bligh and Dyer extraction (19).

Quantitation of Phospholipid Species

The quantitation of the individual phospholipid species was carried out in 2 ways. First, an aliquot (200-300 μg) of the total lipid extract was analyzed by TLC on Silica Gel H using solvent system I. The various phospholipids were visualized and assayed for lipid phosphorus by the method of Rouser et al. (21). This TLC system did not resolve phosphatidylserine from phosphatidylinositol. In the second method, HPLC (22) was utilized to separate the individual species. An aliquot (200-400 μg) of the total lipid extract was injected onto a μPorasil column (3.9 mm × 30 cm, Waters) with a flow rate of 2.2 ml/min at 50 C. The eluant solvent system consisted of isopropanol/hexane (8:6, v/v) containing 1.5% H_2O. After 5 min, the H_2O was increased to 9% over a period of 20 min. The column was then eluted isocratically for an additional 10 min. The resolved lipid species were detected by absorbance at 206 nm. The peaks were collected and quantitated by assay for phosphorus (21).

Separation and Quantitation of 1,2-Diacyl-, 1-*O*-Alkyl-2-acyl- and 1-*O*-Alk-1′-enyl-2-acyl-GPC and -GPE

The various lipid classes were analyzed as described earlier (23). Briefly, the purified choline- and ethanolamine-linked fractions (1-3 mg) were treated with HCl gas for 5 min to hydrolyze the 1-*O*-alk-1′-enyl groups. The cleaved aldehyde, the lysophospholipid, and the unreacted phospholipid were purified by preparative TLC in solvent system I. The amount of lysophospholipid determined by lipid phosphorus analysis (21) was taken as the 1-*O*-alk-1′-enyl content.

The unhydrolyzed phospholipid was treated with phospholipase C and the products acetylated (24) to yield 1,2-diacyl-3-acetylglycerol and 1-*O*-alkyl-2-acyl-3-acetylglycerol. These two species were separated by preparative TLC on Silica Gel G using solvent system II. An aliquot of the phospholipase C-treated lipid (diglycerides) was analyzed by TLC on Silica Gel G using solvent system III. In this solvent system, 1,2-diacyl- and 1,3-diacylglycerides comigrate and are resolved from 1-*O*-alkyl-2-acyl- and 1-*O*-alkyl-3-acylglycerides, which also comigrate (25). Analysis of the diglycerides in system III and the acetylated derivatives in system II showed similar relative intensities of the 1,2-diacyl and 1-*O*-alkyl-2-acyl bands. This confirmed that the product identified as 1-*O*-alkyl-2-acyl-*sn*-glycerol was not 1,3-diacyl-*sn*-glycerol formed by acyl migration. Fatty acid methyl esters were

prepared (23) from the purified acetylglycerides and from the lysophospholipid formed by acid treatment of the plasmalogens; the methyl esters were analyzed by GLC using 15:0 methyl ester as an internal standard. Each mole of methyl ester was taken to represent 1 mole of 1-*O*-alkyl-2-acyl-, 1 mole of 1-*O*-alk-1'-enyl-2-acyl- or 0.5 mole of 1,2-diacyl-GPC or -GPE.

Determination of 1-*O*-Alkyl Chain Distribution

An aliquot (1-2 mg) of the unreacted phospholipid from acid treatment was reduced with Vitride as described earlier (26). The 1-*O*-alkylglycerol product was purified by preparative TLC on Silica Gel G using solvent system IV, and the isopropylidene derivative was prepared as described earlier (23). The 1-*O*-alkyl chain distribution, which was determined by GLC-mass spectroscopy (GLC-MS), was based on peak area percentages.

Determination of 1-*O*-Alk-1'-enyl Chain Distribution

Aldehydes formed by acid treatment of the plasmalogens were reduced with Vitride to the corresponding alcohols (26). The fatty alcohols were then acetylated in the same manner as the diglycerides (24) and analyzed by GLC-MS as described below. The 1-*O*-alk-1'-enyl chain distribution was based on peak area percentages.

GLC and GLC-MS Analyses

Analysis of fatty acid methyl esters was performed on a Varian 3700 gas chromatograph with a CDS-111 data processor. The methyl esters were separated on a 60-m column coated with OV-351. Column temperature ranged from 175 to 245 C with a programmed rate of 10 C/min for the first 5 min, 0 C/min for 20 min, and 2 C/min for another 10 min. The injector and detector temperatures were both 250 C, and the helium flow rate was 1 ml/min.

The isopropylidene derivatives of alkylglycerol and the fatty alcohol acetates were both analyzed on a Ribermag R10-10 quadrupole mass analyzer using a PDP-8A minicomputer for data acquisition and reduction. Software for this system is from R.D.S. Nermag. The isopropylidene derivatives were separated on a DB-1 WCOT fused-silica column with a helium velocity of 35 cm/sec. Column temperature ranged from 170 to 270 C at a programmed rate of 5 C/min. The injector, source, and interface temperatures were 250 C, 200 C and 280 C, respectively. Filament current was 0.3 mA and the electron voltage was 70 eV. The fatty alcohol acetates were separated on a SE-52 WCOT fused-silica column with a helium velocity of 40 cm/sec. Column temperature ranged from 125 to 225 C, beginning with a 5-min hold and followed by a programmed rate of 3 C/min. The injector, source, and interface temperatures were 200 C, 170 C and 225 C, respectively. Filament current for this analysis was also 0.3 mA and the electron voltage was 30 eV.

RESULTS AND DISCUSSION

The phospholipids of human platelets were analyzed by TLC and HPLC as described in Methods. As shown in Table 1, the major phospholipid species were PC (38%), PE (25%) and sphingomyelin (18%), with smaller amounts of phosphatidylserine (11%) and phosphatidylinositol (4%). These findings are in close agreement with previously published data on human platelet phospholipid distributions (27-29). As shown in Table 2, the PC fraction of human platelets contained 82% 1,2-diacyl-, 10% 1-*O*-alkyl-2-acyl- and 9% 1-*O*-alk-1'-enyl-2-acyl-GPC. Although this 1-*O*-alkyl content is not as high as in other blood cell types (23,30,31), it can be calculated based on the total phospholipid/10^8 cells (38 nmol), the percentage of PC (38%), the 1-*O*-alkyl-2-acyl-GPC content (10%) and the amount of PAF synthesized by 10^8 ionophore-stimulated platelets (0.5 pmol) (7), that an ca. 2800-fold excess of PAF precursor exists in the cell. In contrast to the ether lipid content of the PC fraction, the PE fraction contained a larger portion of 1-*O*-alk-1'-enyl-2-acyl-GPE (60%), a small amount of the 1-*O*-alkyl-2-acyl species (4%) and intermediate levels of 1,2-diacyl-GPE (36%). Earlier work by several authors has been reported on the PE plasmalogen content of platelets, with values ranging from 30 to 60% of ethanolamine-linked phospholipids (32,33).

The fatty chain distribution of platelet PC and PE is shown in Tables 3 and 4, respectively. The major fatty acyl residues in both the PC and PE pools were 16:0, 18:0, 18:1, 18:2 and 20:4, which agrees with previously published data on platelet fatty acid content (27,29,34). Arachidonate comprised 25% and 64% of all fatty acids associated with PC and PE, respectively. Of all PC-linked 20:4, 17% was found in the 1-*O*-alkyl-2-acyl-GPC pool. When compared to the 1-*O*-alkyl content (10%) of the choline-linked phospholipids, a 1.7-fold enrichment of 20:4 in the 1-*O*-alkyl fraction can be computed. A high content of 1-*O*-alkyl-2-arachidonyl-GPC has also been seen in human neutrophils (Mueller et al., submitted for publication) and rabbit alveolar macrophages (31). In contrast, 65% of all PE-associated 20:4 was found in the plasmalogen fraction indicating a slight enrichment, since plasmalogens comprised 60% of the PE fraction. Another interesting characteristic of platelet fatty acyl composition is the high

TABLE 1

Phospholipid Composition of Human Platelets

	mol % phosphorus (N=4)[a]
Sphingomyelin	17.7 ± 2.3
Choline-containing phosphoglycerides	38.0 ± 1.6
Phosphatidylinositol	4.4 ± 0.5
Phosphatidylserine	10.8 ± 1.3
Ethanolamine-containing phosphoglycerides	25.3 ± 1.8

[a]The data are presented as the mean ± standard deviation of 4 separate determinations done on the combined total lipid extract from the platelets in 11 units of blood. There were ca. 38 nmol of phosphorus and 176 μg of total lipid per 1×10^8 platelets.

TABLE 2

Ether Class Composition of Choline- and Ethanolamine-Containing Phosphoglycerides

	Choline-containing phosphoglycerides (mol %) (N = 3)[a]	Ethanolamine-containing phosphoglycerides (mol %) (N = 3)[a]
1,2-Diacyl	81.8 ± 2.6	36.1 ± 0.3
1-*O*-Alkyl-2-acyl	9.7 ± 0.3	3.5 ± 0.1
1-*O*-Alk-1′-enyl-2-acyl	8.8 ± 2.4	

[a]The data are presented as the mean ± standard deviation of 3 separate determinations done on the purified platelet PC and PE fractions.

content of 22:4, 22:5 and 22:6. These 3 fatty acids collectively comprised 18% and 24% of the fatty acids esterified in 1-*O*-alkyl-2-acyl-GPE and 1-*O*-alk-1′-enyl-2-acyl-GPE, respectively. The relatively high amounts of 20:4 and 22-carbon unsaturated fatty acids in the ethanolamine-containing phospholipids have been seen by others (27,29,34).

The 1-*O*-alkyl chain distribution of platelet PC was determined by GLC-mass spectroscopy as described in Methods. The predominant alkyl chains in the *sn*-1 position of 1-*O*-alkyl-2-acyl-GPC were 16:0 (44%) and 18:0 (37%), with smaller amounts of 18:1 (16%) and 20:0 (4%). Human neutrophils (Mueller et al., submitted for publication), guinea-pig neutrophils and macrophages (30), and rabbit alveolar macrophages (31) have a similar allocation of alkyl chains, although no 20:0 was seen in the nonhuman cells. Rabbit peritoneal neutrophils, however, contain substantial portions of 20:0 (16%) and 22:0 (9%) in the *sn*-1 position of 1-*O*-alkyl-2-acyl-GPC (23). Due to the low content of 1-*O*-alkyl-2-acyl-GPE, the alkyl chain distribution was not determined in this fraction.

The 1-*O*-alk-1′-enyl chain distribution of PC and PE was also determined by GLC-MS as described in Methods. The major vinyl-ether chains in the *sn*-1 position of PC and PE plasmalogen were 16:0, 18:0, 18:1 and 18:2. These results are comparable to those reported earlier, although no 18:2 was seen (29,34).

The present study demonstrates that human platelets contain sufficient 1-*O*-alkyl-2-acyl-GPC to support the synthesis of PAF by a deacylation-

TABLE 3

Fatty Chain Distribution of Choline-Containing Phosphoglycerides

	1,2-Diacyl (mol %)	1-*O*-Alkyl-2-acyl (mol %)		1-*O*-Alk-1′-enyl-2-acyl (mol %)	
	Position 1+2 (N = 3)[a]	Position 1 (N = 1)[a,b]	Position 2 (N = 3)[a]	Position 1 (N = 1)[a,b]	Position 2 (N = 3)[a]
16:0	32.4 ± 0.5	43.6	20.0 ± 2.3	34.0	20.7 ± 2.2
16:1 (Δ^9)	1.4 ± 0.2	-	1.3 ± 0.1	-	1.1 ± 0.1
18:0	14.2 ± 0.3	36.6	4.7 ± 2.5	52.0	10.6 ± 1.2
18:1 (Δ^9)	25.1 ± 0.3	15.6	12.8 ± 2.9	9.2	23.0 ± 1.8
18:2 (n-6)	9.2 ± 0.2	-	6.3 + 1.1	4.8	9.4 + 0.1
20:0	0.7 ± 0.1	4.1	0.8 ± 1.0	-	0.8 ± 0.1
20:1 (Δ^{11})	0.8 ± 0.0	-	0.7 ± 0.8	-	1.4 ± 0.5
20:3 (n-6)	1.5 ± 0.1	-	2.4 ± 0.2	-	2.3 ± 0.1
20:4 (n-6)	11.6 ± 0.3	-	43.7 ± 4.8	-	25.1 ± 1.9
22:4 (n-6)	0.8 ± 0.1	-	1.5 + 0.2	-	1.1 ± 0.1
22:5[c]	0.6 ± 0.1	-	2.4 ± 1.4	-	1.2 ± 0.7
22:6 (n-3)	0.5 ± 0.0	-	1.3 ± 0.3	-	0.9 ± 0.2
Other[d]	1.2	-	2.0	-	2.5

[a]The data are presented as the mean ± standard deviation of N separate determinations done on the purified PC fraction.

[b]Data are given as area % rather than mol %.

[c]Both n-3 and n-6 families were seen.

[d]Other fatty acids which were detected but constituted less than 1% each of the total were 14:0, 14:1, 17:0, 18:3, 20:2, 20:5, 22:0, 22:1, 24:0 and 24:1.

TABLE 4

Fatty Chain Distribution of Ethanolamine-Containing Phosphoglycerides

	1,2-Diacyl (mol %)	1-*O*-Alkyl-2-acyl (mol %)		1-*O*-Alk-1'-enyl-2-acyl (mol %)	
	Position 1 + 2 (N = 3)[a]	Position 1	Position 2 (N = 3)[a]	Position 1 (N = 1)[a,b]	Position 2 (N = 3)
14:0	0.1 ± 0.1	N.D.	1.7 ± 0.5	-	0.2 ± 0.2
16:0	7.6 ± 1.3	-	14.5 ± 5.2	27.6	1.0 ± 0.5
16:1 (Δ^{9})	0.6 ± 0.4	-	2.0 ± 0.1	-	0.5 ± 0.1
18:0	35.4 ± 5.5	-	14.9 ± 2.2	40.5	0.9 ± 0.4
18:1 (Δ^{9})	15.4 ± 0.4	-	11.1 ± 1.6	30.2	2.6 ± 0.7
18:2 (n-6)	3.9 ± 0.5	-	2.7 ± 0.5	1.7	1.5 ± 0.3
20:0	0.7 ± 0.3	-	3.4 ± 3.5	-	-
20:1 (Δ^{11})	0.5 ± 0.2	-	5.7 ± 3.3	-	0.1 ± 0.0
20:3 (n-6)	0.8 ± 0.1	-	3.6 ± 1.4	-	0.6 ± 0.2
20:4 (n-6)	30.0 ± 6.3	-	20.4 ± 3.3	-	68.3 ± 4.4
22:4 (n-6)	1.9 ± 0.3	-	8.9 ± 1.2	-	12.2 ± 1.0
22:5[c]	1.2 ± 0.4	-	5.6 ± 0.7	-	7.6 ± 1.2
22:6 (n-3)	1.2 ± 0.3	-	2.5 ± 1.3	-	3.5 ± 0.5
Other[d]	0.8	-	3.2	-	1.0

[a]The data are presented as the mean ± standard deviation of N determinations done on the purified PE fraction.

[b]Data are given as area % rather than mol %.

[c]Both n-3 and n-6 families were seen.

[d]Other fatty acids which were detected but constituted less than 1% each were 14:1, 17:0, 18:3, 20:2, 20:5, 22:0, 22:1 and 24:0.

reacylation mechanism, and that there is a substantial amount of arachidonate associated with this fraction. During review of this manuscript, Natarajan et al. (35) reported similar but somewhat lower levels (4.5% based on $LiAlH_4$ reduction) of alkyl-linked species in the PC fraction of human platelet lipids; acyl compositions were not reported. The presence of 1-*O*-alkyl-2-arachidonyl-GPC is of interest since the action of phospholipase A_2 would yield precursors for both PAF and products of the arachidonate cascade. Whether or not their formation is tightly coupled metabolically remains to be determined, but the presence of this molecular species makes the idea an attractive possibility.

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Effect of Specific Dietary Fatty Acids on Lipogenesis in the Livers and Mammary Glands of Lactating Mice

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ABSTRACT

The effects of linoleic, linolenic and columbinic acids fed as 4% of a high carbohydrate (50% glucose) diet on the activities and the amounts of several enzymes associated with fatty acid synthesis in livers and mammary glands of lactating mice were compared with those for stearic and oleic acids. Fatty acid synthesis, measured in vivo, was significantly lower in livers of mice ingesting all 3 polyunsaturated fatty acids (PUFA), whereas in mammary glands synthesis was lower only in mice receiving columbinic acid. The activities of fatty acid synthetase (FAS) and acetyl CoA carboxylase were significantly reduced in liver by all 3 PUFA, as were activities of glucose-6-phosphate dehydrogenase, malic enzyme (ME) and citrate cleavage enzyme (CCE), also associated with lipogenesis. In mammary gland, on the other hand, the activities of these enzymes were unaffected by dietary PUFA. The tissue contents of FAS, ME and CCE, measured by rocket immunoelectrophoresis, were found to be significantly reduced in liver by linoleate, linolenate and columbinate but were not significantly altered in mammary gland. The decrease in hepatic lipogenesis observed was principally due to a decrease in the amounts of these enzymes induced by the dietary PUFA but the inhibition in mammary gland caused by columbinate could not be accounted for by a reduction in enzyme contents and therefore may be due to allosteric effects which occur when fatty acid synthesis is measured with ^3H_2O. The fatty acid composition in liver and mammary gland of dams and in liver and kidney of pups completely reflected dietary fatty acids. Columbinate made up ca. 20% of the total fatty acids in both tissues of the columbinic acid-fed mice and ca. 15% in the pup tissues. This suggests that columbinate is incorporated into milk lipids of dams and is easily absorbed by pups. The elevated ratios of 16/16:1 and 18/18:1 in liver and mammary gland of dams and liver and kidney of the pups from dams fed linoleate, linolenate and columbinate suggest that each of these polyunsaturated fatty acids in the diet can inhibit the activity of Δ9 desaturase.

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Hepatic lipogenesis has long been recognized to be under dietary control. Indeed, such metabolic regulation occurs in a great variety of mammals (1-6), fish (7) and birds (8,9). Thus, fat-free diets rich in monosaccharides promote the de novo synthesis of fatty acids (10,11), whereas those that contain fats, particularly those rich in polyunsaturated fatty acids, inhibit this process (12,13). Although there is general agreement that this phenomenon plays a major role in liver, the regulatory aspects of dietary fats on lipogenesis in other tissues is less certain. Some investigators (14) have reported that lipogenesis in adipose tissue also responds to dietary manipulation of the host animal, but such suggestions have not been as widely confirmed nor accepted as those in liver (15). The situation with neoplasms is somewhat different. There is overall agreement that hepatomas of various origins do not respond (16-18). Indeed, liver neoplasms that have either high or low lipogenic capacities failed to show an effect of dietary fat on fatty acid synthesis (18). The unique nature of adult liver in this aspect of metabolic control is further emphasized by findings, previously reported from this laboratory, that lipogenesis is not influenced in either fetal (19) or neonatal livers (20) by the dietary fat intake of pregnant or newly lactating mice.

In 1969, we showed that diets which contain 15% corn oil when fed to either virgin or lactating mice for as few as 3 days elicited substantial decreases in (a) the ability of the liver to produce fatty acids from either acetate or pyruvate, and (b) the activities of many enzymes concerned with de novo fatty acid synthesis (21). The extreme sensitivity of liver to respond was emphasized in that study, which demonstrated that such dietary treatment failed to produce changes in the metabolic parameters examined in the mammary glands of either virgin or lactating mice. Thus, we suggested at that time that fatty acid synthesis in neither mammary gland adipose cells nor mammary gland parenchymal cells could be influenced by the dietary fat fed to mice in the same way as in hepatocytes. Subsequently, however, Romsos et al. (22), using the in vivo conversion of tritium from ^3H_2O into fatty acids (23,24) as a measure of fatty acid synthesis, reported that high-fat diets fed to lactating mice for 5 days could depress lipogenesis in liver, mammary glands and adipose tissue. These workers reported that, whereas dietary fat decreased lipogenesis in liver 4-fold, the effect on mammary glands was much less and amounted to only 2-fold. The fat content of the diet employed was more than 3 times greater than that used in the studies reported (21) from this laboratory (48% vs 15%).

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In another study, this time with lactating rats, Grigor and Warren (25) fed the animals diets containing 20% fat for 7 days before measuring lipogenesis in both liver and mammary tissue by the same in vivo ^3H_2O technique. These workers found, in mammary gland, that the lipogenic rate for rats fed a diet containing peanut oil was only one-fifth that for rats fed a fat-free diet. Lipogenesis in the liver of the lactating rat was also depressed, but synthesis appeared to be less sensitive to fat in the diet than was synthesis in the mammary gland, as only a 2-fold inhibition was shown in the liver tissue. Thus, in the study with lactating rats which employed 20% fat-containing diets, the mammary glands appeared to be more sensitive to this dietary constituent than did the livers of the same animals, a finding which is the reverse of the situation reported by Romsos et al. (22) with mice. To explore further the question of tissue sensitivity to dietary fat and to shed light on the question of which types of fatty acids are most effective, we reinvestigated this problem. In experiments reported here, we used pure fatty acids with different degrees of unsaturation and investigated their effects on lipogenesis as well as on the activities and the contents of specific enzymes concerned with fatty acid synthesis, in mammary glands and livers both taken from lactating mice.

MATERIALS AND METHODS

Animals

Only BALB/c mice were used in this study. They were originally obtained from Simonsen Laboratories, Gilroy, CA, and are now bred and maintained in our vivarium. They were routinely fed a nutritionally complete stock diet (Berkeley Diet Mouse Breeder Food) purchased from Feedstuffs Processing Co. (San Francisco, CA), which contains 9% fat. The diet and water were allowed to the animals ad libitum in a temperature-controlled room (20-23 C) with a 12-hr light and a 12-hr dark cycle, the light period starting at 07:00 hr.

Female mice that were actively lactating and suckling at least 5 pups for 10 days were then fed one of the specially prepared diets for 7 additional days. The number of pups was controlled, since it has been known for some time that suckling and milk removal plays a significant role in the metabolism and lipogenic capacity of the gland during lactation (26-29). We did not want this variable to complicate interpretation of the data. The dams continued to suckle their pups for the entire period before being killed. Each dam was studied during its 17th day postpartum.

Diets

All test diets were based on a fat-free dry mixture which contained, by weight, 50% glucose; 24% vitamin-free casein; 6% salt mixture, U.S. Pharmacopia XIV; 0.01% zinc carbonate; 2.2% vitamin mixture (ICN Nutritional Biochemicals Corp., Cleveland, OH; vitamin diet fortification mix); 0.3% methionine; 13.5% cellulose; and 0.01% butylated hydroxytoluene. The fat-containing diets were made immediately before use by adding each pure fatty acid to the dry mixture at a level of 4% by weight. The fat-free diet contained 4% additional cellulose. Pure oleic, linoleic and linolenic acids (99+%) were purchased from NuChek-Prep, Inc., Elysian, MN, and pure stearic acid (96.7% stearic, 1.9% palmitic and 1.4% shorter chain fatty acids) was obtained from Eastman Organic Chemicals, Rochester, NY. Pure columbinic acid (91.1% columbinic, 7.6% linoleic, and 1.3% shorter chain fatty acids) were a gift from Dr. U.M.T. Houtsmuller, Unilever Research, Vlaardingen, Netherlands.

At the end of each experiment, the diets were analyzed for their constituent fatty acids as well as for evidence of possible peroxidation. Each dietary fat was isolated and yielded the same chromatographic pattern in gas chromatographic analysis as it did before incorporation into the diets. Malondialdehyde determinations (30) performed with each diet showed that those containing polyunsaturated fatty acids did not have any more of this peroxidation product than did those diets which contained either saturated or monounsaturated fatty acids.

in vivo Lipogenesis

Usually starting at 09:00 hr, 1 mCi of ^3H_2O in 0.1 ml of 0.15 M sodium chloride was injected intraperitoneally and the dams killed exactly 15 min later. During this period, the pups were allowed free access to the dams so that suckling continued until the dams were killed. Immediately after the mice were killed by cervical dislocation, the required tissues were removed from the mice, washed in ice-cold saline, blotted dry, and distributed according to the requirements of the experiment. For lipogenesis studies, precisely weighed pieces of tissue (usually 500 mg) were immediately saponified in 1 ml of 15% KOH in 50% ethanol at 85 C for 18 hr under reflux. After removal of the nonsaponifiable material by extraction with petroleum ether (31), the mixtures were acidified and the fatty acids extracted with hexane (21).
(21).

The hexane extract was washed twice with water and finally dried over anhydrous sodium sulfate. Aliquots of the fatty acid containing hexane solution were then taken for measurement of tritium activity in a scintillation spectrometer as given previously (21). Lipogenesis is reported as μmol tritrated water incorporated/g tissue/15 min.

Analysis of Tissue Fatty Acids

In most experiments, fatty acids were isolated

from the tissues, converted to methyl esters, and quantitatively identified by gas liquid chromatography from correspondence of their retention times with those of known standards according to methods described previously (27). In those experiments in which tritiated fatty acids formed from in vivo lipogenesis were studied, they were separated by urea-silicic acid column chromatography according to the method described by Kumar et al. (32). To be certain that complete separation of the desired fatty acids was achieved, we used [1-^{14}C]dodecanoic acid, [^{3}H]decanoic acid and octanoic acid as markers for each column run before separating the mixtures of fatty acids synthesized in vivo by the mammary gland.

Preparation of Tissue Cytosols and Measurement of Enzyme Activities

The required tissues were homogenized in exactly 3 vol of 0.25 M sucrose at 2 C and centrifugally separated into cytosol and microsomes after removing the cell debris and mitochondria (27). The individual cytosol fraction from each tissue was used for assay of enzyme activity as well as for the determination of enzyme amount as given below. The assay procedures for fatty acid synthetase (33), acetyl CoA carboxylase (21), glucose-6-phosphate dehydrogenase (34), 6-phosphogluconate dehydrogenase (35), malic enzyme (36) and citrate cleavage enzyme (37) have been reported. Units of enzyme activity are given as nmol substrate converted to product per min at 30 C under the conditions of the specific assay used. Protein was determined by the method of Lowry et al. (38). Each assay was performed at 2 different protein concentrations and the results of the 2 closely agreeing values (5%) were averaged.

Preparation of Antisera Specific for (a) Fatty Acid Synthetase, (b) Malic Enzyme, and (c) Citrate Cleavage Enzyme

Each enzyme was purified to homogeneity as evidenced by a single protein band on SDS-polyacrylamide gel electrophoresis. For this purpose, we used the cytosols from the livers of mice fed a 50% glucose fat-free diet (21) for a minimum of 7 days. The electrophoretically pure fatty acid synthetase had a specific activity of 1200 units/mg protein (39); pure malic enzyme exhibited a specific activity of 40,000 units/mg protein (40), and pure citrate cleavage enzyme possessed a specific activity of 6,000 units/mg protein (41). Specific antisera to each enzyme were prepared in rabbits and purified as previously described (39-41). They were stored at −70 C until used.

Rocket Immunoelectrophoresis

The method employed was essentially that described by Weeke (42) in which we used a Pharmacia flat bed, temperature-controlled electrophoresis apparatus (Pharmacia Fine Chemicals, Piscataway, NJ). One percent agarose gels (22 × 11 × 0.1 cm) containing either 0.78% antifatty acid synthetase antibody, 1.3% antimalic enzyme antibody or 2.3% anticitrate cleavage enzyme antibody were formed in barbital/glycine/Tris-HCl buffer, ionic strength I = 0.02μ (41). A similar buffer, but with I = 0.04μ, was used as the electrode solution. Cytosols from the livers or the mammary glands were diluted 3:1 (v/v) with barbital/glycine/Tris-HCl buffer, I = 0.08μ, and 8-μl samples were applied to wells 3.5 mm in diameter.

To confirm the proportionality of rocket height to amount of enzyme protein, each sample was run at several dilutions. In each experiment with each enzyme studied, all cytosol samples were run on the same gel utilizing several concentrations of pure enzyme. Electrophoresis was allowed to proceed by applying a voltage of 4 V/cm overnight at 10 C, after which the plates were washed in running tap water, pressed dry and finally stained for protein with Coomassie brilliant blue R-250 (42). Rocket heights were measured from the center of each well to the top of each peak. Using this technique, we could show a linear relationship between rocket height and enzyme content for concentrations of fatty acid synthetase between 0.4 and 2.0 mg/ml, of malic enzyme between 50 and 500 μg/ml, and of citrate cleavage enzyme between 0.9 and 2.3 mg/ml.

RESULTS AND DISCUSSION

The history of the mice used in these experiments is given in Table 1. Although there were no statistically significant differences in the amounts of diet eaten or in either the body or liver weights of the lactating mice in most of the various diet groups, those which consumed the 4% linolenic acid-containing diet appeared to be somewhat lower in both body and liver weight and to have eaten less of the diet than did the mice fed the other fat-containing diets. These small weight differences observed in the dams were not detected in the suckling pups.

When in vivo fatty acid synthesis was studied in the livers of these lactating mice, it was clear that in those mice ingesting the diets containing the polyunsaturated fatty acids, i.e., linoleic, linolenic and columbinic, lipogenesis was significantly depressed when compared to those eating the stearic or oleic acid diets (Table 2). Indeed, a close correlation was observed between the levels of lipogenesis and the activities of fatty acid synthetase and acetyl CoA carboxylase. Thus, in all cases of decreased hepatic lipogenesis, the activities of these 2 enzymes were also depressed below the levels observed in the livers of those lactating mice fed the stearic and oleic acid-containing diets. Furthermore, when the activities of glucose-6-phosphate dehydrogenase,

TABLE 1

Body and Organ Weights of Lactating BALB/c Mice and Pups Fed Diets Containing Various 18-Carbon Fatty Acids[a]

	Dams				Pups		
Dietary fatty acid[b]	No.[c]	Food eaten (g/day)	Body weight (g)	Liver % body weight	No. per dam	Body weight (g)	Liver % body weight
18:0	4	14.6±5.7	31.3±2.6	7.80±0.41	5.8±3.0	7.9±0.9	3.50±0.56
9-18:1	4	13.5±3.2	30.4±2.7	8.06±0.46	5.1±0.4	8.6±1.8	4.62±0.21
9,12-18:2	12	14.5±4.7	31.5±3.5	7.61±0.77	5.7±0.3	8.6±2.0	4.21±0.76
9,12,15-18:3	5	11.5±3.6	26.9±3.0	6.44±0.61	5.0±0	7.8±0.5	4.85±0.35
5*t*,9,12-18:3	4	14.9±3.3	27.5±4.4	7.30±0.50	5.2±1.6	7.9±1.1	3.61±0.50

[a]Results are presented as means±SD.
[b]Fed as 4% of the diet.
[c]Number of lactating dams used per group.
[d]$p<0.01$, compared to 9-18:1 diet.

TABLE 2

Effect of Dietary Fatty Acids on in vivo Lipogenesis and Enzyme Activities in Livers of Lactating BALB/c Mice[a]

			Enzyme activity (nmol/min/mg)[d]					
Dietary fatty acid[b]	No. of mice	Fatty acid synthesis[c]	FAS	ACC	G6PDH	6PGDH	ME	CCE
18:0	4	43.0±10.4	90.8±10.4	17.5±2.6	182.8±36.6	14.4±1.9	362.7±61.6	71.7±14.1
9-18:1	7	43.3±10.8	89.0±16.5	20.9±6.1	198.1±36.7	11.2±2.7	302.8±52.4	73.1±14.0
9,12-18:2	12	20.4±11.7[e]	67.1±10.0[e]	15.3±2.6[f]	109.7±31.9[e]	8.9±1.0	214.7±31.2[e]	45.3± 4.7[e]
9,12,15-18:3	8	19.3± 8.7[e]	29.8± 4.8[e]	7.1±2.7[e]	63.3±28.5[e]	8.2±1.1[f]	158.0±31.3[e]	17.8± 3.2[e]
5*t*,9,12-18:3	8	9.8± 7.2[e]	42.4±21.5[e]	8.8±3.9[e]	31.8±10.9[e]	9.6±2.5[b]	126.4±15.2[e]	18.9± 8.2[e]

[a]Results are presented as means±SD.
[b]Fed as 4% of the diet.
[c]Given as μmol water incorporated/g tissue/15 min.
[d]Given per mg cytosol protein; abbreviations used are FAS for fatty acid synthetase, ACC for acetyl CoA carboxylase, G6PDH for glucose-6-phosphate dehydrogenase, 6PGDH for 6-phosphogluconate dehydrogenase, ME for malic enzyme, and CCE for citrate cleavage enzyme.
[e]$p<0.01$, compared to 9-18:1 diet.
[f]$p<0.02$, compared to 9-18:1 diet.

malic enzyme and citrate cleavage enzyme in the livers of the lactating mice fed linoleic, linolenic and columbinic acid-containing diets were compared to those same enzyme activities in the livers of the dams fed the stearic and oleic acid diets, a considerable depression was also evident (Table 2). On the other hand, in the mammary gland, the only significant decrease from the levels observed with either the stearic or the oleic acid diet-fed dams was seen with in vivo lipogenesis in the columbinic acid diet-fed lactating mice (Table 3). Hence, although mammary gland lipogenesis in the columbinic acid-fed group was lower than that in any of the other groups, the activities of the mammary gland lipogenic enzymes, i.e., fatty acid synthetase and acetyl CoA carboxylase, as well as those of other enzymes usually associated with lipogenesis, i.e., glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme and citrate cleavage enzyme, were not significantly affected by this polyunsaturated fatty acid in the diet when compared to either the saturated- or monounsaturated-18-carbon fatty acids.

It should be noted that even though the level of tritium labeled fatty acids found in the mammary gland (Table 3) was greater than that found in the liver (Table 2) and the time after ^3H_2O injection was short, so as to minimize redistribution of labeled fatty acids possibly synthesized in other tissues, we could not be certain that all of the fatty acids isolated from the gland were indeed synthesized by the gland. However, during lactation, the gland produces an unique pattern of fatty acids which is characterized by the presence of medium chain length fatty acids. Indeed, it is probably the only tissue in the body which produces these fatty acids in substantial quantities. Hence, our observations that the patterns of fatty acids synthesized from ^3H_2O in all dietary groups were not different (Table 4) adds to our confidence in the data and is consistent with the view that synthesis of fatty acids by the gland was unaffected by the dietary manipu-

TABLE 3

Effect of Dietary Fatty Acids on in vivo Lipogenesis and Enzyme Activities in Mammary Glands of Lactating BALB/c Mice[a]

Dietary fatty acid[b]	No. of mice	Fatty acid synthesis[c]	Enzyme activity (nmol/min/mg)[d] FAS	ACC	G6PDH	6PGDH	ME	CCE
18:0	4	58.3±13.3	214±41	50.5± 5.2	605± 79	64.6±5.4	881± 85	242±67
9-18:1	7	67.0±20.0	175±27	40.5± 7.1	478±161	56.9±5.1	635± 99	196±56
9,12-18:2	12	62.6±20.5	190±38	53.8±12.0[e]	587±112	63.5±5.7[f]	683±130	169±40
9,12,15-18:3	8	61.9±18.8	169±37	34.1± 5.8	519± 68	55.4±5.7	604± 66	164±44
5*t*,9,12-18:3	5	27.8±12.2[e]	150±28	43.5± 3.2	553± 80	60.9±7.8	643± 42	148±16

[a]Results are presented as means±SD.
[b]Fed as 4% of the diet.
[c]Given as μmol water incorporated/g tissue/15 min.
[d]Given per mg cytosol protein; abbreviations used are FAS for fatty acid synthetase, ACC for acetyl CoA carboxylase, G6PDH for glucose-6-phosphate dehydrogenase, 6PGDH for 6-phosphogluconate dehydrogenase, ME for malic enzyme, and CCE for citrate cleavage enzyme.
[e]$p<0.01$, compared to 9-18:1 diet.
[f]$p<0.05$, compared to 9-18:1 diet.

TABLE 4

Effect of Dietary Fatty Acids on the Distribution of [^{3}H]Fatty Acids Synthesized by Lactating BALB/c Mouse Mammary Glands in vivo from [^{3}H]Water

Tissue fatty acid	Dietary fatty acid[a,b] 18:0 (4)[c]	9-18:1 (3)	9,12-18:2 (5)	9,12,15-18:3 (3)	5*t*,9,12-18:3 (4)
8:0	2.5±1.4	2.0± 0.7	1.3± 0.2	1.3±0.4	2.7±0.7
10:0	10.2±4.0	12.6± 5.4	11.0± 3.7	10.8±1.4	14.6±2.7
12:0	19.3±4.5	24.0± 8.3	17.6± 2.7	23.1±2.1	18.7±0.9
14:0 and longer	62.1±8.8	60.5±14.3	60.4±11.7	63.5±4.5	61.5±2.7
Between peaks	5.9±2.6	0.9± 0.7	4.2± 3.1	1.3±0.6	2.5±1.2

[a]Fed as 4% of the diet.
[b]Results are presented as the means of the total recovery of [^{3}H]fatty acids synthesized±SD.
[c]Number of mice used per group given in parentheses.

lations. Thus, both metabolic (lipogenesis) and enzymic data indicate that all of the dietary polyunsaturated fatty acids studied here depressed lipogenesis in the liver and the mechanism appears to involve a decrease in the activity of specific lipogenic enzymes. In the mammary gland, the situation is quite different. In the gland, inhibition of lipogenesis was not caused by linoleate or linolenate but only by columbinate. Furthermore, in this tissue, columbinate did not significantly decrease the activity levels of fatty acid synthetase or acetyl CoA carboxylase.

To explore further the reasons for the influence of these dietary fatty acids on hepatic and mammary gland lipogenesis, we investigated their effects on the amounts of specific enzymes present in these tissues taken from lactating mice. The enzymes chosen for such studies were fatty acid synthetase, malic enzyme and citrate cleavage enzyme.

It is clear from the data presented in Table 5 that, although the amounts of each of these enzymes in the livers of the animals fed the polyunsaturated fatty acid containing diets (linoleic, linolenic and columbinic acids) were lower than in those fed the saturated or monounsaturated fatty acids, the quantities of these enzymes in the mammary glands did not change. We have, therefore, concluded that the decrease in lipogenesis observed in the liver (Table 2) was probably due to a lowering in the amount of these enzymes induced by the polyunsaturated fats in the diet. On the other hand, the absence of change in either the level of activity or the amount of enzyme in the mammary gland does not explain why columbinic acid but not linoleic or linolenic acids depressed in vivo fatty acid synthesis (Table 3). It is, of course, possible that columbinic acid either (a) acts as an allosteric inhibitor of an enzyme concerned with lipogenesis as measured by the ^3H_2O technique while not affecting the amount of the enzyme or its activity when measured under the optimum conditions of our in vitro assays

TABLE 5

Effect of Dietary Fatty Acids on Enzyme Contents of Mammary Glands and Livers of Lactating BALB/c Mice[a]

Dietary fatty acid[b]	Tissue					
	Liver			Mammary gland		
	FAS[c]	ME	CCE	FAS	ME	CCE
18:0	26.5±3.2	33.8±4.1	19.0±1.5	75.6± 3.9	62.0±20.9	74.7± 4.1
9-18:1	21.9±2.2	26.7±4.5	16.4±3.6	87.2± 6.2	62.0± 4.4	66.5±15.4
9,12-18:2	17.2±1.4[d]	10.8±4.1[d]	11.6±2.3	80.9±14.3	67.8± 4.9	75.9±15.7
9,12,15-18:3	13.2±1.8[d]	10.0±5.3[d]	4.1±2.1[d]	75.4± 7.6	54.0± 9.5	66.0± 5.4
5*t*,9,12-18:3	15.9±2.5[d]	7.3±3.1[d]	6.2±2.3[d]	83.8± 3.5	58.3± 7.4	71.9± 5.7

[a]Results presented as means±SD; μg enzyme protein/mg cytosol protein.
[b]Fed as 4% of diet.
[c]See Tables 2 and 3 for abbreviations.
[d]$p<0.05$, compared to 9-18:1 diet.

TABLE 6

Effect of Dietary Fatty Acids on Fatty Acid Composition of the Livers of Lactating BALB/c Mice[a]

Fatty acid	Dietary fatty acid[b]				
	18:0 (4)[c]	9-18:1 (5)	9,12-18:2 (8)	9,12,15-18:3 (5)	5*t*,9,12-18:3 (4)
14:0	0.49±0.08	0.75±0.09	0.46±0.18	0.31±0.03	0.53±0.43
16:0	21.03±1.21	21.41±1.46	22.55±2.14	23.44±1.00	22.17±1.98
9-16:1	4.73±0.33	6.30±1.11	2.49±0.65	2.44±0.65	1.69±0.45
18:0	10.18±1.30	4.76±0.21	12.78±2.45	15.15±0.46	16.77±2.78
9-18:1	41.80±1.59	55.60±5.75	24.40±5.05	17.01±1.41	13.85±3.81
9,12-18:2	2.87±0.66	1.27±0.21	13.54±1.84	2.39±0.74	2.86±0.75
5*t*,9,12-18:3	0	0	0	0	21.79±1.87
9,12,15-18:3	0.90±0.12	1.49±0.38	0.49±0.23	6.84±0.70	0.27±0.08
8,11-20:2	0	0	0.29±0.04	0	0.08±0.02
5,8,11-20:3	6.40±2.18	3.92±0.62	0.35±0.02	0.06±0.01	0.69±0.49
8,11,14-20:3	0.70±0.17	0	1.59±0.38	0.88±0.08	1.44±0.54
5,8,11,14-20:4	5.35±0.90	1.89±0.17	13.77±3.46	6.02±1.10	7.53±1.24
5,8,11,14,17-20:5	0.40±0.10	0	0.24±0.21	12.26±2.67	3.60±0.95
7,10,13,16-22:4	0.56±0.17	0	0	0.50±0.20	0
unid[d]	1.01±0.16	0.59±0.06	3.40±0.26	0	3.00±0.86
7,10,13,16,19-22:5	0.34±0.35	0	0.34±0.41	3.33±0.16	0
4,7,10,13,16,19-22:6	3.26±0.69	2.09±0.18	2.33±0.53	9.33±0.84	4.12±0.96

[a]Results presented as the mean percent of total fatty acids±SD.
[b]Fed as 4% of the diet.
[c]Number of animals used per group given in parentheses.
[d]Unidentified fatty acid.

procedures, or (b) acts on other enzymes which contribute to the pool of lipogenic precursors rather than on the specific enzymes chosen for investigation.

As a result of chromatographic studies on the fatty acid composition of liver (Table 6) and mammary gland (Table 7) excised from the lactating dams fed the various 18-carbon fatty acids as the sole source of lipid, it is clear that the patterns obtained completely reflect the dietary fatty acids administered. For example, in the case of liver (Table 6), when the dietary lipid was oleic acid the proportion of this monounsaturated acid in the total lipids was higher than when the diet contained linoleic, linolenic or columbinic acids. It is worthy of note that the levels of 5,8,11-20:3 were higher when stearic and oleic acids were fed than when any of the polyunsaturated 18-carbon fatty acids were given in the diet, a finding in accord with a great many other studies, indicating the conversion of 9-18:1 to 5,8,11-20:3 under conditions where linoleate is omitted from the diet. In addition, when linoleate was absent from the diet, this fatty acid as well as its metabolic product, arachidonate, were found to be at depressed levels. The polyunsaturated fatty acid content of the livers from those dams fed saturated- and monounsaturated-18-carbon fatty acids were low (11-22%), whereas

these acids were high (35-45%) in the livers of the dams fed diets containing linoleic, linolenic or columbinic acids.

We could find no evidence for fatty acids derived from columbinate other than columbinate itself in the liver lipids of the mice fed this fatty acid (Table 6). It would appear, therefore, that whereas the enzyme systems present in the liver can elongate and desaturate fatty acids with *cis*-9,12, double bonds, the presence of the *trans* double bond in columbinate (5*t*,9,12-18:3) prevents its elongation and desaturation even though it does not prevent esterification and incorporation into complex lipids (43).

The influence of the various dietary fatty acids on the fatty acid composition of the mammary glands of lactating dams is given in Table 7. In general, the proportion of each fatty acid in the total fatty acids found in the gland was similar to that found in the liver for each fatty acid fed.

Previously, we showed that the pattern of the milk fatty acids reflects the dietary lipid composition and thus we were not surprised to find that the fatty acid composition of the livers of the suckling pups (Table 8) was found to be in complete accord with our observations made with the dams' livers (Table 6). These data emphasize that columbinic acid as well as the other acids are secreted into the milk and are freely absorbed by the suckling pups. Indeed, analysis of the fatty acid composition of the pup kidneys (Table 9) fully confirms such conclusions.

The influence of dietary fatty acids fed to the lactating dams on the values for the ratios of 16/16:1, 18/18:1 and 18:2/20:4 for liver and mammary gland of the dams and liver and kidney of the pups are given in Table 10. It is clear that dietary stearic and oleic acids produced different effects than did dietary linoleic, linolenic and columbinic acids (Table 10). Since the source of 16:1 in these animals was completely endogenous (there was none in the diet), these ratios suggest that each of the polyunsaturated fatty acids used was capable of inhibiting the activity of Δ9 desaturase, the enzyme responsible for conversion of palmitate to palmitoleate. Although we cannot be certain of this conclusion until the activity of the hepatic enzyme involved is measured, we are confident of this interpretation since the value for the ratio 18/18:1 in the livers of the mice fed the 18:0 diet was also found to be higher than those for the livers excised from mice in the polyunsaturated fatty acid-fed groups. The situation is less clear in the kidneys of the pups. With respect to the liver, from either the dams or the pups and to mammary glands from the dams, the conversion of 18:2 to 20:4 does not seem to be affected by dietary stearic, oleic, linolenic or columbinic acids to any appreciable extent, although each of the fatty acids produced a decrease in this conversion when

TABLE 7

Effect of Dietary Fatty Acids on Fatty Acid Composition of the Mammary Glands of Lactating BALB/c Mice[a]

	Dietary fatty acid[b]				
Fatty acid	18:0 (3)[c]	9-18:1 (5)	9,12-18:2 (8)	9,12,15-18:3 (5)	5*t*,9,12-18:3 (4)
8:0	0.45 ± 0.14	0.18 ± 0.04	0.28 ± 0.17	0.18 ± 0.17	0.36 ± 0.10
10:0	7.02 ± 1.06	4.28 ± 0.66	3.85 ± 0.28	6.35 ± 1.23	7.66 ± 1.17
12:0	11.86 ± 1.14	6.91 ± 2.53	9.82 ± 2.86	10.16 ± 1.67	10.51 ± 1.02
14:0	14.20 ± 1.20	9.95 ± 3.04	12.71 ± 3.00	14.43 ± 1.63	12.73 ± 1.19
16:0	22.41 ± 2.26	20.18 ± 2.10	20.37 ± 2.79	23.85 ± 4.76	18.82 ± 1.54
9-16:1	4.16 ± 0.59	5.05 ± 0.65	2.88 ± 0.78	3.21 ± 0.31	2.18 ± 0.68
18:0	4.17 ± 1.05	3.60 ± 0.73	4.21 ± 0.83	4.09 ± 0.92	5.32 ± 1.37
9-18:1	28.92 ± 1.71	46.04 ± 5.65	18.27 ± 3.06	12.13 ± 2.31	12.10 ± 2.90
9,12-18:2	1.09 ± 0.20	0.48 ± 0.17	18.01 ± 3.83	0.71 ± 0.28	2.42 ± 0.52
5*t*,9,12-18:3	0	0	0	0	20.78 ± 1.20
9,12,15-18:3	2.11 ± 0.65	2.07 ± 0.49	0.83 ± 0.36	15.46 ± 2.13	0.99 ± 0.40
8,11-20:2	0	0	1.36 ± 0.16	0.02 ± 0.00	0.59 ± 0.51
5,8,11-20:3	0.83 ± 0.36	0.76 ± 0.24	0	0	0
8,11,14-20:3	0.11 ± 0.11	0.14 ± 0.02	1.26 ± 0.32	0.09 ± 0.02	1.86 ± 0.30
5,8,11,14-20:4	0.98 ± 0.23	0.82 ± 0.35	2.56 ± 0.39	1.82 ± 0.32	1.24 ± 0.47
unid[d]	0	0	0	1.28 ± 0.25	0
5,8,11,14,17-20:5	0.71 ± 0.06	0	0.19 ± 0.07	3.27 ± 1.06	1.63 ± 0.80
7,10,13,16-22:4	0.97 ± 0.33	0	1.32 ± 0.41	0	1.12 ± 0.07
7,10,13,16,19-22:5	0	0	0	2.12 ± 0.76	0
4,7,10,13,16,19-22:6	0	0	0	0.84 ± 0.35	0

[a]Results presented as the mean percent of total fatty acids ± SD.
[b]Fed as 4% of the diet.
[c]Number of animals used per group given in parentheses.
[d]Unidentified fatty acid.

TABLE 8

Effect of Dietary Fatty Acids Fed to Lactating BALB c Dams on the Fatty Acid Composition of the Livers of the Suckling Pups[a]

Fatty acid	Dietary fatty acid[b] 18:0 (3)[c]	9-18:1 (7)	9,12-18:2 (11)	9,12,15-18:3 (5)	5t,9,12-18:3 (4)
12:0	0.45 ± 0.16	0.48 ± 0.00	0.49 ± 0.07	0	0.51 ± 0.17
14:0	1.88 ± 0.60	1.96 ± 0.51	1.77 ± 0.39	1.46 ± 0.36	1.17 ± 0.16
16:0	23.76 ± 2.02	22.70 ± 1.28	22.44 ± 2.03	22.31 ± 1.25	26.29 ± 3.02
9-16:1	2.98 ± 0.59	2.62 ± 0.49	1.37 ± 0.16	1.62 ± 0.22	1.41 ± 0.29
18:0	10.03 ± 1.09	7.47 ± 1.36	9.10 ± 1.54	11.19 ± 1.99	14.46 ± 2.63
9-18:1	31.06 ± 2.76	36.80 ± 5.46	11.67 ± 1.67	12.08 ± 1.45	9.67 ± 1.59
9,12-18:2	5.89 ± 1.45	7.14 ± 1.43	24.33 ± 2.59	6.65 ± 1.65	6.06 ± 1.56
5t,9,12-18:3	0	0	0	0	15.42 ± 4.15
9,12,15-18:3	0.83 ± 0.12	1.22 ± 0.16	0.28 ± 0.09	10.95 ± 4.35	0.21 ± 0.11
8,11-20:2	0.14 ± 0.05	0.21 ± 0.06	1.02 ± 0.15	0.21 ± 0.05	0.27 ± 0.04
5,8,11-20:3	1.96 ± 0.36	1.15 ± 0.45	0.02 ± 0.02	0	0
8,11,14-20:3	0.85 ± 0.31	0.86 ± 0.21	2.03 ± 0.21	0.85 ± 0.19	0.75 ± 0.02
5,8,11,14-20:4	10.32 ± 1.89	9.00 ± 1.26	14.52 ± 1.68	8.96 ± 2.01	8.40 ± 3.59
5,8,11,14,17-20:5	0.52 ± 0.06	0.35 ± 0.14	0.10 ± 0.01	1.28 ± 0.48	0.89 ± 0.23
7,10,13,16-22:4	0.89 ± 0.16	0.52 ± 0.12	2.22 ± 0.33	0.57 ± 0.16	1.25 ± 0.16
unid[d]	1.06 ± 0.18	0.40 ± 0.30	1.79 ± 0.21	0	0.88 ± 0.05
7,10,13,16,19-22:5	0.52 ± 0.11	0.53 ± 0.42	0.30 ± 0.22	4.14 ± 0.76	0.77 ± 0.27
4,7,10,13,16,19-22:6	6.84 ± 0.76	6.96 ± 1.45	6.62 ± 0.74	9.31 ± 1.03	8.00 ± 3.97

[a]Results presented as the mean percent of total fatty acids ± SD.
[b]Fed as 4% of the diet.
[c]Number of animals used per group given in parentheses.
[d]Unidentified fatty acid.

TABLE 9

Effect of Dietary Fatty Acids Fed to Lactating BALB c Dams on the Fatty Acid Composition of the Kidneys of the Suckling Pups[a]

Fatty acid	Dietary fatty acid[b] 9-18:1 (3)[c]	9,12-18:2 (4)	5t,9,12-18:3 (3)
8:0	1.54 ± 1.28	3.14 ± 2.71	3.05 ± 0.80
10:0	0.62 ± 0.35	0.51 ± 0.54	1.58 ± 1.16
12:0	2.26 ± 0.60	1.97 ± 1.24	2.57 ± 0.40
unid[d]	0	0	2.00 ± 0.17
14:0	5.83 ± 1.30	5.16 ± 2.28	6.33 ± 0.25
16:0	21.85 ± 0.67	19.28 ± 3.45	19.84 ± 4.28
9-16:1	2.69 ± 0.34	2.57 ± 0.94	3.14 ± 0.69
18:0	9.32 ± 1.19	9.10 ± 3.53	8.31 ± 3.35
9-18:1	24.49 ± 2.32	17.62 ± 2.90	17.17 ± 3.90
9,12-18:2	8.86 ± 1.97	13.70 ± 2.69	7.00 ± 2.15
5t,9,12-18:3	0	0	6.28 ± 0.98
9,12,15-18:3	1.26 ± 0.13	0.85 ± 0.38	0.72 ± 0.32
8,11-20:2	0.56 ± 0.17	1.07 ± 0.22	0.44 ± 0.09
5,8,11-20:3	0.36 ± 0.19	0	0
8,11,14-20:3	0.97 ± 0.17	1.49 ± 0.10	1.34 ± 0.18
5,8,11,14-20:4	12.06 ± 1.89	15.53 ± 2.35	12.29 ± 0.96
5,8,11,14,17-20:5	0.55 ± 0.05	0.56 ± 0.12	0.88 ± 0.21
7,10,13,16-22:4	1.00 ± 0.14	1.53 ± 0.25	1.18 ± 0.06
unid[d]	0.26 ± 0.07	0.49 ± 0.05	0.45 ± 0.11
7,10,13,16,19-22:5	0.59 ± 0.06	0.67 ± 0.11	0.62 ± 0.03
4,7,10,13,16,19-22:6	5.20 ± 1.50	4.59 ± 0.55	4.36 ± 0.50

[a]Results presented as the mean percent of total fatty acids ± SD.
[b]Fed as 4% of the diet.
[c]Number of animals used per group given in parentheses.
[d]Unidentified fatty acid.

compared to linoleic acid.

In confirmation of the observations made in liver, the values for the ratios of saturated to monounsaturated fatty acids (16/16:1 and 18/18:1) in mammary glands were also found to be lower when stearic and oleic acids were fed than when linoleic, linolenic or columbinic acids were included in the diets (Tables 7 and 10). Whether this represents depressed Δ9 desaturase in the gland (44) or is the result of the lowered hepatic Δ9 desaturase is not proven by these data but must await measurement of the specific enzymes in each tissue under these dietary conditions.

During the preparation of this work for publication, Grigor et al. (45) reported the results of an additional study on the regulation of lipogenic capacity in lactating rats fed fat-free and 20% peanut oil-containing diets, in which they rigorously controlled the number of suckling pups. They now conclude that "the activities of the mammary gland and liver enzymes are regulated independently, with the mammary gland enzymes being specifically affected by changes in milk demand whereas the liver enzymes are affected specifically by dietary changes." These workers (45) further state that, in contrast to the liver enzymes, the activities of the mammary gland fatty acid synthetase and glucose-6-phosphate dehydrogenase are not significantly altered by fat feeding. Thus, their new work which shows the relative insensitivity of the mammary gland enzymes to dietary fat manipulation of

TABLE 10

Specific Fatty Acid Ratios in Tissues of Lactating Mice and Suckling Pups as a Result of Different Dietary Fatty Acids Fed to the Dams[a]

Tissue		Dietary fatty acid[b]	Ratio		
Organ	Source		16/16:1	18/18:1	18:2/20:4
Liver	Dam	18:0	4.46±0.32[c]	0.25±0.04[c]	0.55±0.14
		9-18:1	3.46±0.52	0.09±0.05	0.66±0.14
		9,12-18:2	9.56±2.30[c]	0.57±0.23[c]	1.00±0.08
		9,12,15-18:3	10.19±2.66[c]	0.99±0.50[c]	0.40±0.12[c]
		5*t*,9,12-18:3	13.83±3.42[c]	1.31±0.48[c]	0.38±0.10[c]
Liver	Pup	18:0	8.10±0.93	0.33±0.06[c]	0.57±0.06
		9-18:1	8.65±1.57	0.20±0.07	0.85±0.30
		9,12-18:2	16.62±2.82[c]	0.81±0.23[c]	1.71±0.36[c]
		9,12,15-18:3	14.00±2.14	0.95±0.24	0.74±0.06
		5*t*,9,12-18:3	19.32±4.64[c]	1.51±0.24[c]	0.57±0.15
Mammary gland	Dam	18:0	5.40±0.24[c]	0.15±0.03[c]	1.15±0.30[c]
		9-18:1	4.05±0.72	0.08±0.04	0.62±0.13
		9,12-18:2	7.67±2.67[c]	0.25±0.09[c]	7.37±2.71[c]
		9,12,15-18:3	7.49±1.69[c]	0.34±0.07[c]	0.43±0.28
		5*t*,9,12-18:3	9.18±2.35	0.48±0.23	2.10±0.61[c]
Kidney	Pup	18:0	ND[d]	ND	ND
		9-18:1	8.20±1.00	0.38±0.08	0.74±0.14
		9,12-18:2	8.32±3.00	0.56±0.29	0.91±0.29
		9,12,15-18:3	ND	ND	ND
		5*t*,9,12-18:3	6.70±2.64	0.53±0.28	0.58±0.123

[a]Results presented as means ± SD; data from Tables 6-9.
[b]Fed as 4% of the diet.
[c]$p < 0.05$ compared to 9-18:1 diet.
[d]ND signifies not done.

the host confirms our observations made in mice (21).

The failure of dietary fat to affect mammary gland fatty acid synthetase while so strongly affecting the liver synthetase cannot be caused by possible differences in the enzyme's structure or function. Since the synthetases from both tissues are immunologically similar, have the same molecular weight and also possess common catalytic properties (46), they therefore appear to be identical proteins. Hence, the mechanism for this effect must lie in the pathways or processes whereby the enzymes are synthesized in each tissue, for, as shown here, the amount of enzyme in the liver, as opposed to that in the mammary gland, is exquisitely sensitive to the type of dietary fat. Dietary 18-carbon polyunsaturated fatty acids with double bonds in the 9- and 12-positions (or fatty acids derived from them such as arachidonate) are either not converted to compounds which depress the synthesis of lipogenic enzymes (39) or the process itself is missing in the mammary glands of lactating mice.

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Phospholipid Studies of Marine Organisms: V.[1] New α-Methoxy Acids from *Higginsia tethyoides*

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ABSTRACT

The phospholipids of the demosponge *Higginsia tethyoides* are shown to have at least 16 long-chain α-methoxy acids, which represent a new class of fatty acids. Among them are the saturated α-methoxy acids containing 19-24 carbon atoms. The monounsaturated compounds are 2-OMe-Δ^{17}-24:1, 2-OMe-Δ^{18}-25:1, 2-OMe-Δ^{19}-26:1 and 2-OMe-Δ^{21}-28:1. The major diunsaturated ones were shown to be 2-OMe-$\Delta^{5,19}$-26:2 and 2-OMe-$\Delta^{5,21}$-28:2. Small amounts of 2-OMe-23:1, 2-OMe-26:3; 2-OMe-27:1 and 2-OMe-28:3 were also encountered. Structures of the minor monounsaturated compounds were tentatively assigned as 2-methoxy-16-tricosenoic acid and 2-methoxy-20-heptacosenoic acids. The double bonds of the fatty acids show all-*cis* configuration. Circular dichroism measurements indicate an R-configuration for the α-methoxy acids. The major component of the total phospholipid acid mixture is 5,9,23-triacontatrienoic acid. Possible biosynthetic routes to these unusual phospholipid acids are discussed. The major phospholipids were phosphatidylethanolamine, phosphatidylglycerol and phosphatidylserine. The distribution of fatty acids among the phospholipids was also investigated.

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INTRODUCTION

The reports of a large number of novel sterols with unusual side-chain elongation and branching in marine invertebrates and algae (2,3) raise interesting questions concerning membrane structures of these organisms. Recent research carried out in various laboratories has shown that some other lipophilic compounds such as carotenoids and tetracyclic sesterpenoids are present in primitive marine organisms (4-6). In addition, Litchfield and coworkers (7-11) have encountered new fatty acids in different species of sponges. These "demospongic" acids contain 24-30 carbons and usually feature $\Delta^{5,9}$ diunsaturation, sometimes together with one more double bond near the chain terminus. Our investigation of marine organisms has already revealed a large number of straight chain and methyl branched C_{24-30} phospholipid acids featuring typical $\Delta^{5,9}$ diunsaturation or Δ^{5} monounsaturation in the sponges *Aplysina fistularis* (12), *Petrosia ficiformis* (13) and *Strongylophora durissima* (Dasgupta, A., Ayanoglu, E., and Djerassi, C., manuscript in preparation), all of which also contain high levels of sterols with "unusual" side chains (12,14-17). Another unusual feature is that 5,9-hexacosadienoic acid ($\Delta^{5,9}$-26:2) was found to be virtually the sole acid in the PS and PE fractions of the Mediterranean sponge *Axinella verrucosa* (Ayanoglu, E., and Djerassi, C., unpublished data), in contrast to conventional phospholipids in which the two acyl fragments are different.

In a recent communication, we have also reported (1) the occurrence of a new compound, (2*R*,21*Z*)-2-methoxy-21-octacosenoic acid, as the second major nonpolar component in the phospholipids of the sponge *Higginsia tethyoides*, following 5,9,23-triacontatrienoic acid ($\Delta^{5,9,21}$-30:3), a previously reported (8) demospongic acid. Consequently, we undertook a more extensive search for such α-methoxy acids, which do not appear to have been encountered previously in nature, and report herewith the isolation and identification of an entire series of such acids in the phospholipids of the African sponge *Higginsia tethyoides*.

EXPERIMENTAL

H. tethyoides sponge colonies were collected near Joal (ca. 100 km from Dakar, Senegal). The lyophilized samples were extracted with cold $CHCl_3$/MeOH (1:1, v/v). Separation of the total phospholipids from other lipids using silicic acid column chromatography has already been described (12). The samples were kept under argon at −10C in solutions containing 0.002% BHT. Silica Gel H was used as thin layer chromatography (TLC) adsorbent for separations of fatty acid derivatives as well as phospholipids. Assignments based on the comparisons with codeveloped standards were verified by the use of ninhydrin (PE, PS), Dragendorff (PC, SM) and periodate-Schiff (DPG, PG, PI) reagents. Molybdenum Blue and ceric sulfate

[1]For preceding paper, see reference 1.

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Abbreviations: BHT, butylated hydroxytoluene; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

were the general reagents for the phospholipids and fatty-acid derivatives, respectively, and preparative TLC plates were sprayed with Rhodamine 6G as the nondestructive color reagent (18,19). Quantitative estimation of phospholipids was carried out using a spectrometric phosphorus assay (20). Phospholipids were chromatographed in chloroform/methanol/28% NH_4OH (65:35:8, v/v/v). General developing solvent systems for the fatty acid derivatives were hexane/ether (8:2, v/v) and (3:7, v/v). For argentic TLC, 750 μ layers of silica gel were impregnated with 15% (by weight) silver nitrate, and the plates were developed in hexane/dichloromethane (8:2).

The following instruments and conditions were used for other chromatographic separations and for obtaining physical data.

Capillary gas chromatography (GC). Carlo Erba series 4160 Fractovap chromatograph equipped with a fused silica column (30 m × 0.32 nm) coated with SE-54 (J & W scientific, Inc.), a Model 400 LT programmer, a cooled on-column injection system and a flame ionization detector were used. The initial oven temperature was 70C or 140C for fatty acid methyl esters and 200C for pyrrolidides. Final temperature was 290C. The program temperature was either set to 3.0 or 10.0C/min.

High performance liquid chromatography (HPLC). A 50 cm × 9 mm Whatman ODS-2 reversed-phase column or a 25 cm × 10 mm Altex Ultrasphere column, a Waters M-6000A pump, a Valco loop injector and a Waters R401 refractometer detector were used.

Gas chromatography-mass spectrometry (GC-MS). Either a Finnigan MAT-44 GC-MS system using a spiral glass column (1.80 m × 2.0 mm), coated with 3% OV-17 on GCQ, or a Ribermag GC-MS-DS system, combining a Ribermag R 10-10 quadrupole mass spectrometer with a Carlo Erba series 4160 Fractovap chromatograph containing a fused silica column (28 m × 0.32 mm) with SE-54 (J & W Scientific, Inc.) was used.

Nuclear magnetic resonance (NMR) spectra. A Varian Associates HA-100 NMR instrument was used. Deuterated chloroform was used as solvent and shift values are given in ppm (δ).

Infrared (IR) spectra. A Beckman Acculab spectrophotometer was used. The samples were measured in chloroform.

Circular dichroism (CD) spectra. A Jasco Model J-40 instrument was used, with all measurements being conducted in hexane or in cyclohexane.

Transesterification of the phospholipid acids to obtain methyl esters was carried out by sodium hydroxide in methanol and hydrochloric acid in methanol (21). Boron trifluoride in methanol was used for the same purpose (22). The individual phospholipid classes were analyzed for the fatty acid content by preparative TLC. Each phospholipid band was scraped into a test tube, digested with methanolic boron trifluoride for transesterification (23) and the resulting methyl esters then analyzed by GC. Aliquots of fatty acid methyl esters were hydrogenated in methanol with platinum (IV) oxide (8 hr, normal pressure, room temperature). The methyl esters were converted to N-acylpyrrolidide derivatives in pyrrolidine/acetic acid (10:1, v/v, 1 hr, 100C) for the location of double bonds as well as branching. $LiAlH_4$ reduction was carried out in dry tetrahydrofuran (2 hr, reflux temperature). $NaIO_4/KMnO_4$ oxidative degradation was achieved in *tert*-butanol (24) (5-7 hr, room temperature). Ozonization in BF_3/MeOH was also performed (25) for the conversion of double bonds to methyl esters and gave almost quantitative yields. For this purpose, the sample was subjected to ozonization using 14% BF_3 in methanol as reactive solvent for 1 min at room temperature and the reaction mixture was heated at 100C in a capped vial for 1 hr. The standard fatty acid methyl ester samples were obtained from Supelco (Supelco Park, Bellefonte, PA) or from Applied Science (Milton Ray Co. Laboratory Group, State College, PA) for comparison purposes.

RESULTS AND DISCUSSION

Quantitative analysis of the phospholipids using solvent elution and spectrophotometric analysis of each TLC spot indicated that the 4 classes listed in Table 1 comprise ca. 86% of the mixture and that PC were essentially absent. The remaining 14% was distributed among several minor spots on the chromatogram.

The capillary GC analysis of the total phospholipid fatty acid mixture allowed equivalent chain length (ECL) values to be assigned to each major peak, on the basis of their retention times, relative to methyl esters (26). This analysis indicated the presence of ca. 61 detectable peaks, of which 21 comprised 87% of the total (Table 2). Many of the peaks, especially the unsubstituted "conventional" ones, were simply identified by comparing their

TABLE 1

The Major Phospholipids of *H. tethyoides*[a]

Phospholipid class	Mol %[a]
Phosphatidylethanolamine	31
Phosphatidylglycerol	24
Phosphatidylserine	26
Phosphatidylinositol	5

[a]Average of 3 replicates; percentages are based on relative phosphate content.

TABLE 2

Identified Major Fatty Acids from the Phospholipids of *H. tethyoides*[a]

Compound	ECL[b]	Fatty acid	Percent (by wt) in phospholipids	Distribution in phospholipid classes[c,d]		
				PI/PS	PG	PE
1	14.00	Tetradecanoic (n-14:0, myristic)	0.7	0.8	0.8	0.5
2	14.99	4,8,12-Trimethyltridecanoic (4-Me,8-Me,12-Me-13:0)	8.1	5.5	10.8	8.3
3	15.73	9-Hexadecenoic (Δ^9-16:1, palmitoleic)	2.2	1.7	5.6	0.5
4	16.00	Hexadecanoic (n-16:0, palmitic)	2.6	1.3	7.1	-
5	16.78	10-Heptadecenoic (Δ^{10}-17:1)	0.5	-	0.9	-
6	17.46	5,9-Octadecadienoic ($\Delta^{5,9}$-18:2)	0.6	0.6	1.2	-
7	17.75	11-Octadecenoic (Δ^{11}-18:1, vaccenic)	2.8	1.5	4.9	2.4
8	18.00	Octadecanoic (n-18:0)	2.3	1.9	3.7	1.6
9	19.19	5,8,11,14-Eicosatetraenoic ($\Delta^{5,8,11,14}$-20:4)	10.1	2.7	34.5	2.3
10	19.39	5,8,11-Eicosatrienoic ($\Delta^{5,8,11}$-20:3)	1.2	-	3.5	-
11	21.16	2-Methoxycosanoic (2-OMe-20:0)	0.5	0.7	-	0.5
12	23.17	2-Methoxydocosanoic (2-OMe-22:0)	2.2	2.5	-	3.1
13	24.17	2-Methoxytricosanoic (2-OMe-23:0)	0.6	1.0	-	0.5
14	24.91	2-Methoxy-17-tetracosenoic (2-OMe-Δ^{17}-24:1)	1.9	2.8	0.5	2.0
15	25.17	2-Methoxytetracosanoic (2-OMe-24:0)	3.2	3.4	0.6	4.7
16	25.92	2-Methoxy-18-pentacosenoic (2-OMe-Δ^{18}-25:1)	0.5	0.5	-	0.9
17	26.77	2-Methoxy-5,19-hexacosadienoic (2-OMe-$\Delta^{5,19}$-26:2)	2.7	3.1	-	3.8
18	26.92	2-Methoxy-19-hexacosenoic (2-OMe-Δ^{19}-26:1)	1.3	2.0	-	1.4
19	28.78	2-Methoxy-7,21-octacosadienoic (2-OMe-$\Delta^{7,21}$-28:2)	2.7	3.4	0.6	3.8
20	28.93	2-Methoxy-21-octacosenoic (2-OMe-Δ^{21}-28:1)	13.7	16.3	3.8	17.3
21	29.43	5,9,23-tricontatrienoic ($\Delta^{5,9,23}$-30:3)	34.2	40.8	15.9	42.0

[a]Identified minor (<0.5%) fatty acids: **(22)** n-16:3 (ECL 15.33); **(23)** n-17:2 (ECL 16.46); **(24)** n-17:0 (ECL 17.00); **(25)** 18:1 (ECL 17.75); **(26)** 2-OMe-19:0 (ECL 20.16); **(27)** 2-OMe-21:0 (ECL 22.16); **(28)** 2-OMe-**D**^{16}-23:1 (ECL 23.92); **(29)** 2-OMe-26:3 (ECL 26.62); **(30)** 2-OMe-**D**^{20}-27:1 (ECL 27.92); **(31)** 2-OMe-28:3 (ECL 28.63); **(32)** 30:2 (ECL 29.58).

[b]Equivalent chain length values were calculated from the methyl esters of the corresponding acids.

[c]Averages from three determinations; - implies that the acid is less than 0.5%.

[d]Full names of phospholipids are given in the text.

ECL values with those of known esters and coinjection with standard samples. A capillary GC analysis of a hydrogenated aliquot of the methyl esters provided more information for identification. Pyrrolidides of the original and hydrogenated fatty acids were also investigated by capillary GC-MS, especially for the location of double bonds and branching. The spacings between major peaks (associated with the carbonyl-bearing fragments) were considered for the location of double bonds. In general, the presence of 12 instead of 14 amu between the most intense peaks of fragments containing n-1 and n carbon atoms in the acid moiety indicates the presence of a double bond between carbon n and n+1 in the fatty acid derivative (27). Thus, for example (Fig. 1), the mass spectrum of the pyrrolidide of the major (34.2%) compound, 5,9,23-triacontatrienoic acid, exhibited spacings of 12 amu between the C_4 (m/z 140) and C_5 (m/z 152); between the C_8 (m/z 194) and C_9 (m/z 206); and between the C_{22} (m/z 388) and C_{23} (m/z 400) fragments, and also exhibited peaks of higher intensities corresponding to the C_3 (m/z 126), C_7 (m/z 180), C_{11} (m/z 234), C_{21} (m/z 374) and C_{25} (m/z 428) fragments formed by allylic cleavages around each double bond. Coinjection with an authentic sample (12) gave only one peak on capillary GC.

Only one branched chain fatty acid, 4,8,12-trimethyltridecanoic acid (**2**) (8.1%), was encountered in the phospholipids of *H. tethyoides*. This compound is probably of zooplanktonic origin and has been found in marine mammals (28). All of the major fatty acids were straight chain saturated or unsaturated compounds.

The methoxy fatty acid methyl esters were easily separated (1) from the conventional fatty acid methyl esters by open silica gel column chromatography. The column was first eluted with hexane and then with hexane/ether (95:5, v/v) to collect "conventional" fatty acid methyl ester fractions. Further elution of the column with hexane/ether (92:8, v/v) yielded the methoxy fatty acid derivatives. The mixture which gave only one spot on TLC with a lower Rf value (0.75 in hexane/ether [8:2, v/v]) than that of unsubstituted fatty acid derivatives (Rf 0.85) contained the previously reported (1) α-methoxy fatty acid **20** as the major component according to capillary GC analysis and coinjection. A capillary GC-MS analysis of this mixture showed that all compounds had a characteristic ion at m/z 104 (1) corresponding to a

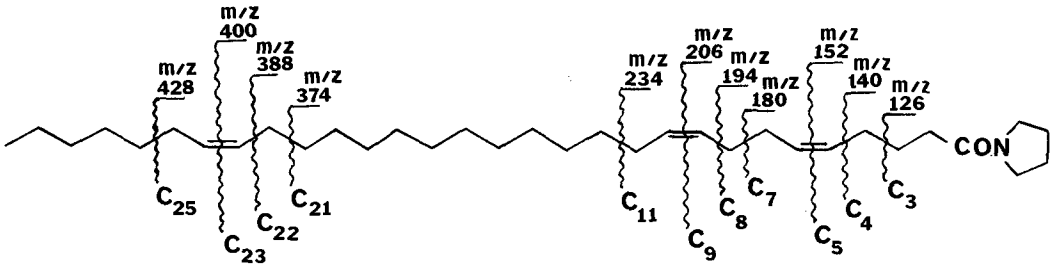


FIG. 1. The major diagnostic mass spectral fragmentations of N-(5,9,23-triacontatrienoyl)pyrrolidone (compound **21**, Table 2).

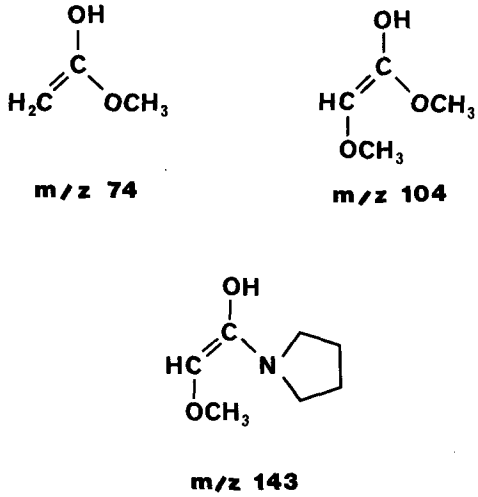
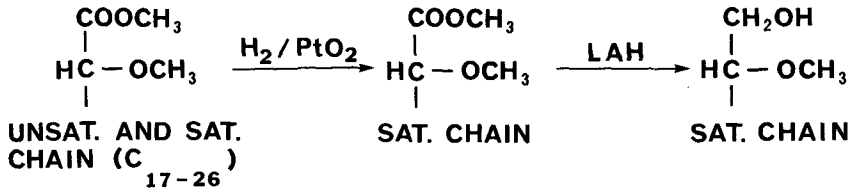


FIG. 2. McLafferty rearrangement ions arising from α-unsubstituted (m/z 74) and α-methoxy (m/z 104) methyl esters and α-methoxy pyrrolidides (m/z 143).

McLafferty rearrangement (Fig. 2) (29), due to the presence of a methoxy or hydroxymethyl group. A TLC comparison indicated that standard hydroxy fatty acids give a much shorter Rf value (0.4) than the α-methoxy acid **21**, because of the free hydroxyl substituent.

An attempt to obtain the corresponding α-hydroxy acids directly from the mixture with hydroiodic acid for identification purposes gave high yields of side products. A reaction procedure (Fig. 3) was then pursued which was monitored by TLC (hexane/ether [8:2, v/v]) and NMR in order to convert the α-methoxy acids to the α-unsubstituted compounds with one less carbon atom. Hydrogenation of the mixture did not cause a detectable change in the Rf value, but the olefinic proton signals previously observed at 5.350 ppm had disappeared. The $LiAlH_4$ reduction products gave a shorter Rf value (0.45) and the NMR methyl ester signal at 3.755 ppm had also disappeared. The cleavage of the methyl ether bond was observed by the formation of a polar TLC spot (Rf: 0.15) and by the absence of the formerly present methyl ether signal at 3.380 ppm. $NaIO_4/KMnO_4$ oxidative



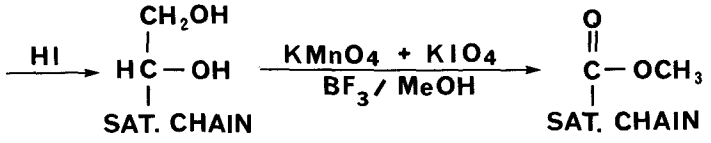


FIG. 3. Conversion of unsaturated and saturated α-methoxy acids to the corresponding α-unsubstituted analogs with one less carbon atom.

degradation of the 1,2-diols followed by methyl esterification yielded the straight chain C_{18}-C_{27} acid derivatives which were identical with standard samples in all respects. This result confirms the occurrence of C_{19}-C_{28} α-methoxy acids in our original mixture.

A direct application of the methoxy acids to HPLC columns did not provide satisfactory separation. For this reason, the components of the mixture were first fractionated according to their degrees of unsaturation using silver nitrate impregnated preparative plates. The least polar TLC fraction comprised methyl esters of compounds **26**, **11**, **27**, **12**, **13** and **15** in the order of increasing ECL values. These derivatives did not change on hydrogenation and exhibited molecular ion peaks at m/z 342, 356, 370, 384, 398 and 412, respectively, requiring no degrees of unsaturation. Purification of the major α-methoxy saturated acid **15** was achieved by HPLC (Whatman column, MeOH/H_2O [98:2, v/v], 33 min). The molecular ion (M^+ 412) and other expected (1) prominent peaks (m/z 380 [M^+ − MeOH], m/z 353 [$M^+ - COOCH_3$], m/z 321 [M^+ − ($COOCH_3$ + MeOH)] and m/z 104 [McLafferty rearrangement, Fig. 2]) of the mass spectrum of this compound indicated it to be 2-methoxytetracosanoic acid. The typical absorptions at 0.883 (3H, t, terminal CH_3), 1.259 (s, CH_2 chain), 3.381 (3H, s, α-methoxy CH_3) and 3.756 (3H, s, methoxycarbonyl CH_3) ppm in the NMR spectrum, together with heretofore mentioned chemical as well as chromatographic data also confirmed its structure. A CD spectrum of the purified methyl ester in cyclohexane exhibited a strong negative Cotton effect at 205-210 nm (θ 18,000) and a weaker positive one at ca. 240 nm (θ 2,000) similar to the reported (1) CD spectrum of (2*R*,21*Z*)-2-methoxy-21-octacosenoic acid, thus supporting the assignment of an R configuration. The low quantities of the other α-methoxy compounds in pure form prevented CD measurement of each ester, but since the major saturated (**15**) and unsaturated (**20**) acid esters and various enriched fractions (including 95% pure diunsaturated **19**) showed almost identical CD spectra, we feel safe in assuming that all α-methoxy acids in our mixture have the R configuration.

The other saturated α-methoxy acid (**26**, **11**, **27**, **12** and **13**) esters recovered from the same argentic TLC band again gave typical M^+ − MeOH, $M^+ - COOCH_3$ and M^+ − ($COOCH_3$ + MeOH) mass spectral fragment ions together with the m/z 104 McLafferty rearrangement peak. Their pyrrolidides exhibited the molecular ions of a homologous series at m/z 381, 395, 409, 423, 437 and 451, always with a McLafferty peak (Fig. 2) at m/z 143, ensuring their α-methoxy saturated structures. These results clearly showed the presence of 2*R*-methoxy nonadecanoic (**26**), eicosanoic (**11**), heneicosanoic (**27**), docosanoic (**12**) and tricosanoic (**13**) acids in our mixture. Compounds **11**, **12**, and **15** which contain even numbers of carbon atoms in their chains were found to be more abundant (Table 2).

The second band from the argentic TLC contained monoenic compounds as expected and yielded the previously reported (1) 2-methoxy-21-octacosenoic acid (**20**) as the major one according to capillary GC coinjection and GC-MS analysis. Three more compounds (**14**, **16** and **18**) were recovered from the same band in relatively smaller amounts, in addition to some minor (<0.5%) peaks. The pyrrolidide derivatives of **14**, **16** and **18** exhibited molecular ion peaks at m/z 449, 463 and 477, respectively, together with the expected m/z 143 peak for each acid. The presence of a methoxy group at C_2 led to additional peaks in the mass spectra of the pyrrolidide derivatives and prevented unambiguous location of the double bonds. For this reason, the 3 acids were subjected to HPLC using an Altex column (MeOH/H_2O [97:3, v/v], 2.5 ml/min) for the separation and double bond degradation of each individual compound.

The molecular ion peak at m/z 438 of the acid **18** (HPLC retention time: 39 min) shifted to m/z 440 in accordance with a monoolefinic α-methoxy C_{26} structure, after hydrogenation. The distinctive $M^+ - CH_3OH$ (m/z 406), $M^+ - COOCH_3$ (m/z 379) and M^+ − ($COOCH_3$ + CH_3OH) (m/z 347) peaks were also shifted 2 amu but the m/z 104 rearrangement peak (Fig. 2) remained unchanged. The dicarboxylic acid methyl ester obtained from the oxidative double bond cleavage of compound **18** displayed its molecular ion at m/z 386 with an intense $M^+ - COOCH_3$ peak at m/z 327. The mass spectrum also furnished the diagnostic McLafferty rearrangement ions at m/z 74 and m/z 104 (Fig. 2) corresponding to α-unsubstituted and α-methoxy methyl esters. The other prominent peaks were m/z 354 (M^+ − 32), m/z 295 (M^+ − 91) and m/z 263 (M^+ − [32+91]) showing the structure of the degradation product to be 2-methoxynonadecanedioic acid. This result determines the location of the double bond at C_{19} of the parent acid **18**. IR spectra of this compound and other isolated mono- and diolefinic acids gave no prominent absorption (7,11) at 980-968 cm^{-1}, indicating a *cis* rather than *trans* orientation in each case. The monoolefinic acid **18** is, therefore, established as (2*R*,19*Z*)-2-methoxy-19-hexacosenoic acid.

The other 2 monoenic methoxy acids **14** and **16** (HPLC retention times 31 and 35.5 min, respectively) had molecular ion peaks at m/z 410 and at m/z 424. These peaks were shifted 2 amu to m/z 412 and 426, along with the characteristic $M^+ - 32$, $M^+ - 59$, and $M^+ - 91$ peaks on hydrogenation as expected, but the m/z 104 peaks did not change. Their structures were illustrated to be 2-methoxy-17-tetracosenoic acid (**14**) and 2-methoxy-18-pentacosenoic acid (**16**) since the double bond

cleavage products were analogs of the above-mentioned dicarboxylic acid with two and one less carbon atoms, namely, 2-methoxyheptanedioic and 2-methoxyoctanedioic acids. The R and *cis* configurations were assigned in each acid for the α-substitution and the double bonds, respectively, based on the CD and IR spectral evidence.

Finally, the structures of the 2 new diunsaturated phospholipid components **17** and **19** (Table 2) were established as (2*R*,5*Z*,19*Z*)-2-methoxy-5,19-hexacosadienoic (**17**, M^+ 436) and (2*R*,7*Z*,21*Z*)-2-methoxy-7,21-octacosadienoic (**19**, M^+ 464) acids. In addition to the general mass, NMR, IR and CD characteristics mentioned earlier, the following observations confirmed the structural assignments. (a) The chemical reactions outlined in Figure 3 yielded straight chain pentacosanoic and heptacosanoic acids. (b) The pyrrolidides of the parent compounds showed molecular ion peaks at m/z 475 and m/z 503 together with the diagnostic m/z 143 ion (Fig. 2) for each compound. (c) The hydrogenation products of the methyl esters gave M^+ 440 and M^+ 468, respectively, ensuring the presence of diunsaturation, rather than cyclization. (d) When subjected to oxidative degradation, both compounds yielded the same mono- and α-unsubstituted dicarboxylic acids, showing $\Delta^{5,19}$ and $\Delta^{7,21}$ double bond patterns for **17** and **19**, respectively.

The widespread occurrence of $\Delta^{5,9}$ demospongic acids and their biosynthesis in the sponges are well known (13,30). Double bonds (e.g., Δ^{19}, Δ^{23}, etc.) near the chain terminus of various demospongic acids are also reported (8,12). Recently we have encountered Δ^5 fatty acids of this class in some marine organisms such as *Strongylophora durissima*. Incorporation studies using ^{14}C-acetate in the sponge *Microciona prolifera* revealed that the sponge synthesizes its demospongic acids by the chain elongation of conventional short chain length precursors followed by $\Delta^{5,9}$ desaturation (30). Therefore, it was proposed (8) that 5,9,23-triacontatrienoic acid (also the major acid **21** in *H. tethyoides*) arises from 9-hexadecenoic acid (compound **3** in our organism):

$$\Delta^9\text{-16:1} \rightarrow \Delta^{11}\text{-18:1} \rightarrow\rightarrow\rightarrow\rightarrow\rightarrow \Delta^{23}\text{-30:1} \rightarrow \Delta^{9,23}\text{-30:2} \rightarrow \Delta^{5,9,23}\text{-30:3}$$

To our knowledge, a biosynthetic route to α-methoxy substituted phospholipid acids is not known. One possible pathway would be by hydroxylation of C_2 of each acid followed by biomethylation. On the other hand, since the unsaturated C_{24}, C_{26} and C_{28} acids **14**, **18** and **20** have their double bonds at C_{17}, C_{19} and C_{21}, respectively, α-unsubstituted precursors of **14** and **18** may also be biosynthetic precursors of the major α-methoxy acid **20**. A similar biosynthetic pattern can also be drawn for compounds **17** ($\Delta^{5,19}$) and **19** ($\Delta^{7,21}$). The same analogy leads us to propose the structure of the minor monounsaturated C_{23} (**28**) and C_{27} (**30**) acids (M^+ 396 and 452, hydrogenation: M^+ 398 and 454) as 2-methoxy-16-tricosenoic acid and 2-methoxy-20-heptacosenoic acid, respectively. They probably arise from the odd numbered 10-heptadecenoic acid (5). 2-Methoxy-18-pentacosenoic acid (16) also fits into such a chain elongation process between 28 and 30. Whether the methoxyl introduction is performed by the sponge or some bacterial or symbiont component associated with the animal remains to be established.

The presence of unusually long-chain saturated and unsaturated 2-methoxy fatty acids in the phospholipids of a primitive marine organism is unprecedented and raises interesting questions about their biological function in the membranes in addition to the above discussed biosynthetic pathways. Various experiments have shown that chain length, unsaturation and the presence of other functional groups cause significant differences in membrane properties of phospholipids such as fluidity, compressibility and permeability (31,32). Theoretical studies and calculations are in general agreement with these results and it seems possible to draw some general rules (32,33).

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METHODS

Use of Antioxidants in the Analysis of Vitamins A and E in Mammalian Plasma by High Performance Liquid Chromatography

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ABSTRACT

A simple, sensitive, quantitative method for the simultaneous assay of retinol, α-tocopherol and γ-tocopherol in rat, guinea pig, monkey and human plasma was developed by using high performance liquid chromatography. It was found that antioxidant was required to stabilize the fat-soluble vitamins in the plasma of rats. The effect of several antioxidants on the recovery of fat-soluble vitamins was evaluated. Results showed that 0.125% butylated hydroxytoluene (BHT) in ethanol and 0.025% BHT in heptane yielded recoveries >95% in 0.1 ml plasma.

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The application of high performance liquid chromatography (HPLC) to the quantitation of fat-soluble vitamins in blood is rapidly gaining acceptance as a sensitive and rapid analytical tool (1-5). The methods to date have been developed principally for application to blood samples from humans, rabbits and horses. The isomers of vitamin E in human and horse serum (6) and in human and rabbit plasma (7) have been separated by HPLC. Bieri et al. (8), de Leenheer et al. (9) and Driskell et al. (10) have reported HPLC methods for analyzing α-tocopherol and retinol simultaneously in human plasma samples. Since rats, guinea pigs and monkeys are used extensively as animal models in biological research, we developed and report here a method for the simultaneous measurement of retinol, α-tocopherol, and γ-tocopherol in the plasma of these species as well as humans.

Our investigation showed that an antioxidant was required to stabilize these vitamins in rat plasma during the extraction process. We compared the efficacy of several antioxidants in the present study and report an extraction procedure which permits excellent recoveries of these vitamins from plasma samples of rats, guinea pigs, monkeys and humans.

MATERIAL AND METHODS

Reagents

All-*trans*-retinol (R), all-*trans*-retinyl acetate (R-A), cholecalciferol (D_3),d-α-tocopherol (α-T) and d-γ-tocopherol (γ-T) were purchased from Eastman Kodak Co. (Rochester, NY). Liquid chromatography-grade heptane and methanol were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). All other reagents and chemicals were of analytical-reagent grade or the highest purity obtainable.

Standard solutions of the vitamins were prepared in 100% ethanol and stored at −17 C in the following concentrations (μg/ml): (A) internal standard (R-A) 2; (B) spike mix: R 2; D_3 .8; α-T 1.4; γ-T 2.

High Performance Liquid Chromatography (HPLC)

The HPLC equipment consisted of a Model 6000A solvent delivery system, U6K universal liquid chromatograph injector, a 3 mm × 22 mm guard column packed with Bondapak C_{18}-Corasil (37-50 μ), and a 3.9 mm × 30 cm analytical column of μBondapak C_{18} (10μ) packing material, all from Waters Associates (Milford, MA). Ultraviolet absorption was monitored in a Spectromonitor III variable wavelength UV detector from Laboratory Data Control (Riviera Beach, FL). Automatic integration of peak area was performed by a Model 730 Data Module microprocessor-controlled integrator-recorder (Waters Associates).

The chromatographic conditions comprised a mobile phase of methanol/water (93:7, v/v), flow rate of 1.0 ml/min, UV detector set at 292 nm, 0.01 absorption units full scale (AUFS) and recorder chart speed at 1.0 cm/min.

Plasma Extraction

Blood samples were obtained by cardiac puncture from small animal species and by venipuncture in monkeys and adult human subjects (3 men and 3 women) into heparinized tubes. All animals and human subjects were consuming normal diets. Plasma was separated by centrifugation at 4 C for 10 min at 2500 rpm (1060 × g).

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Vitamins were extracted from duplicates of each plasma sample under subdued light according to the method of Bieri et al. (8), with several modifications. 100-μl aliquot of plasma was transferred into a 6 × 50 mm glass test tube by micropipet with glass tip. After adding 50 μl of internal standard (A) and 50 μl of 0.125% butylated hydroxytoluene (BHT) in ethanol, the mixture was stirred on a vortex mixer for 20 sec. The fat-soluble vitamins were then extracted with 200 μl of heptane containing 0.025% BHT by agitating on a vortex mixer for 1 min. The tubes were centrifuged at 2500 rpm (1060 × g), 4 C, for 15 min. A 100-μl aliquot of the heptane layer was transferred to a 12 × 75 mm tube, and evaporated in a sample-concentrator vacuum centrifuge (Savant Instruments, Inc., Hicksville, NY) for 5 min. The residue was dissolved in 100 μl of methanol, of which 50 μl were injected into the HPLC system.

Effect of Antioxidants

The effect of 3 different antioxidants on the recovery of fat-soluble vitamins was studied in rat and human plasma. All studies were performed on triplicates of each sample. In the initial study, to 100 μl of pooled rat plasma and 50 μl of internal standard (A) or spike mix (B) were added 50 μl of 70% ethanol containing one of the following: no antioxidant; 10% ascorbic acid (AA); or 13% (saturated) AA. These samples were extracted with 200 μl of heptane containing no antioxidant. A fourth group was extracted with 10% AA in 70% ethanol and 0.005% BHT in the heptane phase.

In a second study using rat plasma samples, the effect of increased concentration of BHT in the extraction medium was evaluated in combination with pyrogallol (PG) or BHT in the ethanolic phase. To 100 μl of pooled rat plasma and 50 μl of internal standard (A) or spike mix (B) were added 50 μl of ethanol containing either no antioxidant, 3% PG, or 0.125% BHT. Tubes were agitated on a vortex mixer for 20 sec and extracted with 200 μl of heptane containing 0.025% BHT.

A final comparison was made with pooled human plasma in the following manner: to 100 μl of plasma were added 50 μl of either 10% AA in 70% ethanol or 0.125% BHT in absolute ethanol. These groups were extracted with 200 μl of heptane containing 0.025% BHT and treated as above.

RESULTS

High Performance Liquid Chromatography

A chromatogram of a mixture of standards demonstrating the separation of R, R-A, D_3, α-T and γ-T is presented in Figure 1. Retention times of analyses performed on the same day or after a 21-day interval were reproducible within ± 2.0%. (Table 1). The detector response for peak areas was linear ($r^2 > 0.99$) for all the vitamins in the following range of concentrations: R = 0.005-0.500 μg; α-T = 0.125-1.00 μg; and γ-T = 0.060-0.500 μg. Peak area is plotted against vitamin concentration for R and the tocopherols in Figure 2.

Analysis of the 5-compound vitamin mixture was complete in less than 30 min under isocratic conditions, avoiding reequilibration delay between runs. Vitamin D_3 can also be determined in this system as demonstrated by its satisfactory resolution from γ-T. However, since its maximum UV absorption is at 265 nm, the sensitivity at 292 nm (the λ max for α-T and γ-T) is not adequate to detect the minute amounts of D_3 which may be present in plasma samples.

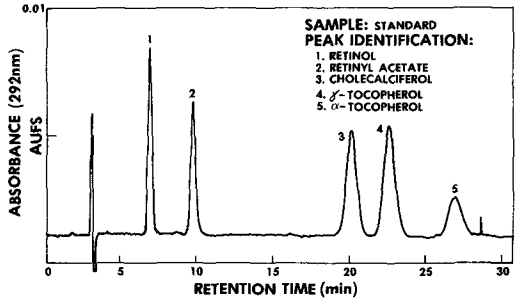

FIG. 1. HPLC chromatogram of a mixture of standard fat-soluble vitamins. Conditions: methanol/water (93:7, v/v), flow rate, 1.0 ml/min.; μBondapak C_{18} column (3.9 mm × 30 cm, 10 μ particle size) with Bondapak C_{18} Corasil guard column (3 mm × 22 mm, 37-50 μ particle size). Amounts injected: retinol = 0.025 μg, retinyl acetate = 0.025 μg, cholecalciferol = 0.200 μg, γ-tocopherol = 0.3125 μg, and α-tocopherol = 0.250 μg.

TABLE 1

Retention Time Reproducibility

Compound	Day 1 Mean[b] ± SD	Day 1 CV[c](%)	Day 2[a] Mean[b] ± SD	Day 2[a] CV[c](%)
Retinol	6.62 ± 0.04	0.60	6.62 ± 0.02	0.30
Retinyl acetate	9.00 ± 0.08	0.84	9.01 ± 0.04	0.44
γ-Tocopherol	19.63 ± 0.39	1.99	19.68 ± 0.24	1.22
α-Tocopherol	23.03 ± 0.47	2.04	23.10 ± 0.28	1.21

Chromatographic conditions: μBondapak C_{18} column (3.9 mm × 30 cm) and precolumn of Bondapak C_{18} Corasil (3 mm × 22 mm), 1 ml/min of methanol/H_2O (93:7, v/v), UV detector at 292 nm, 0.01 AUFS and chart speed of 1 cm/min.

[a]The analyses on day 2 were performed 3 weeks after day 1.

[b]Within-day average of 6 injections of either standard vitamin mix or vitamin-spiked plasma, retention time, min. Capacity factors (k') for the respective peaks were retinol = 1.1, retinyl acetate = 1.9, γ-tocopherol = 5.2, and α-tocopherol = 6.3.

[c]Coefficient of variation.

Figure 3 represents an analysis of rat plasma extracted with BHT in the ethanol and heptane phases. The retention time and capacity factor (k') for the BHT peak are 5.65 min and 0.59, respectively, providing adequate separation from retinol with a retention time of 7.60 min and k' of 1.1.

Effects of Antioxidants

The effects of the antioxidants on recovery of standards added to rat and human plasma samples are summarized in Table 2. In rat plasma, there was interference at the retention time of retinol when no antioxidant was added, and only 34% of the added tocopherol standard was recovered. The addition of AA to the ethanol phase slightly improved the α-T recovery to 65%. The combination of AA in the ethanol and BHT in the heptane phase did not improve recoveries.

Omission of AA from the ethanolic phase and the simultaneous increase of BHT from 0.005% to 0.025% in the heptane phase provided better recoveries than when AA was added. The presence of 0.025% BHT in heptane appeared to be adequate to produce acceptable recoveries (from 92% to 96%), either with or without the simultaneous use of PG or BHT in the ethanolic phase.

Extraction of human plasma without the use of an antioxidant resulted in recoveries from 84 to 98%. Samples treated with AA had recoveries of greater than 100% in all cases, whereas the use of BHT in both phases resulted in values closer to 100%.

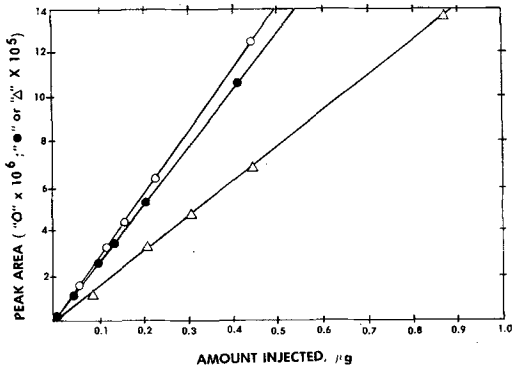

FIG. 2. Standard curves for retinol (o), α-tocopherol (△), and γ-tocopherol (.), peak area vs weight (μg). Conditions as in Figure 1.

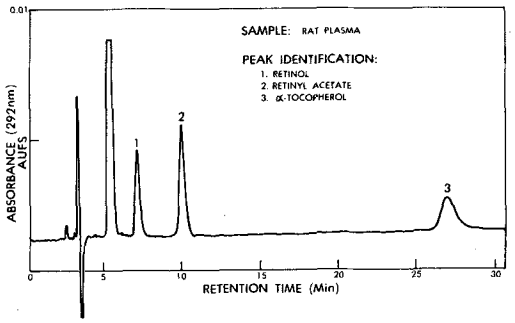

FIG. 3. HPLC chromatogram of heptane extract of rat plasma, using 0.125% BHT as antioxidant. Chromatographic conditions as in Figure 1. Injection amount: 50 μl.

TABLE 2

Effect of Antioxidants on Recovery of Fat-Soluble Vitamins in Rat and Human Plasma

Antioxidant			Percent recovery of added standard					
					Tocopherols			
Species	Ethanol phase	Heptane phase	Retinol	(CV[a])	α	(CV)	γ	(CV)
Rat	0	0	216	(6)	34	(5)	35	(6)
Rat	10% AA[b]	0	99	(3)	65	(24)	84	(15)
Rat	13% AA	0	89	(5)	66	(2)	96	(0.3)
Rat	10% AA	0.005% BHT[c]	111	(6)	37	(30)	59	(13)
Rat	0	0.025% BHT	95	(0.3)	96	(3)	94	(2)
Rat	3% PG[d]	0.025% BHT	109	(1)	92	(5)	94	(3)
Rat	0.125% BHT	0.025% BHT	98	(2)	109	(1)	100	(1)
Human	0	0	86	(7)	84	(17)	98	(5)
Human	10% AA	0.025% BHT	106	(3)	111	(9)	123	(6)
Human	0.125% BHT	0.025% BHT	99	(3)	101	(3)	114	(3)

[a]Coefficient of variation, %.
[b]Ascorbic acid.
[c]Butylated hydroxytoluene.
[d]Pyrogallol.

Plasma Vitamin A and E Levels

The amounts of R, α-T and γ-T found in human and various animal species' plasma are summarized in Table 3. No γ-T was detected in rat or guinea pig plasma, but significant amounts were found in monkey (rhesus *Macaca mulatta*) and human plasma.

DISCUSSION

This procedure was developed to permit the simultaneous determination of R, α-T and γ-T in the plasma of humans and various animal species without requiring modifications of extraction procedures or chromatographic conditions from species to species. The separation presented here was also designed for future application to the analysis of these vitamins plus D_2 and/or D_3 in animal tissue extracts and food samples in our laboratory. Preliminary results showed that these types of samples contain a number of nonsaponifiable compounds and require an extended run time beyond that which would be adequate to separate the vitamins alone (unpublished results). We chose to use a single internal standard (R-A) in order to avoid an even longer analysis time if a second internal standard were included, such as α-tocopheryl acetate used by Bieri et al. (8). Driskell et al. (10) also found that the use of R-A as the only internal standard in their system provided the same degree of precision and accuracy as when both R-A and tocopheryl acetate were used.

Heptane was used instead of the customary hexane for the extraction medium on the basis of Bieri's observation (8) that it contained less interfering material. Our study showed no differences between recoveries with heptane or hexane. We also omitted the use of ethyl ether employed by Bieri et al. (8) to redissolve the extracted vitamins for injection into the HPLC system, since our method produced acceptable recoveries without it.

Other investigators (1,5,8,10) have reported the interference of β-tocopherol (β-T) with γ-T in HPLC separations. Pure standards of β-T were not available at the time this investigation was carried out; therefore, we cannot say if it coelutes with γ-T in our system. However, in view of the low natural occurrence of β-T in foods, and the fact that many of the values reported for β-T in foods are compromised by the mutual interference of γ-T and β-T (14), it is unlikely that β-T is present in detectable amounts in the samples we analyzed.

The use of antioxidants during the extraction of plasma samples is usually limited to saponification procedures. Tangney et al. (7) used 3% PG in the ethanol phase and 0.025% BHT in hexane when extracting tocopherols from rabbit and human plasma and obtained recoveries ranging from 86 to 99%. In the current investigation, when human plasma was extracted without antioxidants (unsaponified), recoveries ranged from 84 to 98%. However, extraction of tocopherols from rat plasma without antioxidant reduced the recovery to only 34% of added α-T, and the recovery of R was inflated by unidentified interference.

The addition of ascorbic acid at 10% or 13% in ethanol, when used as the only antioxidant or in combination with BHT in the heptane phase, improved, but did not provide acceptable recoveries (α-T $< 66\%$). The use of BHT in heptane alone or in combination with PG or BHT in ethanol gave maximum recoveries. The method selected for use in this laboratory was the addition of BHT to both ethanol and heptane phases, providing for optimum recoveries ($>95\%$) in both rat and human plasma.

The applicability of this method was demonstrated in plasma samples obtained from rats, guinea pigs, monkeys and humans (Table 3). Human plasma values agreed with others reported in the literature as shown in Table 4. The only exception was the higher amount of γ-T found in this study, which may be a reflection of increased consumption by the participants of vegetable oils and nuts which contain significant amounts of γ-T. Since β-T and γ-T coelute, there also may be some contribution of β-T to the γ-T values, particularly if the consumption of whole wheat products, which contain β-T, is very high; however, in systems where β-T is completely separated from γ-T (6,11), levels of only 0-0.02 mg/dl were found in plasma.

The method described in this report provides a simple, sensitive quantitative system for R, α-T and γ-T analysis which is applicable to different animal species' and human plasma without modification. Recoveries of $>90\%$ are obtained from as little as 0.1 ml plasma. Although the total run time for our analysis is somewhat longer than previous reports for the simultaneous determination of retinol and tocopherol, our procedure has the advantage of also determining vitamin D_2 or D_3 in the same run. Improved recoveries of added tocopherols were obtained by varying the amount and kind of antioxidant used.

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Reference to a brand or firm name does not imply endorsement by the U.S. Department of Agriculture over others that may be suitable.

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TABLE 3

Retinol and Tocopherol Levels in Rat, Guinea Pig, Monkey and Human Plasma

Species	N[a]	Retinol (μg/ml)	Tocopherols α (μg/ml)	Tocopherols γ (μg/ml)	Tocopherols Total (μg/ml)
Rat	6	0.43 ± 0.11	3.43 ± 1.32	ND[b]	3.43
Guinea Pig	7	0.25 ± 0.03	0.57 ± 0.23	ND[b]	0.57
Monkey	5	0.38 ± 0.04	8.09 ± 1.24	0.97 ± 0.20	9.06
Human	6	0.68 ± 0.13	9.22 ± 1.63	3.00 ± 1.22	11.62 ± 0.29

Values expressed are means ± standard deviation, duplicate analyses of each individual.
[a]Number of individuals.
[b]None detected.

TABLE 4

Comparison of Literature Values for Human Serum or Plasma Retinol and Tocopherol Content

Range of values[a]								
		Tocopherols (mg/dl)						
Retinol (μg/dl)	N	α	β	γ	δ	Total	N	Reference
NA	NA	0.66-1.50	0-0.02	0.07-0.27	0-0.03	NR	12	11
NA	NA	0.59-0.99	0-0.01	0.06-0.08	ND	0.66-1.07	3	6
25-85	14	0.42-1.68	NR	NR	NA	NR	14	8
21-47	133	NA	NA	NA	NA	0.50-1.60	329	12
31-88	6	NA	NA	NA	NA	NA	6	13
27-100	25	0.55-1.98	NA	NA	NA	0.55-1.98	25	9
54-89	6	0.67-1.08	NA	0.15-0.44	NA	0.67-1.44	6	This report

[a]N = number of individuals, NR = not reported, ND = not detected, NA = not analyzed.

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COMMUNICATIONS

Lipolysis of Corn, Peanut and Randomized Peanut Oils

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ABSTRACT

Corn oil, peanut oil and randomized peanut oil exhibit different atherogenic potentials; peanut oil being more atherogenic than the other oils. This study was conducted to ascertain if the atherogenicity of these oils was related to their rates of lipolysis. Using both pancreatic lipase and milk lipoprotein lipase (LPL), it was shown that the rated of lipolysis were corn oil > peanut oil > randomized peanut oil. The rates of lipolysis are not related to atherogenicity and may be affected by the distribution of long-chain saturated fatty acids in the component triglycerides.
Lipids 18:842-844, 1983.

INTRODUCTION

Peanut oil is unexpectedly atherogenic for rats (1), rabbits (2,3) and monkeys (4,5). Peanut oil differs from most other vegetable oils in that it can contain up to 6% of long-chain saturated fatty acids: arachidic, behenic and, in some cases, lignoceric. These fatty acids are always present in the *sn*-3 position of peanut oil triglycerides (6). Randomization of peanut oil provides a fat whose fatty acid composition is identical to the original oil but whose triglyceride structure is different. Randomized peanut oil, which has all of its component fatty acids distributed evenly throughout the triglyceride, is markedly less atherogenic than peanut oil in rabbits (7) and monkeys (5). On the possibility that the differences in atherogenicity may be reflected in rates of lipolysis, we have examined rates of lipolysis of peanut, randomized peanut and corn oils by pancreatic and milk lipase.

EXPERIMENTAL PROCEDURES

Peanut oil and randomized peanut oil were generously provided by Standard Brands, Inc., Wilton, CT. The corn oil was a gift of Best Foods, CPC International, Union, NJ. A base-catalyzed randomization of the peanut oil was carried out. The oil was mixed with 0.3% (w/w) sodium methoxide and heated to 60-80 C under nitrogen for 30 min. The catalyst was neutralized with HOAc. The oil was washed with water until neutral and dried by heating to 120 C under vacuum. Randomization was performed by the Procter and Gamble Co., Cincinnati, OH. The fatty acid composition (%) of the peanut and randomized peanut oils was: 16:0 : 7.0 and 7.3; 18:0 : 1.1 and 1.1; 18:1 : 62.3 and 63.3; 18:2 : 24.8 and 23.6; 18:3 : 0.9 and 0.9; 20:0 : 1.2 and 1.2; and 22:0 : 3.0 and 3.0, respectively. All the fatty acids of randomized peanut oil were present in the 2-position to 31-34% of their total concentration. The corn oil contained 8.5% of 16:0; 0.8% of 18:0; 22.2% of 18:1; 68.5% of 18:2 and a trace of 18:3. In nature, the principal fatty acids found in the 2 position of either peanut or corn oil are 16:0, 18:1 and 18:2.

The oils were hydrolyzed by pancreatic lipase from pancreatin (ICN Nutritional Biochemicals) using the methods of Mattson (8,9). The digestion mixture of 50 mg oil in 2.5 ml Tris buffer (pH 8.0), 0.2 ml of 22% $CaCl_2$ and 0.3 ml of 0.1% sodium cholate was incubated at 37 C for 30 min before adding 12 mg of pancreatin suspended in 1 ml of buffer. Incubation was carried out at 37 C in a Dubnoff shaker. Lipolysis was stopped by acidification with 6 N HCl. The reaction mixture was extracted with diethyl ether and the extract washed with water, dried over anhydrous Na_2SO_4 and taken to dryness under N_2. The lipid extract was separated by thin layer chromatography (TLC) using hexane/ether/acetic acid (70:30:1) and the free fatty acids quantitated by charring (10).

Milk lipoprotein lipase (LPL) was prepared according to the method of Bier and Havel (11) from fresh milk generously donated by Abbott Dairies, Philadelphia, PA. Lipolytic activity was determined using a modification of the method of Boberg and Carlson (12). An emulsion of a specific oil was prepared by mixing 100 mg

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oil, 50 mg Trition X-100, 20 mg a albumin and 10 ml ammonia buffer (pH 8.6). The mixture was placed in an ice bath and sonicated at mixmum setting (Bronson Sonicator) for 5 min. One ml of the oil emulsion (10 mg of oil), 7 ml of ammonia buffer (pH 8.6) and 1 ml of rabbit serum were incubated for 30 min at 37 C before adding 1 ml of enzyme solution. The reaction was allowed to proceed at 37 C in a Dubnoff shaker and was terminated by addition of 2.5 ml of isopropyl alcohol/4 N H_2SO_4 (40:1). Then 2 ml of water was added and the lipids extracted with hexane. Fatty acids were isolated and quantitated as described above.

In both studies, samples were taken for analysis at 1, 3, 5, 15, 30 and 60 min. The 1-hr samples were subjected to TLC on Silica Gel G using hexane/ether/acetic acid (50:50:2) to separate 2-monoglycerides. Fats were subjected to transesterification (13) and their fatty acid spectra determined by gas liquid chromatography using a column containing 15% ethylene glycol succinate on 100-120 mesh Gas Chrom P.

RESULTS AND DISCUSSION

The course of hydrolysis by pancreatic lipase and milk LPL is plotted in Figures 1 and 2, respectively. With pancreatic lipase, hydrolysis of corn and peanut oils proceeded in a parallel fashion, but randomized peanut oil was hydrolyzed more slowly. At 60 min, corn oil was 59.2% hydrolyzed; peanut oil, 51.3%; and randomized peanut oil, 43.3%. The gum arabic method emulsification used by Jensen et al. (14) was used in a parallel study and at 30 min, percentage of hydrolysis of corn, peanut and randomized peanut oils was 62.7, 57.9 and 40.3, respectively.

The fatty acid composition of the monoglycerides isolated from the three oils after 60 min is given in Table 1. Milk LPL hydrolysis of corn oil was more complete than that of either of the peanut oils, with randomized peanut oil being hydrolyzed least. After 60 min, percentage of hydrolysis was: corn oil, 71.1; peanut oil, 58.7; and randomized peanut oil, 50.1%. Table 2 shows the fatty acid com-

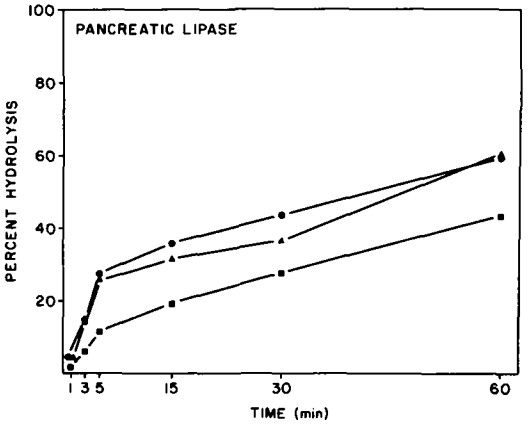

FIG. 1. Rate of hydrolysis of corn oil (●), peanut oil (▲) and randomized oil (■) by pancreatic lipase.

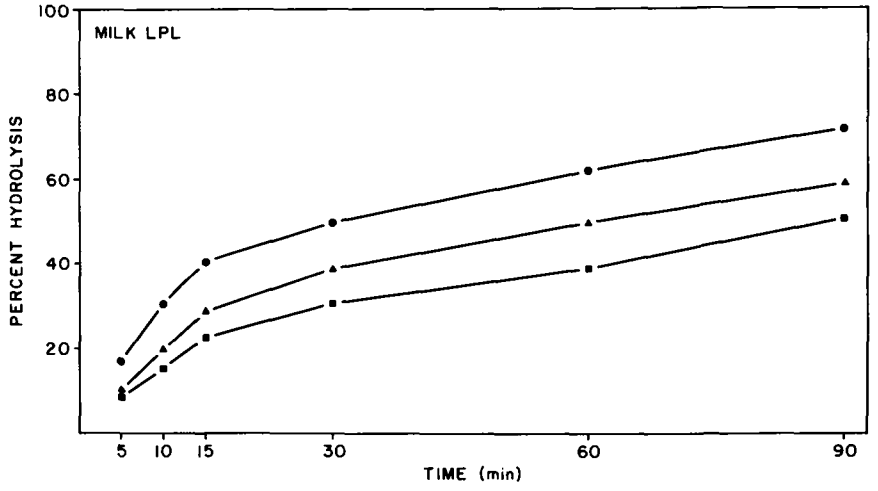

FIG. 2. Rate of hydrolysis of corn oil (●), peanut oil (▲) and randomized peanut oil (■) by milk LPL.

TABLE 1

Fatty Acid Composition (%) of Monoglyceride Obtained after 60-min Exposure of Fat to Pancreatic Lipase

Fatty acid	Peanut oil	Randomized peanut oil	Corn oil
16:0	1.3	11.6	1.4
18:0	–	1.6	trace
18:1	61.8	60.1	18.2
18:2	36.3	19.4	80.2
18:3	–	0.8	–
20:0	–	1.4	–
22:0	–	3.6	–

TABLE 2

Fatty Acid Composition (%) of Monoglyceride Obtained after 60 min of Hydrolysis by Milk LPL

Fatty acid	Peanut oil	Randomized peanut oil	Corn oil
16:0	7.9	27.9	6.4
18:0	0.6	3.7	0.5
18:1	56.3	47.3	23.1
18:2	33.2	13.6	68.2
18:3	–	0.4	–
20:0	–	1.7	–
22:0	–	3.3	–

position of the triglycerides isolated after 60 min.

The monoglycerides obtained from peanut oil after hydrolysis by pancreati lipase contain only palmitic, oleic and linoleic acids. Their weight percentages (9) are 6.2, 33.1 and 48.8, respectively. The same 3 fatty acids are present in the monoglycerides obtained from corn oil and their weight percentages are: palmitic, 5.5; oleic, 27.3; and linoleic, 39.0. The monoglycerides obtained from randomized peanut oil reflect the randomized nature of the fat. Weight percentages of specific fatty acids are: palmitic, 53.0; oleic, 31.6; linoleic, 27.4; arachidic, 38.9; and behenic, 40.0 Patterns of monoglyceride composition after milk lipase hydrolysis for all 3 oils differ from those seen with pancreatic lipase. Palmitic acid content of all 3 sets of monoglycerides is increased. Weight percentage of palmitic acid is 37.6 for peanut oil, 12.7 for randomized peanut oil and 25.1 for corn oil. The substrate specificity of the 2 types of lipase appears to be different but the relative rates of hydrolysis of the three fats are similar.

The results obtained with randomized peanut oil may be due to steric interference with enzyme action caused by the presence of long-chain fatty acids in all 3 positions of the triglyceride. The rate of hydrolysis does not reflect the atherogenic potential of the 3 fats tested. When we compared the atherogenicity of American, African and South American peanut oils (15), their relative atherogenic effects were: American, 1.00; African, 1.10; and South American, 1.18. The ratios of oleic acid to linoleic acid in the 3 fats were: American, 1.65; African, 2.70; and South American, 0.89. The ratios of oleic to linoleic acid in the monoglycerides obtained by pancreatic and milk lipase, respectively, were: peanut oil, 1.70 and 1.70; randomized peanut oil, 3.10 and 3.48; and corn oil, 0.23 and 0.34. Whether the oleic to linoleic acid ratio of peanut oil affects its atherogenicity is under investigation.

ACKNOWLEDGMENTS

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A Comparison of the Positional Distribution of Fatty Acids in Milk Triglycerides of the Extant Monotremes Platypus (*Ornithorhynchus anatinus*) and Echidna (*Tachyglossus aculeatus*)

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ABSTRACT

Milk triglycerides from the platypus were subjected to fatty acid and stereospecific analysis to determine the positional distribution of fatty acids in the triglycerides. Of the major fatty acids, 12:0 was preferentially esterified at the *sn*-3 position, 14:0 and 16:0 were selectively associated with the *sn*-2 position, and 18:0 was located predominantly at the *sn*-1 position. The unsaturated fatty acids, 14:1, 16:1, 18:1, 18:2 and 18:3, were preferentially esterified at the *sn*-3 position. The fatty acid distribution pattern of the platypus, a monotreme, is similar to that of marsupials and eutherians but is in contrast to the only other extant monotreme, the echidna.

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The positional distribution of fatty acids in milk triglycerides from several species of mammals have been reported (1-6). Although fatty acid content at the 3 stereospecific positions may vary from species to species, the distribution patterns are generally similar. Short-chain fatty acids are almost exclusively esterified at the *sn*-3 position; 8:0, 10:0 and 12:0 acids are preferentially esterified at either the *sn*-2 or *sn*-3 position; 14:0 is always located predominantly at the *sn*-2 position and 18:0 is always located predominantly at the *sn*-1 position. The 16:0 acid is selectively associated with either the *sn*-1 or *sn*-2 position and 18:1 is selectively esterified at either the *sn*-1 or *sn*-3 position. Unsaturated acids 18:2 and 18:3 are usually preferentially esterified at the *sn*-3 position. In many cases, there is little difference in fatty acid content at the 2 positions competing for preferential esterification.

An exception to this distribution pattern was noted for the echidna (6), where the triglycerides were found to be symmetrical with the unsaturated fatty acids, 18:1, 18:2 and 18:3, being preferentially esterified at the *sn*-2 position. The echidna and the platypus are the last surviving monotremes and the most primitive of mammals. This paper examines the positional distribution of fatty acids in platypus milk triglycerides to determine if it is the same as that for the echidna.

MATERIALS AND METHODS

Sample

Peak lactation milk was obtained from a platypus taken in the wild from the upper Shoalhaven River in New South Wales. The diet of platypuses in this area consists mainly of insect larvae, notably Trichoptera, Diptera, Coleoptera, Ephemeroptera and Odonata. Subsidary food items include freshwater shrimp (*Paratya australiensis*) and bivalve molluscs (*Sphaerium* sp.) (7).

Lipid was extracted from the milk using chloroform and methanol and stored under nitrogen at -20 C until required for analysis. Triglycerides were obtained from milk lipid by column chromatography using 7% hydrated Florisil (8).

Stereospecific Analysis

The *sn*-1,2(2,3)-diglyceride method of Brockerhoff (9), adapted for mg quantities by Christie and Moore (10), was used with modification. This method, together with the pancreatic lipase deacylation procedure used to obtain monoglycerides, was reported by Parodi (11). In the current study, diglycerides for stereospecific analysis were generated by the Grignard reagent, ethyl magnesium bromide. Results for the *sn*-1 position were obtained by analysis of the lysophosphatide, those for the *sn*-2 position were obtained from monoglycerides by pancreatic lipase deacylation and those for the *sn*-3 position were calculated by difference from the known triglyceride composition. The composition of the *sn*-2,3-diacyl-1-phosphatidylphenols provided a check for the *sn*-3 position.

Fatty Acid Analysis

Triglycerides and partial glycerides were transesterified to methyl esters (12). Phospholipids generated during stereospecific analysis

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were transesterified by the addition of 5 μL of 2.0 N methanolic sodium methoxide and 50 μL of hexane. Methyl esters were analyzed by gas liquid chromatography (11).

RESULTS AND DISCUSSION

The positional distribution of fatty acids in the triglycerides of platypus milk is given in Table 1. Of the major fatty acids, 12:0 was preferentially esterified at the *sn*-3 position, 14:0 and 16:0 were selectively associated with the *sn*-2 position, and 18:0 was located predominantly at the *sn*-1 position. The unsaturated fatty acids 14:1, 16:1, 18:1, 18:2 and 18:3 were preferentially esterified at the *sn*-3 position.

Also included in Table 1 is the positional distribution of fatty acids in the triglycerides of echidna milk reported by Parodi (6). For this species, it is seen that the triglycerides are largely symmetrical with the major saturated acids, 16:0 and 18:0, almost equally distributed between the *sn*-1 and *sn*-3 position, whereas the unsaturated acids, 18:1, 18:2 and 18:3, are preferentially esterified at the *sn*-2 position. Grigor (13) has also shown, using pancreatic lipase deacylation, that echidna milk triglycerides contain C18 unsaturated fatty acids preferentially esterified at the *sn*-2 position, which was not the case for 5 species of marsupials.

The fatty acid distribution pattern in platypus milk triglycerides follows the general pattern for marsupials and eutherians outlined in the introduction. This is in contrast to the echidna where the C18 unsaturated fatty acids are preferentially esterified at the *sn*-2 position. This type of distribution has not been encountered for any other species of mammal. It is more akin to the fatty acid distribution pattern of common vegetable oils (14). Prior to the stereospecific analysis of platypus milk triglycerides reported herein, it was considered that this type of fatty acid distribution pattern may have been characteristic of the milk of monotremes which are the most primitive of mammals.

With the exception of the cow, reported fatty acid distribution patterns for milk triglycerides have been for single samples. It is known that factors such as diet and stage of lactation influence milk fatty acid composition. For the platypus, changes in fatty acid composition may be small (15). Parodi (16,17) studied the fatty acid positional distribution pattern of milk from a cow at different stages of lactation and from a number of cows of different breed from various herds. This pro-

TABLE 1

Positional Distribution of Fatty Acids in Triglycerides from Milk of the Extant Monotremes, Platypus and Echidna

Species	Position	Fatty acid composition (mol %)												
		10:0	12:0	14:0	14:1	15:0	15:1	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3	20:0
Platypus	TG	0.1	3.8	4.6	2.4	1.2	0.8	28.7	14.0	1.7	2.2	5.4	26.4	3.9	4.0	0.8
	sn-1	0.1	2.0	5.5	0.9	0.8	0.3	25.5	12.5	2.3	1.9	11.2	31.1	2.4	1.7	1.8
	sn-2	0.1	4.1	8.0	2.5	1.8	1.1	47.0	13.6	1.7	2.4	3.0	10.7	1.5	2.2	0.3
	sn-3	0.1	5.3	0.2	4.0	0.9	1.0	13.3	15.8	1.2	2.3	2.2	37.4	7.9	8.2	0.2
Echidna[a]	TG	–	–	1.0	0.7	0.4	0.2	22.8	7.4	1.1	0.7	11.1	43.5	9.1	2.0	ND
	sn-1	–	–	1.7	1.3	0.8	0.4	31.5	7.1	1.5	0.7	16.8	33.1	4.1	1.0	ND
	sn-2	–	–	0.9	0.7	0.2	0.1	9.0	7.0	0.4	0.8	2.1	57.6	18.3	2.9	ND
	sn-3	–	–	0.4	0.2	0.1	0.2	27.9	8.0	1.6	0.6	14.3	39.8	4.9	2.0	ND

[a]From Parodi (6).
ND: Not determined.

vided milk samples with a wide range of fatty acid compositions. Over the range examined, highly significant linear relationships were found between content of a fatty acid at all 3 stereospecific positions and the content of the same acid in the intact triglycerides. Thus, the general fatty acid distribution pattern did not change with changing triglyceride fatty acid composition.

Biochemical factors which may influence the positional distribution of fatty acids in milk triglycerides have been discussed (6,16,17).

ACKNOWLEDGMENTS

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Biphasic Action of Platelet-Activating Factor on Isolated Guinea-Pig Ileum

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ABSTRACT

1-0-Hexadecyl-2-0-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor) at 10^{-10}-10^{-9} M induced slow contraction of isolated guinea-pig ileal muscles and the contraction persisted for a long time. At a higher concentration of 10^{-7} M, this phospholipid induced more rapid, but not greater, contraction. At higher concentrations (10^{-6}-10^{-5} M), this phospholipid induced a biphasic response: rapid contraction followed by relaxation. At high concentrations, this compound inhibited acetylcholine-induced contractions. The stimulatory effect of this phospholipid was ca. 300 times that of 1-palmitoyl-2-0-acetyl-*sn*-glycero-3-phosphocholine, while its inhibitory potency on induced contraction was similar to those of 1-palmitoyl-2-0-acetyl-*sn*-glycero-3-phosphocholine and its lyso derivative. It was suggested that the differences in effects on contraction of different concentrations of 1-0-hexadecyl- and 1-palmitoyl-2-0-acetyl-*sn*-glycero-3-phosphocholine were due to the dual effects of these compounds on the ileum: a strong stimuatory effect and a moderate inhibitory effect on contraction.

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Platelet-activating factor (PAF) has recently been identified as 1-0-alkyl-2-0-acetyl-*sn*-glycero-3-phosphocholine (1,2). Subsequent studies showed that PAF caused contraction of isolated muscle from guinea-pig ileum (3,4). On the other hand, lysophosphatidylcholines, which are structurally related to PAF, are known to inhibit the contractions produced by acetylcholine (5-7), histamine (5-7), 5-hydroxytryptamine (6), bradykinin (6) and slow-reacting substance A (6). These opposite effects may be explained by considering that choline phospholipids have both stimulatory and inhibitory effects and that the structural requirements for producing the stimulatory effect are somewhat different from those for the inhibitory one. To investigate this phenomenon, we examined the effects on guinea-pig ileum of a wide range of concentrations of 3 choline phospholipids.

Materials and Methods

Chemicals

1-Palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine (16:0-lyso-PC) was obtained from Sigma Chemical Co. (St. Louis, MO). 1-0-Hexadecyl-2-0-acetyl-*sn*-glycero-3-phosphocholine (16:0-PAF) was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). 1-Palmitoyl-2-0-acetyl-*sn*-glycero-3-phosphocholine (16:0-2:0-PC) was prepared as described previously (8). The phospholipids were dissolved in chloroform/methanol mixture (2:1, v/v) and stored at -20 C until use. The following drugs from the indicated sources were also used: acetylcholine chloride (Daiichi Seiyaku Co.), histamine dihydrochloride (Wako Pure Chemical Industries), and bovine serum albumin (essentially fatty acid-free prepared from fraction V albumin, Sigma).

Measurement of the Response of Ileum Strips

Male guinea-pigs, weighing 300-400 g, were killed by a blow on the head and muscle segments were removed from the ileum 20-30 cm distal to the duodenum. The muscle strips (ca. 2 cm long) were mounted in a 5-ml organ bath containing Tyrode solution maintained at 37 C and bubbled with air. The Tyrode solution had the following composition (in mM): NaCl, 136.8; KCl, 2.7; $NaHCO_3$, 11.9; NaH_2PO_4, 0.4: $MgCl_2$, 1.0; $CaCl_2$, 2.5 and glucose, 5.5 (pH 7.8). Contractions were measured with an isotonic lever with 10-fold magnification and a 1.0 g load. The muscle was incubated for 1-2 hr with repeated washings, until it showed a constant response to acetylcholine, histamine or 40 mM KCl. Choline phospholipids were dissolved with 0.1 ml of Tyrode solution containing 0.1% bovine serum albumin. The final concentration of bovine serum albumin was 0.002%.

RESULTS AND DISCUSSION

Contractile Effect of 16:0-PAF and Its Acyl Analog

The sensitivity of guinea-pig ileum to 16:0-PAF or its acyl analog varied from muscle to

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muscle, but the threshold concentration of 16:0-PAF for an effect was in the range of 10^{-10}-10^{-9} M.

Figure 1 shows typical responses of the muscle to various doses of 16:0-PAF. With a concentration of 10^{-9} M, the muscles contracted very slowly after a lag time, and the contraction did not reach a plateau within 10 min. On increasing the dose to 10^{-8} M, both the rates and levels of contraction increased progressively. With up to 10^{-7} M 16:0-PAF, the rate of contraction induced by 16:0-PAF continued to increase, but the level of contraction remained similar. At higher concentrations (10^{-6}-10^{-5} M), 16:0-PAF evoked a sharp contraction, but this contraction then decreased with time, depending on the doses. The maximal contraction elicited by 16:0-PAF was 30 ± 4% of that produced by acetylcholine. The contractile activity of 16:0-2:0-PC, an acyl analog of 16:0-PAF, was ca. 300 times less than that of 16:0-PAF: its threshold concentration was 10^{-7} – 3 x 10^{-7} M. At 10^{-5} M, 16:0-2:0-PC induced a rapid contraction which was followed by relaxation. The corresponding lyso derivative, 16:0-lyso-PC, had no contractile action at any concentration up to 10^{-5} M. Earlier reports (3,4) have noted that lyso derivatives of PAF do not cause the contraction of guinea-pig ileum. These structure-activity relationships of these choline phospholipids on guinea-pig ileum are consistent with those of their platelet activating potencies (1,9).

Once the ileum had been exposed to 16:0-PAF, it showed no significant response to subsequent addition of the same concentration PAF or a concentration 10 times higher. Even after repeated washing of the ileum, subsequent addition of PAF did not induce contraction, whereas histamine or acetylcholine evoked a normal contraction. These observations are consistent with earlier reports (3,4). Similar desensitization was observed with 10^{-7}-10^{-5} M 16:0-2:0-PC. However, muscle that had been exposed to 7 x 10^{-7} M 16:0-PAF and then washed did not respond to subsequent treatment with 7 x 10^{-7} M 16:0-2:0-PC, whereas the reverse was not the case, as shown in Figure 2. These results suggest that PAF and 16:0-2:0-PC induce contraction by the same mechanism, but that PAF causes stronger stimulation than 16:0-2:0-PC. In recent studies, specific binding sites for PAF have been found on platelets (10) and neutrophils (11). Active choline phospholipids may all induce contraction of the ileum by activation of the same binding sites, and the observed desensitization may occur at these binding sites. If so, 16:0-PAF and its acyl analog may alter the state of these binding sites, resulting in desensitization of the muscle to PAF and its acyl analog. Addition of a high concentration of the potent stimulator, 16:0-PAF, would overcome the desensitization caused by pretreatment with the same concentration of 16:0-2:0-PC.

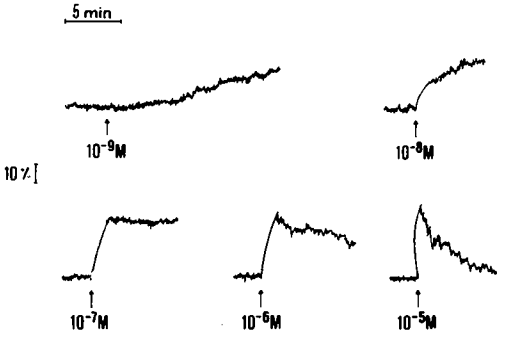

FIG. 1. Typical responses of isolated guinea-pig ileal muscle to various concentrations of 16:0-PAF. Arrows indicate times of addition of 16:0-PAF. The scale on the left represents 10% of the maximal contraction produced by acetylcholine (10^{-4} M).

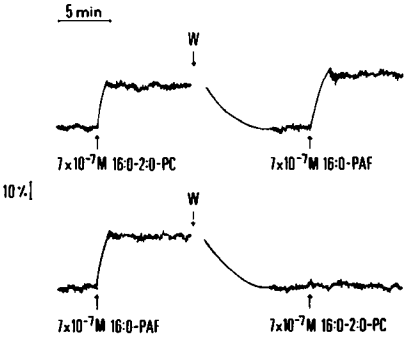

FIG. 2. Desensitization with 16:0-PAF and its acyl analog. Arrows indicate times of additions of 16:0-PAF and its acyl analog or times of washing (W). The scale on the left represents 10% of the maximal contraction produced by acetylcholine (10^{-4} M).

Inhibitory Actions of 16:0-PAF, 16:0-2:0-PC and 16:0-Lyso-PC

The ileum was first stimulated with 2 x 10^{-6} M acetylcholine, which induces ca. 80% of the maximal contraction. When the contractions reached a steady state, various concentrations of 16:0-PAF, 16:0-2:0-PC or 16:0-lyso-PC were added. This resulted in relaxation to different extents depending on the concentrations of these phospholipids added. Because the inhibi-

tory actions of these choline phospholipids were not tachyphylactic, the dose-response curve for each phospholipid could be constructed with a single strip of ileum. Figure 3 shows the log dose-response relationships for these 3 phospholipids. There are no significant differences in their IC_{50} values, unlike the contractile activities of these compounds.

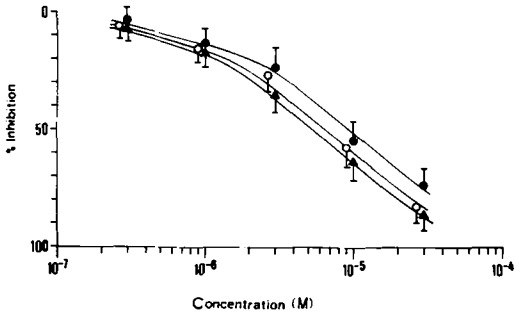

FIG. 3. Log dose-inhibition curves for 16:0-PAF (▲), 16:0-2:0-PC(○) and 16:0-lyso-PC (●). Points and bars indicate means and standard errors (n=7).

Thus, the *sn*-2-acetyl group and 1-0-alkyl bond are not structural requirements for expression of the inhibitory action. Experiments with 10^{-6} M histamine as a stimulant gave similar results to those with acetylcholine (data not shown), suggesting that the inhibitory actions of these choline phospholipids were rather nonspecific for the kind of stimulant. Much higher concentrations of PAF were needed for eliciting the inhibitory effect than for the contractile effect.

Very probably the inhibitory effects of lysophospholipids are related to their surface-active properties, judging from the similar wedge-shaped structures of 16:0-PAF, 16:0-2:0-PC and 16:0-lyso-PC. When higher concentrations of 16:0-PAF, 16:0-2:0-PC and 16:0-lyso-PC were added to muscles contracted by pre-addition of a low concentration of 16:0-PAF or 16:0-2:0-PC, the contraction decreased to the basal tone, and when even higher concentrations of these 3 phospholipids were introduced into the bath, the ileum relaxed from the basal tone. Therefore, the biphasic response of the ileum to 10^{-6}-10^{-5} M PAF is due to inhibition of PAF-induced contraction by its own relaxing effect at higher concentration. 16:0-2:0-PC had similar effects to PAF in a similar concentration range, but 16:0-lyso-PC showed only an inhibitory action at concentrations of up to 10^{-5} M.

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Double-Bond Location in Long-Chain Polyunsaturated Fatty Acids by Chemical Ionization-Mass Spectrometry[1]

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ABSTRACT

For determination of the double-bond position in polyunsaturated C_{24-30} fatty acids from marine organisms, methoxy derivatives were prepared. Diagnostic mass spectral fragment as well as molecular ion intensities were obtained by adjusting the ion source optics in the presence of ammonia at a lower source pressure than used conventionally. A lower detection limit was observed compared to conventional methane chemical ionization, which is a more favorable condition for capillary gas chromatography. Analysis of fatty acids from the sponge *Calyx niceaensis* showed the double-bond position of 8 unsaturated fatty acids, including two new ones. In addition, structural proof is provided for the presence of a new cyclopropane-containing fatty acid: 19,20-methylene-hexacosanoic acid.
Lipids 18:853-858, 1983.

In recent years we have reported the occurrence of unusual phospholipid acids (1-4) which extend the original observations of of Litchfield et al. (5), about a new class of compounds, the "demospongic" acids, from marine organisms. This has encouraged us to screen a large number of organisms, which in turn led us to focus on certain methodological advances applicable to unsaturated fatty acids.

Capillary gas chromatography combined with mass spectrometry has proven to be very useful for the analysis of complex mixtures of fatty acids, especially in the form of their methyl ester and pyrrolidide derivatives (6,7). Fatty acid methyl esters are especially convenient in terms of their chromatographic properties. However, for structure elucidation they are of limited use since only a few diagnostic MS fragment ions can be observed, primarily due to double-bond migration (8). A "post-column" reaction using vinylmethyl ether as a chemical ionization reagent gas has been reported (9), but in our hands (vide infra) this procedure yields diagnostic fragments only for monounsaturated compounds. For the determination of the double-bond position, pyrrolidide derivatives are suitable because of their ease of preparation and the formation of diagnostic fragment ions (7). However, their mass spectra do not show the double-bond position in all cases, and chromatographic peaks often overlap with adjacent peaks of compounds with the same carbon chain length. Therefore, at times, various derivatization methods have also been utilized (10). Among these, permethoxy derivatives of polyunsaturated fatty acid methyl esters, up to C_{22}, were shown to give diagnostic ions for the double-bond position as well as molecular ions using methane and isobutane CI (11). However, no practical data were given concerning possible decreases in sensitivity and the application of this technique to the analysis of natural samples.

After examining various approaches of double-bond location methods, we focused on the polymethoxy derivatization (11). To synthesize such polymethoxy derivatives of C_{24-30} fatty acids, we found it necessary to modify the reported reaction procedure. Furthermore, under our experimental conditions, we found ammonia a "clean" and preferable reagent gas. In this study, we wish to report a combined chemical derivatization and GC-MS approach which is particularly suitable for the analysis of various fatty acids encountered in marine organisms.

EXPERIMENTAL PROCEDURES

Reagents

Oleic (9-octadecenoic acid; C_{18}:1), linoleic (9,12-octadecadienoic; C_{18}:2), 11,14-eicosadienoic, nervonic (15-tetracosadienoic; C_{24}:1) acids and osmium tetroxide were obtained from Sigma Chemical Co., St. Louis, MO.

[1]"Mass Spectrometry in Structural and Stereochemical Problems 262." For preceding paper in this series, see Patterson, D.G., Haley, M.J., Midgley, J., and Djerassi, C., Org. Mass. Spectrom., submitted for publication.

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Abbreviations: GC-MS, gas chromatography-mass spectrometry; CI-MS, chemical ionization-mass spectrometry; VME, vinylmethyl ether; HPLC, high performance liquid chromatography.

Dimethylsulfoxide and methyl iodide were purchased from Aldrich Chemical Co., Milwaukee, WI, and potassium hydride from Alfa Products Corp., Danvers, MA.

C_{24-30} Fatty Acid Standards

5,9-Hexacosadienoic acid ($\Delta^{5,9}$-26:2) was isolated from *Petrosia ficiformis* (2); 2-methoxy-21-octacosenoic acid (2-OMe-Δ^{21}-28:1) and 5,9,23-tricontatrienoic acid ($\Delta^{5,9,23}$-30:3) were isolated from *Higginsia tethyoides* (3). Purification was accomplished by preparative HPLC, using a methanol/water mixture (97:3, v/v) as the eluent and an ODS reversed-phase column (Waters Assoc., Milford, MA).

Synthetic Procedures

Fatty acids were first converted to the hydroxy derivatives using osmium tetroxide (12). Synthesis of polymethoxy derivatives was accomplished according to Suzuki et al. (11) with the difference that solutions were heated to 60 C due to insolubility of the polyhydroxy derivatives of long-chain fatty acids at ambient temperature. After transfer of the solutions containing the hydroxy fatty acids, vials were rinsed with warm methanol. In this way, fatty acid mixtures of ca. 0.5 mg could be handled easily.

Sample Pretreatment

Marine organisms were collected, lyophilized and extracted with cold chloroform/methanol (1:1, v/v). Total phospholipids were separated using silica column chromatography, as described earlier (1-4). The fatty acid methyl esters were prepared using 1.25 N HCl in dry methanol at 60 C. N-Acyl-pyrrolidides were prepared from the methyl esters on treatment with pyrrolidine/acetic acid (10:1, v/v) at 100 C for 1 hr (1).

Instrumentation

A Ribermag GC-MS system was used combining a Ribermag R10-10 quadrupole mass spectrometer with a Carlo Erba on Column injector series 4160 Fractovap gas chromatograph, containing a fused silica column (30 m × 0.32 mm) containing SE 54 (J & W Scientific, Inc.,). The MS ion source was suitable both for electron impact and for chemical ionization MS. For the experiments VME, a bottle of 6.3% VME in nitrogen (Matheson) was connected with a T piece to a vial containing carbon disulfide. With a needle valve, the amount of CS_2 was adjusted so that a base peak at m/z 58 was obtained (VME^+). The source pressure for maximum m/z 58 peak height was 0.3 torr. For conventional ammonia CI the source pressure was 0.5 torr and for "low pressure" conditions as described in this paper 0.2 torr. Pressures were measured on line with an ion gauge (outer diameter 1/8 in.) connecting the standardization compound vial (±15 cm from the source). All spectra were obtained at 70 eV ionizing electron energy and a source temperature of 195 C, unless otherwise specified.

RESULTS AND DISCUSSION

Chemical ionization using VME as the reagent gas, for oleic acid and nervonic acid yielded diagnostic mass spectral fragments (9). However, we abandoned the method because in polyunsaturated acids no specific fragments for double-bond location could be obtained and the addition of CS_2 to a N_2/VME mixture (9) produced decomposition products in the source and gas transfer lines, thus contaminating the instrument. Moreover, many background peaks were observed up to m/z 150, suppressing the lower diagnostic fragments in this mass range. Alkylthio derivatization also showed abundant diagnostic fragments for oleic acid methyl ester (13), but polyunsaturated derivatives gave too long retention times for practical GC analysis.

Trifluoroacetates, obtained from polyhydroxy fatty acid methyl esters are reported to be very convenient for GC analysis (14). However, the mass spectrum of a typical example of 9,10,12,13-tetra-(trifluoroacetyl)-octadecanoic acid methyl ester showed mainly fragments due to loss of trifluoracetyl groups and no specific cleavage of the carbon chain from which the position of the substituents could be deduced (both under IE and CI [NH_3] conditions). We did not prefer TMS derivatives because of the contamination of the mass spectrometer source. Moreover, these compounds are not stable enough for further derivatization. For example, pyrrolidides of the TMS derivatives for the identification of saturated branched or cyclic fatty acids present in a mixture cannot be prepared.

Polymethoxy Derivatives

Standard C_{24-30} fatty acid methyl esters. EI spectra were found to produce abundant diagnostic ions for the determination of the double-bond position in polyunsaturated fatty acid methoxy derivatives, but lacking molecular ions (Fig. 1a). Isobutane and methane CI spectra were reported (11) to show all these fragments, whereas ammonia supposedly displayed mainly molecular ions. However, after working with methane and isobutane CI we

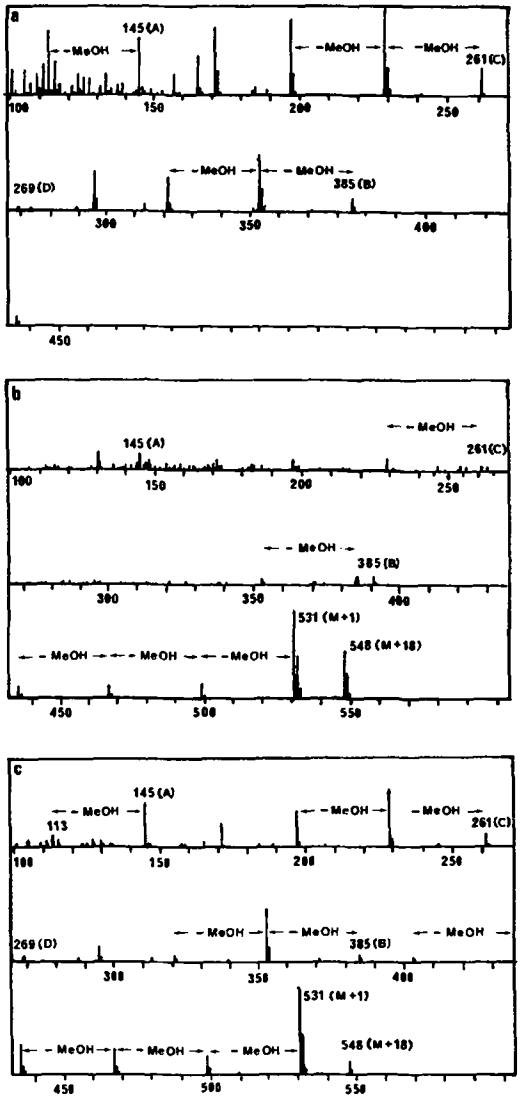


FIG. 1. Mass spectral plots for polymethoxy derivative of 5,9-hexacosadienoic acid methyl ester: (a) electron impact; (b) "normal pressure" NH_3 CI (0.5 torr); (c) "low pressure" NH_3 CI with adjusted ion source optics (see text).

found that the MS sensitivity for EI decreased, but this was never observed with ammonia. Therefore we concluded that ammonia was a cleaner gas for the system. We inserted 5,9-hexacosadienoic acid as its polymethoxy derivative with the probe and adjusted the main diagnostic fragments to their maximum intensities with the ion source optics at a lower pressure of ammonia than conventionally used (0.2 vs 0.5 torr). A comparison of Fig. 1b and 1c shows that both important diagnostic ions as well as molecular ion masses could be obtained under these conditions (conditions A) rather than under normally used pressure. For the polymethoxy derivative of 5,9-hexacosadienoic acid, the absolute intensities of the ions, most indicative for the double-bond position, were twice as high as for methane (11) under "normal pressure" (conditions B). The GC background signal as the most important contribution was 4 times as high for conditions B when compared to A under similar GC-MS conditions (mass range m/z 100-800). This makes conditions A almost an order of magnitude more favorable than conditions B, which is most welcome for low concentration capillary column chromatography. For these conditions, the mass spectra of the polymethoxy derivatives of two other novel long-chain fatty acid methyl esters (2-methoxy-21-octacosenoic and 5,9,23,triacontatrienoic) are presented in Figure 2. A characteristic loss of methanol from the molecular ion and from the fragments formed by the cleavage between the methoxy groups is also noted. Our previous

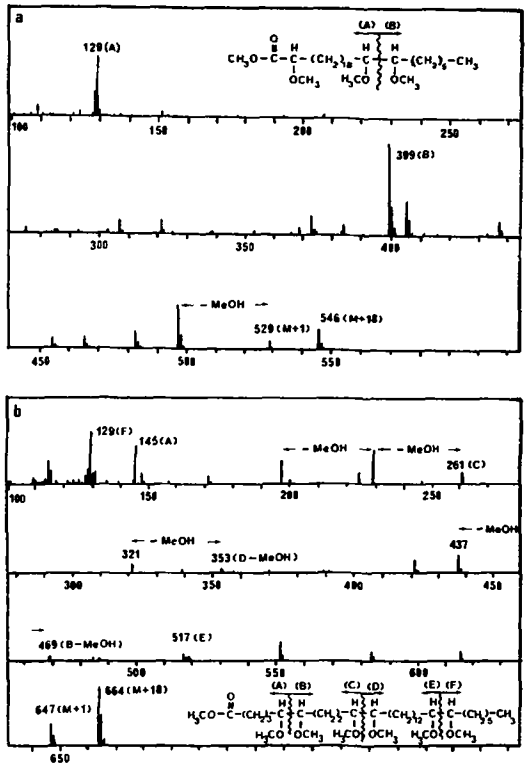


FIG. 2. "Low pressure" NH_3 CI spectral plots of polymethoxy derivatives of (a) 2-methoxy-21-octacosenoic acid methyl ester, and (b) 5,9,23-tricontatrienoic acid methyl ester.

experiments showed (3) that the pyrrolidide derivative of 2-methoxy-21-octacosenoic acid (Fig. 2a) provided a molecular ion, but did not give a clear-cut spectrum suitable for double-bond location. In the case of 5,9,23-triacontatrienoic acid (Fig. 2b), ions A,C,E and F were observed at m/z 145, 261, 517 and 129, respectively. Intense ions due to B and D were not observed, but B-MeOH and D-MeOH were present at m/z 469 and 353, respectively. Subsequent loss of methanol from these ions were exhibited at m/z 437 and 321.

Polymethoxy fatty acid methyl esters from a natural mixture. Using fatty acid methyl esters and pyrrolidides together with electron impact MS-capillary GC, the fatty acid composition of *Calyx niceaensis*, as represented in Table 1, as obtained, including 5 unsaturated fatty acids.

The organism contains a considerable amount of iso and anteiso acids. The presence of this class of phospholipid acids and their possible biosynthetic relations were discussed in an earlier communication (2). The iso series was distinguished from the anteiso series based on equivalent chain length (ECL) values of the methyl esters as well as mass spectral properties of pyrrolidide derivatives (2,7). For synthesis of the polymethoxy derivatives, 1 mg of the fatty acid mixture was treated overnight with 15 mg of osmium tetroxide at 60 C followed by the addition of sodium sulfite and methanol. The methanol was evaporated, the residue was taken up in warm methanol, salt crystals were spun off rapidly and the supernatant was evaporated to dryness in a screw-capped vial. During decanting, all vials were rinsed carefully with warm methanol. Under a stream of dry nitrogen, methylsulfinyl carbanion was added for the permethoxylation. The solvents used throughout the procedure were used in the same relative amounts as reported by Suzuki et al. (11).

GC-MS of the mixture revealed the presence of 6 monounsaturated and 3 diunsaturated fatty acids (Tables 2 and 3), several of which were not detected under the conditions outlined in Table 1. The advantage of the present method is that these polymethoxy derivatives showed a better chromatographic separation from adjacent peaks or compounds with the same carbon chain length compared to their underivatized methyl esters or pyrrolidides—an advantage that is of great importance in detecting these constituents. Among the monounsaturated compounds (Table 2), 4-docosenoic acid (Δ^4-22:1) was recently encountered as a minor constituent of *Mycobacterium tuberculosis* (15), whereas 4-heneicosenoic (Δ^4-21:1) and 4-tricosenoic (Δ^4-23:1) are new compounds. They may also be of bacterial origin or result from homologation by the sponge of a bacterial precursor acid with a Δ^4-double bond.

The presently described technique also allowed us to identify a new nonolefinic acid

TABLE 1

Total Fatty Acid Composition of the Sponge *Calyx niceaensis*[a,b]

Compound	ECL[c]	Fatty acid	Abundance (%)
1	14.62	13-Methyltetradecanoic (iso-15:0)	7.2
2	14.70	12-Methyltetradecanoic (anteiso-15:0)	6.9
3	15.61	14-Methylpentadecanoic (iso-16:0)	3.3
4	15.70	13-Methylpentadecanoic (anteiso-16:0)	1.8
5	16.00	Hexadecanoic (n-16:0)	4.0
6	16.69	15-Methylhexadecanoic (anteiso-17:0)	4.0
7	16.77	9-Heptadecenoic (Δ^9-17:1)	10.9
8	17.00	Heptadecanoic (n-17:0)	4.3
9	17.69	16-Methylheptadecanoic (anteiso-18:0)	2.2
10	17.75	11-Octadecenoic (Δ^{11}-18:1)	3.2
11	18.00	Octadecanoic (n-18:0)	1.1
12	18.62	18-Methyloctadecanoic (iso-19:0)	10.4
13	25.43	5,9-Hexacosadienoic ($\Delta^{5,9}$-26:2)	6.1
14	26.16	25-Methyl-5,9-hexacosadienoic ($\Delta^{5,9}$-iso-27:2)	9.9
15	26.37	24-Methyl-5,9-hexacosadienoic ($\Delta^{5,9}$-anteiso-27:0)	20.1
16	26.71	19,20-Methylene-hexacosanoic	0.6

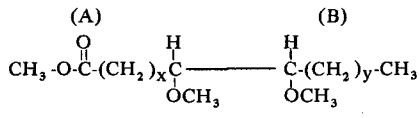
[a]The minor (<0.5%) monounsaturated fatty acids (17-20) which were identified by polymethoxylation are shown in Table 2.

[b]Gas chromatography column: fused silica (30 cm X 0.32 mm) containing SE-54 (J & W Scientific, Inc.), program temperature: 130-290 C, 5.0 C/min.

[c]Equivalent chain length values are those of methyl esters of these acids.

TABLE 2

Polymethoxy Derivatives of Monunsaturated Fatty Acid Methyl Esters in *Calyx niceaensis*



Compound		x	y	m/z (% base peak) M-31	M+1	M+18	A	A-32	B	B-32
6	Δ^9-17:1	7	6	313(100)	345(58)	362(32)	201(46)	168(8)	143(16)	111(16)
7	Δ^{11}-18:1	9	5	327(11)	359(100)	376(3)	229(11)	197(5)	129(21)	------[a]
17	Δ^6-19:1	4	11	341(100)	373(93)	390(8)	159(46)	127(63)	213(6)	181(4)
18	Δ^4-21:1	2	15	369(100)	401(47)	418(8)	131(42)	------[a]	269(5)	------[b]
19	Δ^4-22:1	2	16	383(46)	415(100)	------[b]	131(63)	------[a]	283(3)	------[b]
20	Δ^4-23:1	2	17	397(48)	429(100)	------[b]	131(85)	------[a]	297(19)	------[b]

[a]Not in measured mass range.
[b]Too small (less than 2%).

present in small amounts. Completely interpretable mass spectra of the methyl ester or the pyrrolidide of this compound were not obtained, since it was overshadowed by the presence of the major demospongic acids (14,15) present in the mixture. However, polymethoxylation of the unsaturated demospongic acids yielded much longer retention times and uncovered the presence of a methyl ester with a molecular ion at m/z 422, suggesting a cyclopropane or other cyclic structure. The possibility that it was due to a small amount of an unreacted monounsaturated acid is excluded, since a corresponding polymethoxy derivative is absent in the chromatogram. This mixture was subjected to derivatization with pyrrolidine. The pyrrolidide derivative of the unchanged compound (M^+ 461) showed spacings of 12 amu between the C_{18} (m/z 336) and C_{19} (m/z 348) fragments, indicating the structure as 19,20-methylenehexacosanoic acid (Fig. 3). Cyclopropane-containing C_{16-18} acids such as lactobacillic (11,12-methylene-octadecanoic) (16), dihydromalvalic (8,9-methyleneheptadecanoic) or dihydrosterculic (9,10-methylene-octadecanoic) (17,18) acids are known, but a C_{26} acid with a cyclopropane group has not been encountered until now. Cyclopropyl groups of fatty acids are known (19) to be biosynthesized from monoenic acid and S-adenosylmethionine. We assume that our new acid is either derived by an identical process from the corresponding 19-hexacosenoic acid, or it is a direct chain elongation product of a bacterial cyclopropane acid precursor.

CONCLUSIONS

The described EI-CI conditions using "low pressure" ammonia appear to be of great

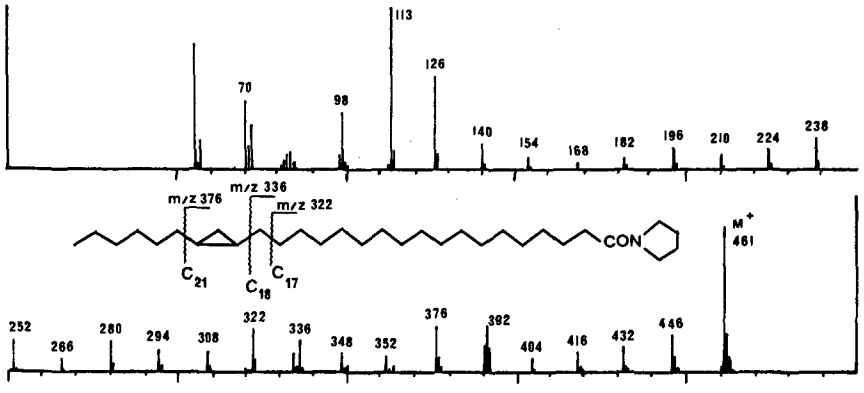
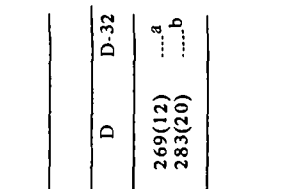


FIG. 3. Mass spectrum of N-(18,19-methylene-hexacosanoyl) pyrrolidine, as found in *Calyx niceasensis*.

TABLE 3

Polymethoxy Derivatives of Diunsaturated Fatty Acid Methyl Esters in *Calyx niceaensis*



Compound	x	z	y	M-31	M+1	M+18	m/z (% base peak) A	A-32	B	B-32	C	C-32	D	D-32
13 $\Delta^{5,9}$-26:2	3	15	2	499(22)	531(100)	548(6)	145(69)	113(56)	385(6)	353(99)	261(17)	229(62)	269(12)	---[a]
14,15[c] $\Delta^{5,9}$-27:2	3	16	2	513(13)	545(43)	563(5)	145(75)	113(21)	399(8)	367(57)	261(17)	229(100)	283(20)	---[b]

[a]Not in measured mass range.
[b]Too small (less than 2%).
[c]These two compounds gave the same double-bond fragmentation pattern.

value in the sensitive detection of unsaturated fatty acids in natural mixtures by capillary GC-MS-CI.

In addition, the methoxy derivatization provides significant differences in the gas chromatographic retention times between unsaturated and saturated acids, including cyclic compounds, which is in some cases superior to pyrrolidide analysis.

ACKNOWLEDGMENTS

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Enhanced Incorporation of Exogenous Arachidonic Acid into Phosphatidylinositol and Other Phospholipids During the Early Stages of Thrombin-Induced Aggregation in Gerbil Platelets[1]

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ABSTRACT

The degradation of platelet phospholipids via phospholipase activity is known to occur during thrombin-induced platelet aggregation. Both phosphatidylinositol and phosphatidylcholine are considered to be sources of the released arachidonic acid which becomes a substrate for prostaglandin and thromboxane A_2 formation. In this work, the effect of thrombin on the incorporation of exogenous arachidonic acid into platelet membrane phospholipids was studied. Suspensions of gerbil platelets were incubated in aggregometer cuvettes with [^{14}C]arachidonic acid in the absence or presence of thrombin, and product formation was monitored by thin layer chromatography and scintillation counting. Within 30 sec, the entry of arachidonic acid into phosphatidylinositol was increased by 165% in thrombin-stimulated platelets over controls. Under identical conditions, the incorporation into phosphatidylcholine was increased by only 57%. These results suggest that the incorporation of exogenous arachidonic acid via lysophosphatidylinositol and lysophosphatidylcholine acyltransferase activities may be intimately associated with thrombin-induced platelet aggregation in the gerbil.
Lipids 18:859-862, 1983.

INTRODUCTION

The stimulation of platelets by agents such as collagen, adenosine diphosphate and thrombin results in the release of arachidonic acid (AA) and its metabolic conversion to prostaglandins and thromboxane (1). This conversion of AA to its metabolites has been associated with platelet aggregation and release reactions (2). Whereas the mechanisms for the release of AA from platelet phospholipids either by phospholipase A_2 (3,4) or by a pathway involving phosphatidylinositol (PI)-specific phospholipase C (5) and diglyceride lipase (6) have been intensively investigated, the incorporation of exogenous AA into platelet phospholipids has received limited attention. The rapid turnover of PI with the concomitant enhanced incorporation of AA into phospholipids has been demonstrated in stimulated adrenocortical cells (7) and in stimulated tumor cells (8), but not in intact platelets. The literature is replete with data on platelets prelabelled with [1-^{14}C]AA over long periods of time (9-11) for the purpose of studying AA-release reactions and metabolite formation.

In view of the fact that AA plays a vital role in platelet function, that the secretory responses of platelets are essentially complete within 30 sec of the initiation of platelet aggregation (5), and that platelets share with other secretory cells the "phosphoinositide effect" (12), it was of considerable interest to investigate the incorporation of exogenous AA into platelet phospholipids during the early stages of gerbil platelet aggregation induced by thrombin. Our finding of enhanced incorporation of exogenous AA into PI within 30 sec concomitant with the activation of platelets with thrombin and their aggregation suggests the possible involvement of such acylation in PI accompanying thrombin-induced aggregation in gerbil platelets.

MATERIALS AND METHODS

Experimental Animals

The male Mongolian gerbils *(Meriones unguiculatus)* used in this study had an average body weight of 65 g and were obtained from High Oak Ranch, Goodwood, Ontario. The animals were housed in pairs as recommended by Hull et al. (13), with access to food (Purina Laboratory Chow) and water ad libitum. Gerbils were fasted for 10 hr prior to blood collection.

Blood Collection

Blood collection was performed on gerbils

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anesthetized with Methoxyflurane (Pitman-Moore, Inc., Washington Crossing, NJ) using 22 gauge 1½-in. needles. Blood (2.5 ml) was collected from each animal into 3-ml plastic syringes containing 0.3 ml of ACD anticoagulant (citric acid, 0.8%; trisodium citrate, 2.2%; dextrose, 2.45%; pH 4.5). The blood was mixed by gently inverting the syringe several times before dispensing the blood into siliconized glass test tubes for centrifugation.

Preparation of Platelet Suspensions

Suspensions of gerbil platelets were prepared by a modification of the method of Lagarde et al. (14) as described by Agwu et al. (15). All procedures were carried out at room temperature using siliconized glassware. The platelet count was determined manually using phase contrast microscopy (16). The final platelet concentration of the platelet suspension was adjusted to 300 x 10^9 platelets/l with buffer prior to aggregometric studies.

Platelet Aggregation

Platelet aggregation responses were recorded at 37 C for 30 sec, 1, 3 and 5 min using an aggregometer with an attached chart recorder (Payton Associated Ltd., Scarborough, Ontario) as described by Agwu et al. (15). Platelet suspensions (1.0 ml) were stirred in a siliconized aggregometer cuvette at 900 rpm for 1 min prior to the addition of 1.4 μ (0.14 μCi) of [1-^{14}C]AA (54.5 mCi/mmol, New England Nuclear, Boston, MA) in ethanol, followed by an immediate stimulation with the aggregating agent. Aggregation was induced by the addition of 0.2 U/ml (final concentration) of topical bovine thrombin (Parke Davis and Co., Ltd., Brockville, Ontario). Aggregation was monitored by changes in light transmission through the platelet suspension. Control tracings were obtained by adding a volume of tris buffer saline (TBS) equal to the volume of thrombin used after the addition of 1.4 μl of [1-^{14}C]AA to the platelet suspension.

Lipid Extraction and Analyses

The incorporation of [1-^{14}C]AA into platelets was terminated by pouring the contents of the cuvette into a test tube containing 0.2 ml of 100 mM sodium ethylenediaminetetraacetic acid (EDTA), mixing quickly on a Vortex mixer and immediately adding 3.8 ml of a chloroform/methanol mixture (1:2 by volume). This was mixed on a Vortex for 1 min before the addition of 1.3 ml of chloroform and mixing for another 1 min. Finally, 1.3 ml of 100 mM sodium EDTA was added and the content of the test tube was mixed for another 30 sec prior to being put in a freezer overnight. There was a clear separation between the lower phase (chloroform layer) and the upper phase (aqueous layer). The chloroform layer was removed with the aid of a pasteur pipette into a small vial and its volume was recorded. The same procedure was repeated for the upper phase. A 100-μl aliquot from the chloroform layer and a 1.0-ml aliquot from the aqueous layer were taken for scintillation counting in Aquasol (New England Nuclear, Boston, MA). The remaining volume of the chloroform layer was subjected to thin layer chromatography.

A 20-μl aliquot of a rat liver lipid extract was added as a carrier to the remaining chloroform layer and the content of the vial was evaporated to dryness under nitrogen, resuspended in 45 μl of chloroform/methanol (2:1 by volume), and spotted across 3.5 cm of 0.4 mm thick Silica Gel H (Analab, Inc., North Haven, CO) plates which had been previously activated. The plate was developed in chloroform/methanol/acetic acid/water (100:50:10:6 by volume). Chromatography tanks were equilibrated for at least 30 min prior to the development of the plates. Following the above procedure, it was possible to obtain reproducible separation of phospholipids, including PI and phosphatidylserine (PS). After air-drying, the plates were sprayed with a dichlorofluorescein solution, placed in a tank filled with ammonia vapor for 1-2 min and observed under UV light. The bands (neutral lipid, phosphatidylethanolamine (PE), PS, PI, phosphatidylcholine (PC) and lyso-PC) were outlined with a needle before being scraped into separate scintillation vials for counting in the presence of Aquasol. The above procedure was also undertaken with 1.0 ml of platelet suspension in which [1-^{14}C]AA was added after terminating the reactions to provide for zero-time controls.

RESULTS

Suspensions of gerbil platelets were quite responsive to 0.2 U/ml of thrombin. Typical aggregation responses of gerbil platelets to the aggregating agent are shown in Figure 1. Thrombin produced irreversible aggregation with maximum aggregation being attained at 3-5 min after platelet stimulation.

The extent of incorporation of [1-^{14}C]AA into all four phospholipid classes increased steadily with time in resting platelets for incubations increasing from 30 sec to 5 min in duration. The percentage incorporation of [1-^{14}C]AA into PC and PE also increased steadily with time in the case of aggregating

platelets (thrombin-induced), although the extent of incorporation into PI and PS approached maximal values within 30 sec (Fig. 1). During the first 30 sec, the percentage incorporation of [1-^{14}C]AA into all phospholipids (PE, PS, PI, PC) was significantly higher in thrombin-stimulated platelets. The percentage stimulation of [1-^{14}C]AA entry into PI was 265% above control (minus thrombin) values within 30 sec, which was considerably greater than that for the other phospholipids (Table 1). At later times, only the incorporation of [1-^{14}C]AA into PE was significantly higher ($P < 0.05$) in activated platelets, and in the case of PI at 5 min, a reversal was observed with a moderately lower incorporation exhibited in the activated platelets after aggregation had reached a plateau (Fig. 1).

DISCUSSION

The involvement of phospholipase A_2 in the release of arachidonic acid from platelet phospholipids with the concomitant production of lysophospholipids has been demonstrated for PC (17) and PE (18). The liberation of AA from PI of thrombin-stimulated platelets occurs via the combined action of phospholipase C plus diglyceride lipase (19) and phospholipase A_2 activity (20) which generates lyso-PI. Combined with the demonstration that human platelets contain an active membrane-bound acyl-CoA:1-acyl-*sn*-glycero-3-phosphorylcholine acyltransferase (21) responsible for the acylation of AA into lyso-PC and that there also exists a preferential acylation of arachidonate at the 2-position of 1-acyl-*sn*-glycero-3-phosphorylinositol in platelet lysates (22), these findings suggest the existence of a mechanism for the incorporation of exogenous AA into intact platelet phospholipids.

The present investigation demonstrates that the stimulation of gerbil platelet suspensions with thrombin results in the enhanced incorporation of exogenous [1-^{14}C]AA into platelet PI within 30 sec of the initiation of platelet aggregation. It also indicates that PE, PS and PC contribute to this increased acquisition of exogenous AA during the early stages of platelet aggregation, although to a smaller degree. Similar results have been demonstrated in the cat adrenocortical cells stimulated with ACTH (7) and in stimulated tumor cells (8). The enhanced entry of exogenous arachidonate into PI, PC and PE of thrombin-stimulated platelets may reflect a greater availability of endogenous lyso-PI, lyso-PC and lyso-PE. The levels of the latter lysophospholipids have been shown to be increased in human platelets shortly after thrombin stimulation (18,20).

The metabolic significance of the early incorporation of exogenous AA into stimulated platelets as observed herein remains unknown.

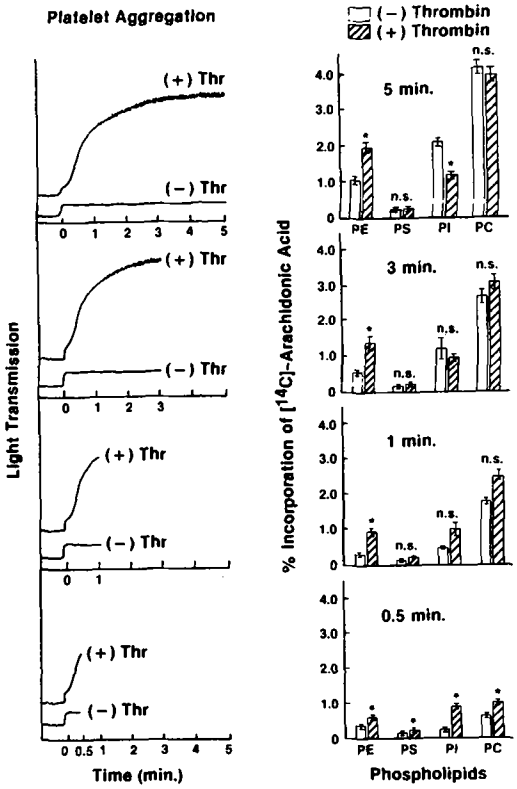

FIG. 1. The concomitant effect of thrombin on platelet aggregation and the incorporation of AA into platelet phospholipids. The aggregation responses of gerbil platelets to 0.2 U/ml thrombin and the corresponding percentage incorporation of [1-^{14}C]AA into PE, PS, PI and PC at 30 sec, 1, 3 and 5 min are shown. The curve tracings in the absence of thrombin are also shown. Details of the experimental procedure are described under Materials and Methods. The asterisk indicates a statistically significant difference between (+) and (−) thrombin tested at P = 0.05 level.

TABLE 1

The Percentage Stimulation of [^{14}C-]Arachidonic Acid Entry into Platelet Phospholipids by Thrombin

Phospholipid	Incubation time (30 sec)
	% Stimulation[a]
PE	66 ± 4
PS	48 ± 8
PI	265 ± 37
PC	57 ± 10

[a]Values are given as means ±SE (n = 3).

It may be speculated that it represents an initial metabolic step in platelet activation or it may serve to replenish membrane phospholipid in arachidonate derived from the plasma pool following phospholipid degradation and the metabolism of AA to prostaglandins and thromboxane (23). The existence of a deacylation-reacylation cycle in activated platelets may provide a unique mechanism for the turnover of AA in membrane phospholipid of activated platelets. However, further studies in this area are needed to elucidate more fully the significance of the altered pattern of AA incorporation into gerbil platelet phospholipids during aggregation.

ACKNOWLEDGMENT

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Temperature Effects on in vitro Lipid Accumulation in Asexual Embryos of *Theobroma cacao* L.

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ABSTRACT

Asexual embryos of cacao were grown for 40-50 days in a modified Murashige and Skoog liquid medium with sucrose concentration increased stepwise from 3 to 27% at temperatures of 10, 17, 20, 23, 26, 29, 32 or 35 C. Temperature influenced overall embryo growth as well as lipid accumulation and composition. Maximum growth occurred at 29 C, whereas maximum lipid and fatty acid accumulation occurred at 26 C. The proportion of individual fatty acids as total fatty acids accumulated per asexual embryo was constant at each temperature but varied with temperature from 10 to 35 C. The fatty acid composition of asexual embryos grown at 26 C was the most similar to that of cocoa butter, the lipid from mature zygotic embryos produced in vivo.
Lipids 18:863-867, 1983.

INTRODUCTION

Theobroma cacao is the source of cocoa butter, the melting characteristics of which make it the preferred seed fat for use in the manufacture of chocolate and other commercial products. At room temperature (20 C) cocoa butter is a solid which starts to soften at 30 C, melting completely at ca. 35 C or slightly below body temperature (1). The melting characteristics of cocoa butter are due to its unique glyceride composition, which has been reported to consist of 2.7-12.5% trisaturated, 67.5-81.3% monounsaturated, 15.3-27.0% diunsaturated and 0.7-2.1% triunsaturated triglycerides (2-7). The glyceride fatty acid composition of cocoa butter consists of 24.4-28.6% palmitic, 34.2-36.2% stearic, 33.4-38.1% oleic and 1.8-3.6% linoleic acid (2-4). According to Chacko and Perkins (2), oleic acid is the principal acyl moiety esterified to carbon 2 of the triglyceride backbone accounting for 79.6-88.7% of the possible fatty acid combinations. Due to the variation in composition of cocoa butter, no accepted biochemical definition exists. The FDA defines cocoa butter as "the edible fat obtained from sound cocoa beans either before of after roasting" (1). The Codex Committee on Cocoa and Chocolate Products defines cocoa butter as "the fat produced from one or more of the following: cocoa beans, cocoa nibs, cocoa mass (chocolate liquor), cocoa cake, expeller cake or cocoa dust (fines) by a mechanical process and/or with the aid of permissible solvents" (8).

Four crystalline forms of cocoa butter are recognized, α, β, β' and γ (1). The γ form has a melting point of 17 C, which rapidly isomerizes to the α form (mp 21-24 C). The α form changes at room temperature into the β' form (mp 27–29 C) which eventually changes into the β form (mp 34-35 C). Additionally, the melting behavior of cocoa butter is influenced by its fatty acid composition (9). Lehrian et al. (10) found that cocoa butter from fruit matured in a microclimate of elevated temperature had more solid fat at 16, 20 and 24 C as well as a greater percentage of saturated triglyceride fatty acid than butter from control fruit. Low temperatures during early fruit development have been shown to result in higher proportions of unsaturated fatty acids in cacao seeds and cocoa butter of lower melting characteristics (11,12).

The influence of temperature on the lipid biochemistry of plants is well documented (12-18); however, its effect on the development of an isolated plant organ free of confounding influences of the whole plant, such as that of an embryo, has not been demonstrated. The proliferation of asexual embryos (19,20) of cacao and their subsequent development in culture (21-23) suggests a potentially valuable system for the study of fatty acid biosynthesis by plant embryos in vitro. Cell suspension and callus culture have been used to study the influence of temperature on lipid biochemistry in vitro (24-26) but cells and tissues have different fatty acid compositions than embryos (27). In this study, we determined the influence of temperature on fatty acid accumulation of asexual embryos of cacao grown in vitro.

The in vitro culture of cacao embryos also

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has been suggested to be an alternative method of producing cocoa butter (23). This investigation attempted to determine the optimum culture temperature for the production of cocoa butter in vitro.

METHODS AND MATERIALS

Asexual embryos were obtained as previously described (19) on a basal solidified agar medium containing 1.5% (w/v) sucrose. The basal medium used throughout the culture of these embryos included Murashige and Skoog salts (28) and the following (in mg/l): casein hydrolysate, 1000; i-inositol, 100; glycine, 2; pyridoxine•HCl, 0.5; nicotinic acid, 0.5; and thiamine•HCl, 0.1. The pH of the media was adjusted to 5.7 before autoclaving.

In the first experiment, embryos were grown in liquid medium on a rolladrum apparatus for 8 days in 1.5% sucrose, and then successively subcultured into media supplemented with higher levels of sucrose after 2 days in 3% sucrose, 2 days each in 9%, 15%, 21% sucrose, and 24 days in 27% sucrose and incubated at 10, 17, 26 and 35 C from day 8 to day 40 of culture. In the second experiment, embryos were grown for 10 days in 3% sucrose, and successively subcultured for 2 days each in 9%, 15%, 21% sucrose, and 29 days in 27% sucrose-supplemented medium and grown at 20, 23, 26, 29, 32 and 35 C during the entire 45 days of culture. The details of cacao embryo culture have been previously described (21,22).

Lipids and fatty acids were extracted and analyzed as previously described (23).

RESULTS AND DISCUSSION

The results of both experiments appeared to complement each other, suggesting that temperature perturbations prior to and after the successive subcultures to higher sucrose-supplemented media do not greatly influence the net accumulation of fatty acid. Thus, the results of both experiments were combined for presentation purposes using a weighted average for treatments at 26 and 35 C.

Temperature markedly affected growth of asexual embryos of cacao. Embryo fresh (FW) and dry weight (DW) increased with increasing temperature between 10 and 29 C and then decreased (Fig. 1). Asexual embryos equivalent in size to zygotic embryos 100 days post-pollination grew after transfer to liquid medium to an average of 1.1 g FW and 0.4 g DW after 45 days in culture at 29 C. Mature zygotic embryos attain ca. 2.0 g FW and ca. 1.0 g DW after a total of 180 days post-pollination (29).

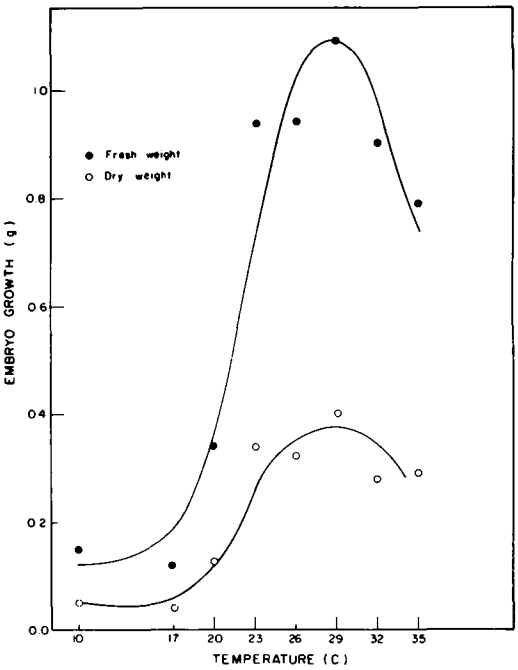

FIG. 1. Effect of temperature on growth of asexual embryos of cacao.

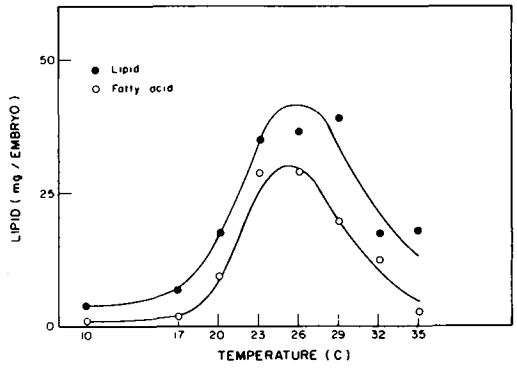

FIG. 2. Effect of temperature on lipids and fatty acid accumulation per asexual embryo of cacao.

Embryo growth was strongly inhibited at 17 C or less (Fig. 1). This is in agreement with the fact that cacao is not grown successfully in areas where the mean monthly minimum temperature is below 15 C (30).

Total lipid and fatty acid accumulation per embryo responded to temperature similarly with a maximum at 26 C (Fig. 2). Between 17 C and 26 C total fatty acid per embryo increased over 1400%, whereas the increase in lipid accumulation per embryo over the same temperature range increased only ca. 500%. The increase in dry weight accumulation from 17

to 26 C was ca. 800%. Due to the disproportionate rates in accumulation of lipid, fatty acid and dry weight per embryo, 26 C was optimum for fatty acid and 17 C for lipid accumulation expressed on a per-gram dry weight basis (Fig. 3).

Pure triglyceride of the types present in cacao lipid should have a fatty acid to lipid (FA/L) ratio equal to ca. 0.90. Since the FA/L ratio of embryos grown at 17 C was 0.13 (Fig. 3), most of the lipid accumulated at 17 C must not be fatty acyl triglycerides. On a dry weight basis then, 17 C could not be considered an optimum temperature for triglyceride lipid accumulation. Embryos grown at 26 C had an FA/L ratio of 0.83, the maximum for this investigation, indicating that 26 C is probably the optimum temperature for triglyceride accumulation in cacao embryos.

The effect of temperature on the relative proportion of individual fatty acids on a gram dry weight basis was complex (Fig. 4). The mole percent of palmitic acid (16:0) declined from 10 to 20 C and then increased up to 35 C, whereas stearic acid (18:0) increased from 10 to 20 C and then declined. Arachidic acid (20:0) showed little response to temperature. Oleic acid (18:1) and linoleic acid (18:2) had complementary responses. Oleic acid had a minimum at 10-17 C and a maximum at 26 C with linoleic acid showing a maximum at 17 C and a minimum at 23-26 C. The response of linolenic acid (18:3) was similar to linoleic acid with a minimum at 23-26 C but the amount of linolenic acid was much less. The relative changes in the mole % of oleic and linoleic acids suggested that increasing temperatures from 17 to 26 C progressively inhibits oleic acid desaturation.

An approach to elucidate the effect of temperature on fatty acid composition would be to determine the rate of fatty acid accumulation over time at each temperature. Although this experiment was not designed as a time course, we had previously determined that total fatty acid accumulation of developing cacao embryos in vivo was linear with the increase in embryo dry weight and that the rate of individual fatty acid accumulation remained constant as fatty acids increased (see Table 5 in ref. 31). The accumulation rate for each fatty acid was determined from the slope (b) of the regression of individual fatty acids to total fatty acids as total fatty acids increased. In the present experiment, asexual embryos cultured in liquid media with high concentration of sucrose formed a population varying widely in fatty acid content at each temperature. If the population of embryos

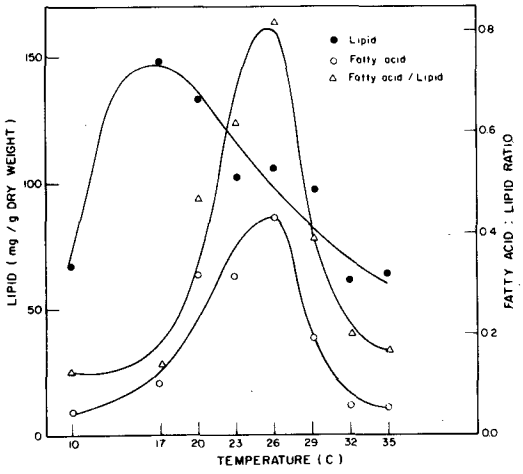

FIG. 3. Effect of temperature on lipids and fatty acid accumulation per gram dry weight and fatty acid/lipid ratio of asexual embryo of cacao.

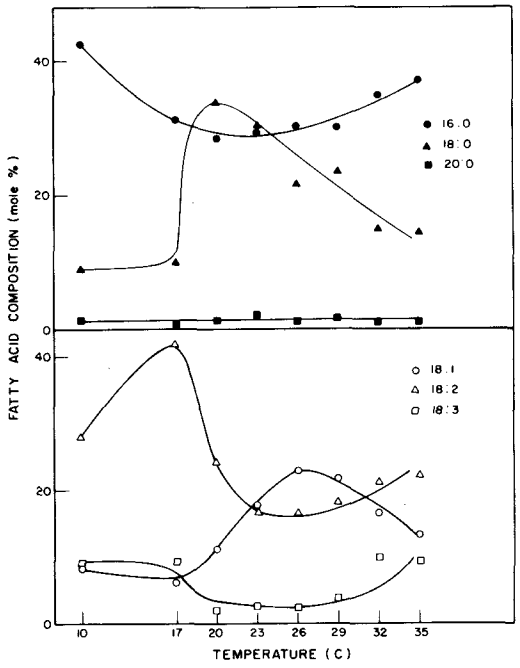

FIG. 4. Effect of temperature on fatty acid composition (mole %) of asexual embryo of cacao.

in each treatment can be considered to be a range of developmental ages, the slope of the regression of individual fatty acids on total fatty acid content also represents an accumulation rate.

Our data indicated that, within each population of asexual embryos, the relationship between individual and total fatty acids was

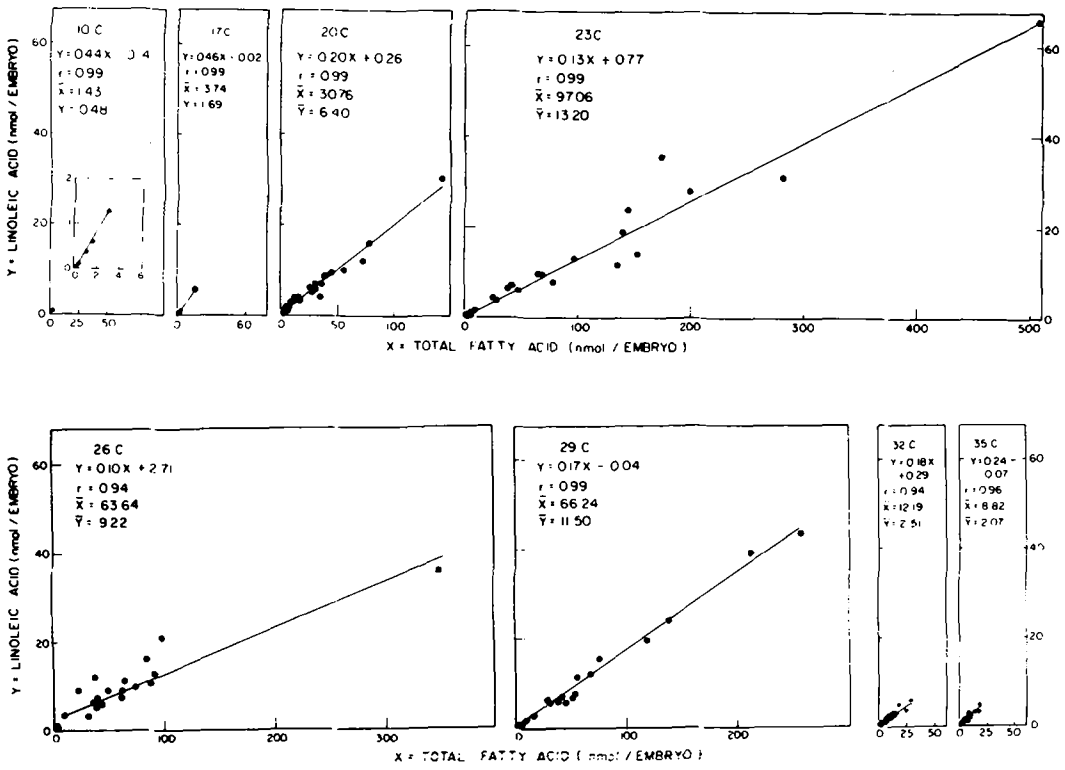

FIG. 5. The relationship between linoleic acid and total fatty acids of asexual embryos of cacao at 10, 17, 20, 23, 26, 29, 32 and 35 C. The slope of the regression line is defined as the rate of accumulation.

linear at each temperature and that the slope of the response (i.e., the accumulation rate) was affected by temperature (Table 1). The accumulation rate of palmitic acid remained relatively constant (0.30-0.26) from 10 to 26 C and then increased to 0.35 at 35 C. Stearic and oleic acid show maxima: 0.39 at 20 C for oleic acid and 0.28 at 26 C for stearic acid. In contrast, linoleic acid had a minimum accumulation rate of 0.10 at 26 C (Fig. 5 and Table 1). This suggests that the percentage of oleic acid being desaturated to linoleic acid decreases with increasing temperature up to 26 C, but increases with increasing temperatures above 26 C. Additionally, this suggests that the percentage of palmitic acid being elongated to form stearic acid increases with increasing temperatures up to 26 C but decreases with temperatures above 26 C.

It has been demonstrated in several plant species that fatty acid desaturation decreases and chain elongation increases with increasing temperature (13-18). Decreasing oxygen solubility with increasing temperature has been proposed as one possibility to account for this phenomenon (32). Although simplistic, this may account for the differences in fatty acid content for embryos grown up to 26 C. The adverse effect of high temperature on asexual embryo growth which also declined above 26 C may be a partial explanation.

The mole percent fatty acid composition of asexual embryos grown at 26 C (palmitic, 30%; stearic, 28%; oleic, 21%; linoleic, 17%; and linolenic acid, 2.8%) was the closest to that of mature field-grown zygotic embryos (palmitic, 29%; stearic, 32%; oleic, 36%; linoleic, 3%; and linolenic acid, 1%) (23). The accumulation rates at 26 C indicated that the mole percent of these fatty acids in cultured asexual embryos eventually will approach 26 % for palmitic, 28% for stearic, 33% for oleic, and 10% for linoleic acid as compared to 29, 32, 36 and 3%, respectively, for cocoa butter (22) if the trend for 45 days in culture continued to maturity. We conclude that asexual embryos cultured in vitro in high sucrose will have a fatty acid composition most similar to that of cocoa butter and mature zygotic embryos at 26 C. Apparently, fatty acid synthesis of asexual embryos cultured in vitro at 26 C mimicks that of zygotic embryos in vivo.

TABLE I

The Rate of Accumulation (b) and Correlation Coefficient (r) of Individual Fatty Acid on Total Fatty Acids (μmol/embryo) with Temperature

Temperature (C)	Palmitic acid		Stearic acid		Oleic acid		Linoleic acid	
	b	r	b	r	b	r	b	r
10	0.30	0.96	0.08	0.85	0.07	0.85	0.44	0.99
17	0.27	0.94	0.07	0.91	0.23	0.98	0.46	0.99
20	0.28	0.99	0.11	0.94	0.39	0.99	0.20	0.99
23	0.28	0.99	0.23	0.96	0.33	0.99	0.13	0.97
26	0.26	0.99	0.28	0.99	0.33	0.99	0.10	0.94
29	0.29	0.99	0.24	0.99	0.24	0.99	0.17	0.99
32	0.34	0.99	0.17	0.97	0.21	0.91	0.18	0.94
35	0.35	0.97	0.19	0.97	0.14	0.87	0.24	0.96

The parameter (b) is the slope of the linear regression of individual fatty acids vs total fatty acids in the population of asexual embryos at each treatment.

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Photosensitized Oxidation of Methyl Linoleate Monohydroperoxides: Hydroperoxy Cyclic Peroxides, Dihydroperoxides, Keto Esters and Volatile Thermal Decomposition Products[1]

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ABSTRACT

Previous studies of lipid secondary oxidation products have been extended to 6-membered hydroperoxy cyclic peroxides from the singlet oxygenation of a mixture of 9- and 13-hydroperoxides from autoxidized methyl linoleate. The oxidation product was fractionated by silicic acid chromatography with diethyl ether/hexane mixtures, and selected fractions were separated by polar phase high performance liquid chromatography. Products characterized by thin layer chromatography, gas liquid chromatography, ultraviolet, infrared, nuclear magnetic resonance and mass spectrometry included: 6-membered cyclic peroxides (13-hydroperoxy-9,12-epidioxy-10- and 9-hydroperoxy-10,13-epidioxy-11-octadecenoates), dihydroperoxides (8,13- and 9,14-dihydroperoxyoctadecadienoates) and keto dienes (9- and 13-oxooctadecadienoates). The 6-membered hydroperoxy cyclic peroxides are apparently formed by 1,4-addition of singlet oxygen to 9- and 13-hydroperoxides with *trans, trans*-conjugated diene systems. Thermal decomposition of the 6-membered hydroperoxy cyclic peroxides at 200 C produced methyl 9-oxononanoate and hexanal as the major volatiles. Other volatiles included 2-pentylfuran, pentane, 4-oxo-2-nonenal, methyl furanoctanoate and methyl 9,12-dioxo-10-dodecenoate. *Lipids* 18:868-876, 1983.

INTRODUCTION

In early studies of autoxidation of linoleate and linolenate, several investigators postulated the formation of 6-membered hydroperoxy cyclic peroxides as secondary products (1-5). However, in more recent investigations (6,7) of linoleate autoxidation, such 6-membered epidioxides were not identified among the secondary oxidation products. In later studies of lipid secondary oxidation products, 5-membered hydroperoxy epidioxides were identified in photosensitized oxidized methyl linoleate (8,9) and linolenate (10,11), and in autoxidized linolenate (12,13). Hydroperoxy bicyclic endoperoxides were also identified in oxidized linolenate (10,14).

A 6-membered hydroperoxy epidioxide, 2-hydroperoxy-3,6-epidioxy-hexane, was prepared from 1-hydroperoxy-4-hexene by free radical cyclization after abstraction of the hydroperoxy proton with di-*tert*-butylperoxy oxalate (15,16). Six-membered hydroperoxy epidioxides apparently form during the autoxidation of squalene (16,17). Also 6-membered epidioxides can be obtained by 1,4-addition of singlet oxygen ($^1O_2$) to 1,3-diene compounds (18-22). Recently, a 6-membered hydroperoxy cyclic compound formed by 1,4-addition from linoleate hydroperoxides was suggested as a precursor of pentyl furaldehyde and pentyl furan (23).

Recent evidence suggests the importance of preventing photosensitized oxidation even in refined oils (23-25). Five-membered hydroperoxy epidioxides from photosensitized oxidized linoleate and linolenate were shown to be important precursors of volatile products formed by thermal decomposition (9,26). The conjugated 9- and 13-hydroperoxy octadecadienoates from autoxidized linoleate can be expected to form cyclic peroxides by oxidation with $^1O_2$. This paper reports our investigation of the reaction of 9- and 13-linoleate monohydroperoxides with $^1O_2$. Six-membered hydroperoxy epidioxides formed as major products were thermally decomposed to examine their possible role as precursors of volatile oxidation products.

EXPERIMENTAL

Materials and Methods

The methyl linoleate (100% by gas liquid chromatography [GLC]) used and the removal of oxidation products before autoxidation

[1]Presented at the 74th annual AOCS meeting, Chicago, 1983.

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were described previously (9). A mixture of 9- and 13-monohydroperoxides was then prepared by silicic acid chromatography of autoxidized methyl linoleate,and the fractions were checked for purity by thin layer chromatography (TLC) (9). Previous procedures were used for preparing derivatives of monohydroperoxy cyclic (13) and dihydroperoxy and keto compounds (10) suitable for gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS) and mass spectrometry (MS). The mixture of linoleate monohydroperoxides, 9- and 13-OOH (500 mg), was treated with $^1O_2$ generated by gaseous O_2 in the presence of methylene blue (10 mg) as sensitizer in a CH_2Cl_2 (80 ml) solution at 0 C for 16 hr in an open tube in the same photochemical apparatus previously described (9). Under these conditions, there was no evidence that methylene blue was bleached or decomposed. Oxidation under these conditions, monitored by TLC and peroxide value determination, gave ca. 80% conversion of monohydroperoxides to secondary oxidation products.

High Performance Liquid Chromatography (HPLC)

A portion of the photosensitized oxidation product (432 mg) was first separated by silicic acid chromatography (9) with diethyl ether/hexane mixtures (given in parentheses) to elute the following fractions: 200 ml (10:90) ketodienes, 100 ml (20:80) + 25 ml (30:70) unreacted monohydroperoxides, 75 ml (30:70) + 75 ml (40:60) 6 membered hydroperoxy cyclic peroxides, 25 ml (40:60) + 100 ml (50:50) + 50 ml (60:40) dihydroperoxides and 50 ml (60/40) + 50 ml (70/30) unidentified polar material + 100 ml diethyl ether/methanol (1:1) residue. The 6-membered hydroperoxy cyclic peroxides and dihydroperoxides were further separated by HPLC, using the same columns and solvent systems to fractionate secondary products in photosensitized oxidized methyl linolenate (10). In the present work, the ultraviolet (UV) detector remained at 215 nm for the entire HPLC separation of dihydroperoxides.

Characterization

The oxidation products were characterized by GC, TLC, UV, infrared (IR), nuclear magnetic resonance (NMR), mass spectrometry (MS) and GC-MS as reported previously (27). In this study for the cyclic peroxides, a Bruker WM-300 WB instrument was used to obtain ^{1}H-NMR spectra at 300 MHz. Analysis of the geometric isomeric composition of linoleate monohydroperoxide was done by HPLC of the hydroxy derivatives obtained after $NaBH_4$ reduction (28). Volatiles were identified by capillary GC-MS using a 0.32 mm x 15 m fused silica column with a polymethylphenyl bonded phase as described previously (26).

RESULTS AND DISCUSSION

Previous work on photosensitized oxidation (methylene blue) of methyl *trans,trans*-8,10-octadecadienoate (29) showed the formation of 6-membered epidioxides by 1,4-addition of $^1O_2$ to a conjugated *trans,trans*-diene. Also, photosensitized oxidation was shown to promote geometric isomerization of conjugated dienoic hydroperoxides from the *cis,trans* to *trans,trans* configuration (30). These studies indicated the possibility that 6-membered hydroperoxy epidioxides may be formed by reaction of $^1O_2$ with conjugated dienoic monohydroperoxides from autoxidized linoleate.

Samples of 9- and 13-linoleate hydroperoxides, photooxidized in the presence of methylene blue, were fractionated by SiO_2 chromatography. Secondary oxidation products were separated and identified functionally by comparison with previously characterized products of photosensitized oxidation (9,10). Quantitative analyses showed the following relative concentrations of secondary products for a sample of 0.4 g linoleate hydroperoxides reacted 16.5 hr with $^1O_2$: 9- and 13-ketodienes (1.2%), unreacted 9- and 13-monohydroperoxides (16.7%), hydroperoxy 6-membered epidioxides (57.9%), dihydroperoxides (17.0%), unidentified polar materials (4.0%) and residue (3.2%). The main products, 6-membered hydroperoxy epidioxides and dihydroperoxides were identified in detail after further purification by HPLC. The ketodienes were identified after silicic acid chromatography.

Six-Membered Hydroperoxy Epidioxides

The silicic acid fraction containing epidioxides was further resolved by polar phase HPLC (Fig. 1) into the pure components, methyl 13-hydroperoxy-9,12-epidioxy-10-octadecenoate (**Ia,b**, Table 1) and methyl 9-hydroperoxy-10,13-epidioxy-11-octadecenoate (**IIa, b**; Table 2), whose structures were established by the spectral and chromatographic studies discussed below. The following relative compositions were estimated by refractive index detection: **Ia**, 21%; **IIa**, 36%; **Ib**, 19.8% and **IIb**, 23.1%.

TLC (silica, diethyl ether/hexane, 60:40) **I, II**; UV inactive and hydroperoxide positive; **Ia**, 0.93; **IIa + Ib**, 0.89 and **IIb**, 0.87 relative to linoleate monohydroperoxides. GC of the

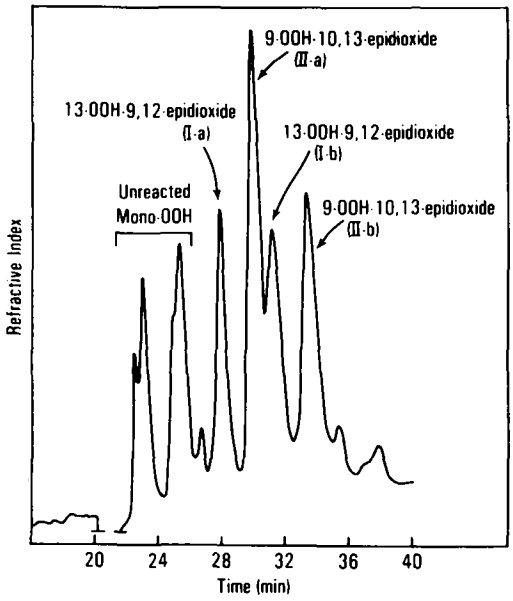


FIG. 1. 10-μm Silica HPLC chromatogram of 6-membered hydroperoxy epidioxides from photosensitized oxidized linoleate monohydroperoxides (flow 4 ml/min; mobile phase, hexane/CH_2Cl_2/ethyl acetate [7:4:1, v/v/v]; refractive index detector, X8; column temperature, 20 C).

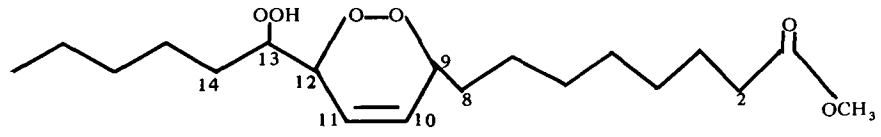
hydrogenated silylated derivatives has the same retentions for **Ia** and **Ib** as 9,12,13-trihydroxystearate, 2.73 and for **IIa** and **IIb** as 9,10,13-trihydroxystearate, 2.67, relative to methyl stearate. IR (CS_2) **I**, **II**: 3,500 (free C-OOH) 3,680-3,220 (bonded C-OOH) and 3,020 (olefinic H) cm^{-1}

MS of the hydrogenated and Ph_3P-reduced derivatives confirmed the general structure of a 6-membered ring with an endocyclic double bond and an exocyclic hydroperoxy group for **I** and **II**. Hydrogenation of **Ia**, **Ib** (Table 1) gave 9,12,13-trihydroxystearate identified by MS (31) after silylation, m/e (rel intensity) **Ia**: M-15, 547 (0.1); M-31, 531 (0.6); M-(90+15), 457 (4); 173 (59); 259 (22); 389 (8); 389-90, 299 (100); and **Ib**: M-15, 547 (0.2); M-31, 531 (1); M-(90+15), 457 (4); 173 (65); 259 (39); 389 (11); 389-90, 299 (100). Hydrogenation of **IIa**, **IIb** (Table 2) gave 9,10,13-trihydroxystearate identified by MS (29) after silylation, m/e (rel intensity) **IIa**: M-15, 547 (0.3); M-31, 531 (1); M-(90+15) 457 (6) ; 173 (45). 259 (83); 303 (55); 213 (100); and **IIb**: M-31, 531 (0.2); M-(90+15), 457 (3); 173 (52); 259 (44); 303 (14); 303-90, 213 (100).

Reduction of **Ia**, **Ib** (Table 1) with Ph_3P produced the unsaturated hydroxyepidioxide derivatives identified by MS after silylation,

TABLE 1

^{1}H-NMR (300 MHz) of 13-Hydroperoxy-9,12-Epidioxy-10-Octadecenoate (I)[a]

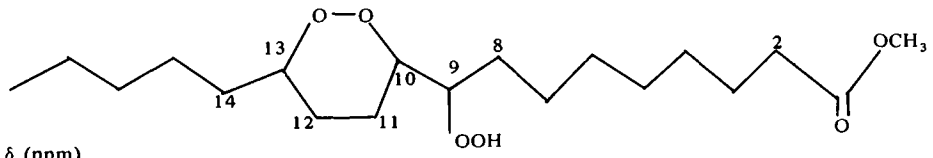


δ (ppm) Ia	δ (ppm) Ib	Multiplicity[b]	Coupling constants Hz[c]	Number of protons	Assignment
8.69	8.75	s		1	OOH
6.01	5.99	s		2	H-10,11
4.61	4.61	m	$J_{12,13}$=4.5	1	H-12
4.54	4.53	m	$J_{9,12}$=2.2	1	H-9
4.14	4.12	m		1	H-13
3.66	3.64	s		3	CH_3O
2.29	2.26	t		2	H-2
1.60	1.56	m		4	H-8,14
1.30	1.32	br		16	H-3-7,15-17
0.89	0.87	t		3	H-18

[a]See Figure 1.
[b]Multiplicity: br=broad, s=singlet, d=doublet, m=multiplet, t=triplet.
[c]Coupling constant for **Ia**.

TABLE 2

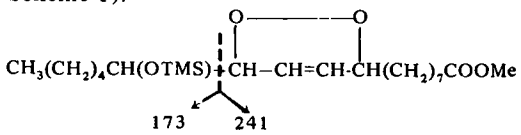
^{1}H-NMR (300 MHz) of 9-Hydroperoxy-10,13-Epidioxy-11-Octadecenoate (**II**)[a]



δ (ppm) IIa	δ (ppm) IIb	Multiplicity[b]	Coupling constants Hz[c]	Number of protons	Assignment
8.76	8.79	s		1	OOH
5.99	6.00	dt	$J_{10,12}=1.3$; $J_{11,12}=10.5$; $J_{12,13}=1.5$	1	H-12
5.90	5.90	ddd	$J_{10,11}=2.7$; $J_{11,13}=1.6$	1	H-11
4.66	4.66	m	$J_{9,10}=7$	1	H-10
4.49	4.48	m	$J_{10,13}=2$	1	H-13
4.17	4.17	m		1	H-9
3.65	3.65	s		3	CH_3O
2.29	2.29	t	J=7.5	2	H-2
1.57	1.57	m		4	H-8,14
1.30	1.31	br		16	H-3-7, 15-17
0.89	0.88	t		3	H-18

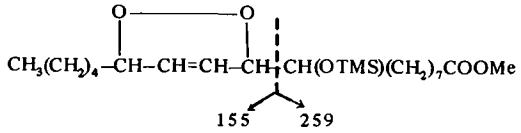
[a]See Figure 1.
[b]Multiplicity: same as Table 1.
[c]Coupling constants for **IIa**.

m/e (rel intensity) **Ia**: M,414 (1); M-16, 398 (2); M-32, 382 (4); 173 (100); 241 (30); and **Ib**: M,414 (0.3); M-16, 398 (1); M-32, 382 (2); M-90, 324 (3); 173 (26) and 241 (2) (see Scheme 1).



SCHEME 1.

Reduction of **IIa**, **IIb** (Table 2) *with* Ph_3P gave the corresponding unsaturated hydroxy epidioxide derivatives identified by MS after silylation, m/e (rel intensity) **IIa**: M,414 (0.7); M-16, 398 (2); M-32, 382 (2); M-90, 324 (2); 259 (56); 155 (58); and **IIb**: M-16, 398 (0.5); M-32, 382 (0.3); M-90, 324 (1); 259 (100); 155 (36) (see Scheme 2).



SCHEME 2.

^{1}H-NMR (300 MHz, $CDCl_3$) for **Ia**, **Ib** (Table 1) and **IIa**, **IIb** (Table 2) provided further evidence for the structures of **I** and **II**. The methine carbon-olefin group-methine carbon structure (-CH-CH=CH-CH-) of the 6-membered epidioxide ring is supported by the long-range coupling constant, 2 Hz, for **Ia** hydrogens 9,12 and **IIa** hydrogens 10, 13. The coupling constant of 4.5 and 7 Hz between the ring methine proton and α hydroperoxy-bearing carbon proton of **Ia** and **IIa**, respectively, serves to distinguish between these positional isomers. Further support for the ring olefin is the *cis* coupling constant, 10.5 Hz, for carbons 11,12 of **IIa**. Other general shifts for the 6-membered hydroperoxy epidioxide structure for **I** and **II** are: 4.17 to 4.12 ppm for the hydroperoxy-bearing carbon methine proton, 4.66-4.61 ppm for the ring methine proton to the α hydroperoxy-bearing carbon, 4.53-4.48 ppm for the other ring methine proton (9), and 6.01-5.90 ppm for the ring olefin protons (29). The small difference in chemical shift between the hydroperoxy-bearing carbons for **Ia** and **Ib** suggested a diastereomeric relationship similar to the shifts found for 5-membered hydroperoxide diastereoisomers (9). However, there was no apparent difference between the same chemical shifts for **IIa,b**. Apparently, **Ia,b** and **IIa,b** are stereoisomers on the basis of their elution order by HPLC relative to the retentions for 5-membered hydroperoxy cyclic peroxides, whose stereochemical structures were established previously (8,9).

These structural studies show that the 6-membered hydroperoxy epidioxides are formed by 1,4-addition of $^1O_2$ to the 1,3-diene system of the linoleate hydroperoxides. The *trans, cis-*

diene hydroperoxides are isomerized in the presence of $^1O_2$ to the *trans,trans* configuration before 1,4-addition (30). Quantitative analyses by HPLC (28) of the dienols produced by $NaBH_4$ reduction of the hydroperoxides before and after cyclization with $^1O_2$ indicated little change in the relative amount of *trans,trans* diene. The *trans,trans* to *cis,trans* ratios of monohydroperoxide mixtures changed by only 2-6% after $^1O_2$ oxidation. These results can be explained according to a scheme in which the photosensitized isomerization (k_1/k_2) from *cis,trans* to *trans,trans* configuration is much faster than the 1,4-cycloaddition (k_3) of $^1O_2$ to the conjugated diene system of linoleate hydroperoxides (Fig. 2).

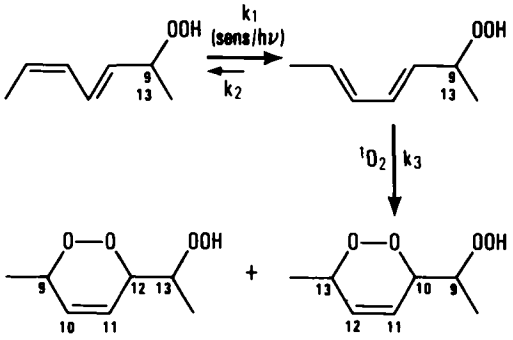


FIG. 2. Scheme for the formation of 6-membered hydroperoxy epidioxides from linoleate monohydroperoxides (sens = sensitizer).

Dihydroperoxides

The appropriate SiO_2 chromatographic fraction was further resolved by polar phase HPLC into positional isomers (Figs. 3 and 4) as described previously for photosensitized oxidized linolenate (10). The HPLC chromatogram (Fig. 3) shows the resolution of dihydroperoxides **III** and **IV** with the following weight percent: **IIIa**, 61.0%; **IIIb**, 9.3%; **IIIc + IVa**, 13.6% and **IVb**, 16.1%.

TLC, all spots peroxide positive and UV active, absolute R_f **IIIa,b**, 0.29; **IIIc, IVa**, 0.24 and **IVb**, 0.11. GC of hydrogenated-silylated derivatives gave retentions relative to methyl stearate: **IIIa,b**, 1.70; **IIIc**, 1.70, **IVa**, 1.78 and **IVb**, 1.78. UV (methanol) **IIIa**: 230 (Em 20,300), **IIIb**: 234 (Em 22,700), **IIIc + IVa**: 230 (Em 22,800), **IVb**, 232 nm (EM 23,300), which indicate conjugated diene functionality. IR (CS_2) **III, IV**: 3,500 (free C-OOH), 3,610-3,200 (bonded C-OOH), 3,010-3,002 (olefinic H), 988-972 (conjugated *trans,trans* unsaturation) cm^{-1}. The UV and IR data show the dihydroperoxides to have *trans,trans* conjugated dienes. Hydrogenation of **III** and **IV** gave 8,13- and 9,14-dihydroxystearates which were identified by MS (31) after silylation, m/e (rel intensity) **IIIa**: M-71, 403 (2); 173 (40); 245 (28) for 8,13-dihydroxystearate; **IIIb**: M-71, 403 (8); 173 (71); 245 (52) for 8,13-dihydroxystearate; **IIIc + IVa** (a di-OOH mixture): M-15, 459 (3); M-57, 417 (17); 159 (75), 259 (74) for 9,14-dihydroxystearate

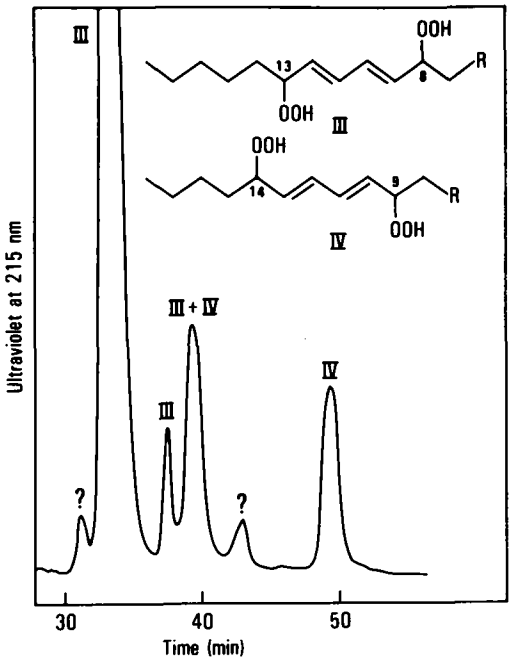


FIG. 3. 6-μm Silica HPLC chromatogram of dihydroperoxides from photosensitized oxidized linoleate monohydroperoxides (flow 20 ml/min: mobile phase, 3% ethanol/hexane [v/v]; UV detector, 0.64 ASU at 215 nm; column temperature, 20 C).

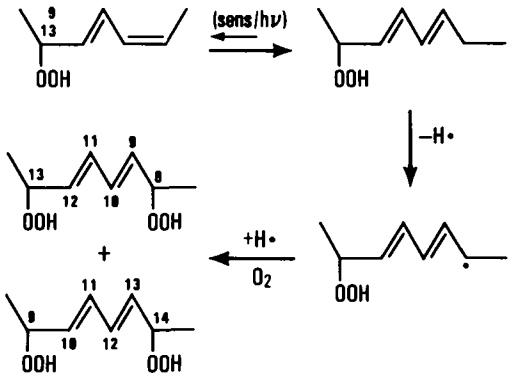


FIG. 4. Scheme for the formation of 8,13- and 9,14-dihydroperoxides from linoleate monohydroperoxides (sens = sensitizer).

and M-71, 403 (9); 173 (51); 245 (40) for 8,13-dihydroxystearate; and **IVb**: M-15, 459 (2); M-57, 417 (20); 159 (66); 259 (55) for 9,14-dihydroxystearate. Reduction of **IIIa** and **IVb** with $NaBH_4$ gave dihydroxy-octadecadienoates identified by MS (8) of the silylated derivatives, m/e (rel intensity) **IIIa**: M-15, 455 (0.4); M-31, 429 (3); M-71, 399 (6); 327 (2); 237 (27); 399 (3); 309 (35) for 8,13-dihydroxyoctadecadienoate (see Scheme 3) and **IVb**: M-15, 455 (1); M-31, 429 (5); M-57, 403 (8); 323 (5); 233 (18); 413 (7); 323 (22) for 9,14-dihydroxyoctadecadienoate (see Scheme 4).

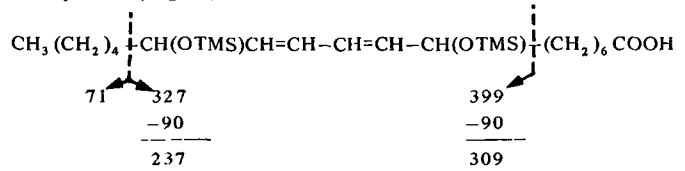
The ^{1}H-NMR data support the UV and IR evidence for olefinic conjugation (6.98-5.25 ppm) and TLC evidence for hydroperoxy functionality (8.25-7.82 ppm, C-OOH; 4.87-4.30 ppm CH-OOH). Based on the MS of the reduced derivatives of **III**, **IV**, the following ^{1}H-NMR assignments are made (^{1}H-NMR, 90 MHz, $CDCl_3$) **IIIa**: 8.22 (br s, 2, OOH), 6.97-5.25 (m, 4, H-9-12), 4.57 (m, 2, H-8,13), 3.67 (s, 3, CO_2CH_3), 2.31 (t, 2, H-2), 2.00-1.50 (m, 4, H-7,14) and 0.93 (t, 3, H-18); **IIIb**: 8.25 (br s, 2, OOH), 6.75-5.25 (m, 4, H-9-12), 4.30 (m, 2, H-8,13), 3.67 (s, 3, CO_2CH_3), 2.31 (t, 2, $CH_2CO_2CH_3$), 1.50-1.80 (m, 4, H-7,14) and 0.92 (t, 3, H-18); **IIIc + IVa**: 8.12 (s, OOH) 6.98-5.25 (m, CH=CH-CH=CH), 4,87 (m, CHOOH), 4.40 (m, CHOOH), 3.67 (s, CO_2CH_3), 2.31 (t, $CH_2CO_2CH_3$), 0.92 (t, CH_3-C); **IVb**: 7.82 (br s, 2, OOH), 6.50-5.25 (m, 4, H-10-13), 4.36 (m, 2, H-9,14), 3.67 (s, 3, CO_2CH_3), 2.32 (t, 2, H-2), 1.83-1.50 (m, 4, H-8-15) and 0.92 (t, 3, H-18).

The 8,13-dihydroperoxy-*trans*-9, *trans*-11- and 9,14-dihydroperoxy-*trans*-10, *trans*-12-octadecadienoates identified here may be free radical side products of the $^1O_2$ reaction formed by H-abstraction on C-8 and C-14 α to the conjugated diene system (Fig. 4). The free radical intermediate apparently is localized on C-8 or C-14 before reacting with O_2. If a delocalized pentadiene radical was formed between C-8 and C-12 or between C-10 and C-14, then 12,13- and 9,10-dihydroperoxides would be expected, but these dihydroperoxides were not detected. In a recent report (32), nonconjugated diene 8- and 14-monohydroperoxides were identified in minor amounts in oxidized methyl linoleate. The products may be rationalized as side products formed by free radical abstraction on α C-8 and C-14 positions of linoleate without delocalization. The expected dihydroperoxides, however, have not been reported in autoxidized linoleate. An alternative mechanism to explain the formation of 8,13- and 9-14-dihydroperoxides in the presence of $^1O_2$ may involve the 6-membered epidioxides undergoing H-abstraction on C-8 or C-14, loss of O_2 and rearrangement of double bonds. Further work is needed to clarify these mechanisms.

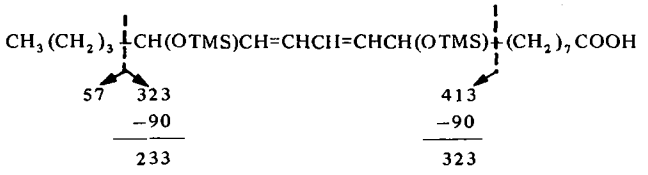
Ketodienes

Another SiO_2 chromatographic fraction was shown to consist of a mixture of methyl 9-oxo-*trans,trans*-10,12- and 13-oxo-*trans,trans*-9-11-octadecadienoates. TLC: absolute R_f, 0.80, UV positive and peroxide negative. UV (methanol) 277 nm (Em 20, 962) is similar to the spectrum previously reported for conjugated ketodiene (33). IR (CS_2) 3,002 (olefinic H), 1,732 (ester carbonyl), 1,681, 1,668, 1,632 (keto carbonyl) 988 (conjugated *trans,trans* unsturation) cm^{-1}. ^{1}H-NMR (90 MHz, $CDCl_3$): 7.50 (m, 1; CH=C-C=O), 6.30-5.62 (m, 3, CH=CH-C=CH-C=O) 3.67 (s, 3, CO_2CH_3), 2.55 (t, 2, C=C-C=OCH_2), 2.36 (t, 2, CH_2CO_2C) 2.15 (m, 2, CH_2C=C) and 0.90 (t, 3, CH_3C). IR and ^{1}H-NMR data are consistent with that reported for *trans,trans* ketodiene (33).

MS gave the the following characteristic



SCHEME 3.



SCHEME 4.

fragments, m/e (rel intensity): M, 308 (32); M-31, 277 (13); 237 (37); 151 (100) for methyl 13-oxo-9,11- and/or 9-oxo-10,12-octadecadienoate. The location of the keto groups and the confirmation of a mixture of 9- and 13-keto compounds is based on MS of hydrogenated derivatives after silylation, m/e (rel intensity): M-31,355 (3); 173 (100); 315 (56) for 13-OTMS-stearate and 259 (48); 229 (43) for 9-OTMS-stearate. These keto dienes may be dehydration side products (6) of monohydroperoxides after their isomerization, catalyzed by $^1O_2$, to the *trans,trans*-configuration.

Volatile Decomposition Products

Methyl 13-hydroperoxy-9,12-epidioxy-10- and 9-hydroperoxy-10,13-epidioxy-11-octadecenoates were each decomposed on a GC injection port at 200 C in a capillary GC-MS system, and the volatiles produced were identified as described previously (26). The fragmentation schemes in Figure 5 account for ca. 94% of the volatiles identified in Table 3. The most important cleavage, A, between the hydroperoxide-bearing carbon and the peroxide ring gave 47% hexanal, 5% methyl 9,12-dioxo-10-dodecenoate and, after dehydration of the ring fragment, 4.7% methyl 8-(2-furyl)octanoate from the 13-hydroperoxy epidioxide (I). The same cleavage gave 55.6% methyl 9-oxononanoate, 5% 2-pentylfuran and 6.5% 4-oxo-2-nonenal from the 9-hydroperoxy epidioxide (II). The second most important cleavage, B, between the ring alkene and the ring oxygen, on the side opposite the hydroperoxide, produced 23% methyl 9-oxononanoate from I. The same cleavage gave 11.4% hexanal, 3.7% methyl

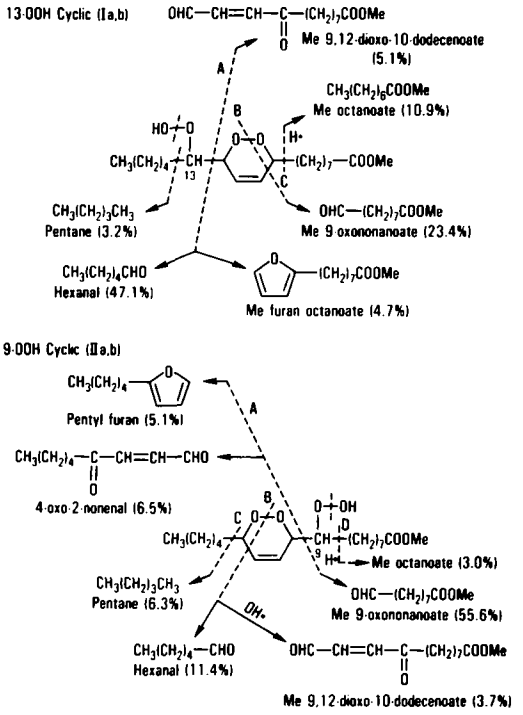


FIG. 5. Scheme for the formation of volatiles by thermal decomposition of 6-membered hydroperoxy epidioxides.

TABLE 3

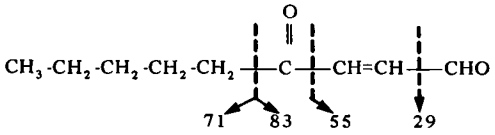
GC-MS Analysis[a] of Volatiles from Thermally Decomposed Hydroperoxy 6-Membered Cyclic Peroxides

Volatile compounds	Relative retention[b]	Relative percent (cleavage, type[c])	
		13-OOH cyclic (I)	9-OOH cyclic (II)
Acetaldehyde	0.05	0.9	0.7
Pentane	0.06	3.2 (D)	6.3 (C)
Hexanal	0.24	47.1 (A)	11.4 (B)
2-Pentylfuran	0.74	0.6	5.1 (A)
Me Octenoate	0.98	0.6	0.1
Me Octanoate	1.00	10.9 (C)	3.0 (D)
Me 8-oxooctanoate	1.31	0.2	–
4-Oxo-2-nonenal[d]	1.32	0.7	6.5 (A)
4-Oxononanal	1.34	–	0.2
9-Oxononanoate	1.47	23.4 (B)	55.6 (A)
Me 8-(2-furyl)octanoate[e]	1.69	4.7 (A)	0.8
Me 10-oxo-8-decenoate	1.72	–	3.3
Me 9,12-dioxo-10-dodecenoate[d]	2.09	5.1 (A)	3.7 (B)
Unidentified	–	2.6	3.3

[a]Quantitation based on flame ionization detection.
[b]Retention relative to methyl octanoate at 10.05 min.
[c]Based on schemes in Figure 5.
[d]Tentative identification.
[e]MS reported by Tressel et al. (34).

9,12-dioxo-10-dodecenoate from **II**. The third important fragmentation, C, between the peroxide ring and the other substituent produced 11% methyl octanoate from **I** and 6% pentane from **II**. Cleavage, D, on the side of the hydroperoxy carbon away from the ring, gave 3% pentane from **I** and 3% methyl octanoate from **II**.

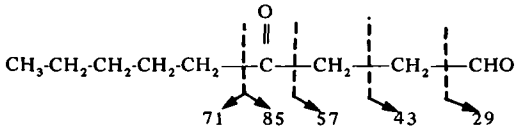
The 1,4-dicarbonyl compounds (Fig. 5, Table 3) detected among the volatiles from cyclic peroxides **I** and **II** apparently have not been identified before. The 4-oxo aldehydes were identified by the following characteristic mass ions: M, 154 (2); M-29, 125 (34); m-55, 99 (14); 83 (85); 71 (13); 55 (69); 29 (29); according to fragmentation in Scheme 5.



4-oxo-2-nonenal

SCHEME 5.

and M-29, 127 (6); 127-H_2O, 109 (16); M-57, 99 (24); 85 (30); 71 (27); 57 (100); 43 (76); and 29 (34); according to fragmentation in Scheme 6.



4-oxononanal

SCHEME 6.

The 4-oxononanal (0.2%) is apparently formed from hydrogen radical abstraction by the nonenal compound. Methyl 9,12-dioxo-10-dodecenoate was identified from the characteristic ion M-31, 209 (31); 197 (7); 153 (2); 139 (2); 125 (11); 111 (15); 97 (30); 83 (30); 55 (100); and 29 (12); according to the fragmentation in Scheme 7.

Substituted furans have been detected in soybean and cottonseed oils (35-38). At least three mechanisms have been postulated for the formation of 2-pentylfuran. Chang et al. (37) postulated but did not identify 4-oxononanal as a precursor which could give 2-pentylfuran by cyclization. Parsons (38) suggested decomposition of linoleate 9-hydroperoxy octadecadienoate and reaction with oxygen to form a vinyl hydroperoxide that can cyclize to form a furan. Frankel (23) suggested that 2-pentylfuran could be formed from decomposition of a 6-membered hydroperoxy epidioxide such as **II** via pentyl furaldehyde. We have identified and proven in this study that **II** is a 2-pentylfuran precursor (Table 3, Fig. 5). Since 4-oxo-2-nonenal is formed from **II**, it also may be a precursor of 2-pentylfuran (37). The 8(2-furyl) octanoate previously identified by Tressel et al. (34) can be formed by decomposition of the 6-membered hydroperoxy epidioxide **I** (Table 3, Fig. 5). Thus, the 6-membered hydroperoxy epidioxides are potential precursors of volatiles including substituted furans, 1,4-dicarbonyl compounds, aldehydes and esters that are known to have an impact on flavor of fats. The thermal fragmentation between the epidioxide and hydroperoxide group agrees with our previous studies of related cyclic peroxides (9,26) and is sufficiently predictable that it could be used as a structural tool for these types of compounds.

ACKNOWLEDGMENTS

We thank S. Hvizdos for assistance with high-performance liquid chromatography.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture over other firms or similar products not mentioned.

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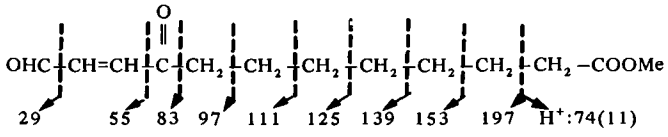

Me 9,12-dioxo-10-dodecenoate

SCHEME 7.

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Elevated Levels of Arachidonic Acid in Fish from Northern Australian Coastal Waters

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ABSTRACT

The fatty acid composition of 10 species of fish caught off the northwest coast of Australia (latitude 17°S) was examined. All species contained high levels of ω6 fatty acids (9.6-23.1% of total fatty acids) with arachidonic acid being the major ω6 fatty acid (5.9-14.8% of fatty acids). Docosatetraenoic and docosapentaenoic acids of the ω6 series accounted for 3-8% of the total fatty acids. The ratio of ω6 to ω3 fatty acids in these fish varied from 0.38 to 0.93, compared with an average ratio of 0.16 for fish from the northern hemisphere (latitude >30°N). The present data and figures from the literature indicate that the marine food chain in the southern hemisphere contains significant quantities of ω6 fatty acids.
Lipids 18:877-881, 1983.

INTRODUCTION

We have recently reported that fish caught off the northwest coast of Australia were rich in arachidonic acid and other ω6 fatty acids (1). Consumption of diets derived almost entirely from these fish for 2 weeks lead to a 3-fold increase in the proportion of arachidonic acid in the plasma lipids of a group of Australian aborigines compared with a smaller elevation in the proportion of the ω3 fatty acids (1).

It is the purpose of this communication to present the detailed fatty acid composition of these species of fish.

MATERIALS AND METHODS

This study was carried out on the northwest coast of Australia (latitude 17°S), 250 km northeast of Broome. In this area there are 10-11 m tides and the fish were caught in nets on both incoming and outgoing tides. The fish were photographed, and the fillets wrapped in aluminum foil and placed in containers of liquid N_2 within 1 hr of being caught. The samples were thus maintained until being analyzed some 4 months later.

Lipids were extracted from 10 g samples of fish with 20 vol of chloroform/methanol (2:1) containing 10 mg/l butylated hydroxytoluene (2). The lipid content was determined gravimetrically and the methyl esters of the fatty acids were prepared by saponification with methanolic KOH followed by esterification with boron trifluoride in methanol (2). The esters were separated using a 45 m × 0.5 mm id support-coated open-tubular (SCOT) glass capillary column coated with OV-275 on Gas Chrom R (Chromalytic Technology, Melbourne, Australia) and also a 70 m × 0.5 mm id SCOT column coated with SILAR 5CP on Gas Chrom R (Chromalytic Technology). The gas chromatograph was fitted with a flame ionization detector. The former column was operated by temperature programming from 135 to 195 C at 2 C/min, and the latter column was operated at 160 to 220 C at 2 C/min. Nitrogen was used as the carrier gas for both columns at a flow rate of 20 cm/sec. Standard methyl esters (Nu-Chek Prep, Elysian, MN) were routinely chromatographed to establish retention times and to determine the response factors for the different esters.

The presence of dimethyl acetals of aldehydes was verified by separation of these from the methyl esters by thin layer chromatography (TLC) on Silica Gel G using toluene as the solvent (3).

RESULTS AND DISCUSSION

The lipid content of the fish analyzed was low and ranged from 0.6 to 3.3 g/100 g wet weight (Table 1).

In most species the saturated fatty acids were the predominant group of fatty acids (Table 2). Palmitic acid was the major component, with stearic acid being the next most abundant. In two species there were significant amounts of myristic and pentadecanoic acids (mullet and queenfish). Dimethyl acetals of aldehydes with 16 and 18 carbons were detected in all species of fish.

Palmitoleic and oleic acids were the major monounsaturated fatty acids; eicosaenoic acid was detected in all species in low amounts and only a trace amount of docosaenoic acid was found.

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TABLE 1

The Muscle Lipid Content of Fish Caught Off the Northwest Coast of Australia (g/100 wet weight)

Common name	Genus	Number analyzed	Average weight (kg)	Lipid content
Sand whiting	Sillago	2	0.1	1.2
Barramundi	Lates	2	7.5	0.6
Spotted bat fish (butterfish)	Drephane	5	0.3	1.7
Red snapper	Lutjanus	2	0.75	1.4
Fork-tailed cat fish	Arius	8	0.75	1.4
Skippy (trevally)	Carangoides	2	0.75	2.0
Queenfish	Scomberoides	3	1.0	2.0
Mullet	Liza	6	1.0	3.0
Trevally	Caranx	5	1.0	1.8
Threadfin salmon	Poladactylus	4	1.0	3.3

The percentage of the ω6 fatty acids ranged from 9.6 to 23.1% of the total fatty acids. Arachidonic acid was the major ω6 fatty acid; however, 22:4 ω6 and 22:5 ω6 were also present in significant amounts. Linoleic and eicosatrienoic acids were found in all species and γ-linolenic (18:3 ω6) acid in two species (mullet and queenfish).

The ω3 fatty acids were the second most important group of components detected. Docosahexaenoic acid was the major fatty acid in this group in all but one of the species analyzed. Eicosapentaenoic and docosapentaenoic acids were also present in substantial amounts. Linolenic acid was found in all species and 18:4 ω3 was detected in levels of ca. 1% in two species (mullet and queenfish).

The low fat content of these fish (Table 1) suggests that the fatty acids were probably derived mainly from muscle phospholipids. This would increase the polyunsaturated fatty acids as a proportion of the total fatty acids.

A considerable amount of information has been published on the fatty acid composition of the marine food chain above latitude 30°N (4,5). These results indicate that this chain or food web is rich in ω3 fatty acids and poor in ω6 fatty acids. Castell (6) has estimated that the average ratio of ω6 to ω3 fatty acids in fatty acids in marine fish from northerly latitudes (30°N) is 0.16 ± 0.1. In the present study, the ω6 to ω3 ratio ranged from 0.38 to 0.93. A typical chromatogram of the fatty acid methyl esters from a fish in this study is shown in Figure 1, and another of an ω3-rich fish (blue grenadier, *Macruronus novaezealandie*), found in the waters between Australia and New Zealand is included for comparison.

Although there has been comparatively little work on the fatty acid composition of the marine food chain in southerly latitudes, the data available indicate that arachidonic acid is a major fatty acid. Two reports from the Indian Ocean (latitude 5°N to 35°S) showed 20:4 ω6 levels from 3 to 14% of total fatty acids in 8 species of fish; in these fish, 20:5 ω3 levels were lower than those of 20:4 ω6 (7,8). Johns et al. (9) have shown that molluscs collected from Australian waters (2 sites, latitudes 16°S and 37°S) contained 20:4 ω6 and 20:5 ω3 in approximately equal proportions; they have also shown that several species of red and brown algae contained high levels of 20:4 ω6 (10). Pearson (11) has reported that lobster caught near Sydney (latitude 34°S) had a lower ratio of 20:4 ω6 to 20:5 ω3 compared with the ratio of these two fatty acids in lobster which had been obtained from tropical waters.

Analysis of four molluscs and one crustacean from New Zealand showed high levels of 20:4 ω6 (13%) in one of the molluscs and the crustacean (12). Pearson (11,13) analyzed 15 species of fresh fish purchased in Sydney and showed that three species (cod, leather jacket and whiting) had arachidonate levels in excess of 5.9% of the total fatty acids; in these species the ratio of 20:4 ω6 to 20:5 ω3 was ca. 1. For all species analyzed, however, the ω6 to ω3 ratio was low (range 0.05-0.24).

Gibson (14) has recently analyzed 20 different fish purchased in Adelaide (mostly local, latitude 35°S) and shown arachidonic acid levels of from 2.5 to 15.8% and ω6 to ω3 ratios which varied from 0.11 to 1.89.

The data presented here together with other data from the southern hemisphere indicate

TABLE 2

Mean Fatty Acid Composition of Muscle Lipids from Fish Caught Off the Northwest Coast of Australia (g/100 g total fatty acids)

Fatty acids n	Sand whiting 2	Barramundi 2	Spotted bat fish 5	Red snapper 2	Cat fish 8	Trevally 2	Queenfish 3	Mullet 6	Skippy 5	Threadfin salmon 4
14:0	1.1	0.6	2.0	1.1	2.1	0.7	4.6	3.9	1.4	3.3
15:0	1.9	0.3	1.4	0.6	0.8	0.7	3.2	5.0	1.0	0.7
16:0 DMA[a]	1.0	3.5	2.0	0.8	2.9	3.4	1.3	1.1	0.8	0.4
16:0	18.3	20.8	21.1	18.8	21.1	15.1	18.8	21.5	20.5	26.7
17:0	1.8	1.0	2.2	1.3	1.4	1.3	1.8	1.8	1.5	1.3
18:0 DMA	0.6	1.3	0.8	1.6	0.7	0.7	1.1	1.4	0.6	0.4
18:0	8.0	9.3	9.7	8.9	10.6	11.6	8.3	6.1	10.5	8.4
20:0	tr[b]	tr	tr	tr	tr	tr	0.2	0.2	0.4	0.4
22:0	0.3	0.3	0.4	tr	tr	tr	0.5	0.8	0.5	0.5
Total saturates	33.0	37.1	39.7	33.1	39.6	33.5	39.8	41.8	37.4	42.1
16:1	2.4	3.5	3.4	2.4	4.3	1.6	9.4	10.4	3.8	7.8
18:1	8.2	13.6	10.1	13.9	14.9	6.8	8.3	6.5	12.1	15.6
20:1	0.4	0.2	1.6	0.9	1.6	0.3	1.4	2.1	0.6	1.0
22:1	tr	–[c]	tr	–	tr	–	–	–	–	–
Total monoenes	11.0	17.3	15.1	17.2	20.8	8.7	19.1	19.0	16.4	24.4
18:2 ω6	1.1	2.0	1.1	1.9	1.8	1.0	1.6	1.3	1.4	0.5
18:3 ω6	–	tr	–	–	–	–	1.1	0.9	–	–
20:3 ω6	0.6	0.4	0.6	0.3	0.6	0.4	0.5	0.5	0.2	0.1
20:4 ω6	14.8	12.3	9.5	11.1	10.9	9.7	6.1	6.6	6.9	5.9
22:4 ω6	4.0	1.8	5.6	2.5	3.0	2.9	1.5	1.3	1.8	1.4
22:5 ω6	2.6	2.9	2.8	2.4	2.2	3.7	2.3	2.2	2.1	1.7
Total ω6	23.1	19.4	19.6	18.2	18.5	17.7	13.1	12.8	12.4	9.6
18:3 ω3	0.3	0.8	0.4	0.4	0.5	0.3	0.7	0.5	0.5	0.4
18:4 ω3	tr	tr	–	–	–	–	1.1	1.0	–	–
20:3 ω3	0.2	–	tr	–	0.2	–	–	–	0.2	0.2
20:5 ω3	9.9	2.8	5.5	4.2	4.7	8.0	6.6	10.4	5.6	4.5

TABLE 2 (continued)

Fatty acids	Sand whiting	Barramundi	Spotted bat fish	Red snapper	Cat fish	Trevally	Queenfish	Mullet	Skippy	Threadfin salmon
22:5 ω3	5.2	3.0	6.7	3.2	3.6	2.4	5.0	4.4	3.9	2.3
22:6 ω3	14.2	18.1	11.1	21.8	10.8	27.5	11.6	6.4	22.5	15.2
Total ω3	29.8	24.7	23.7	29.6	19.8	38.2	25.0	22.7	32.7	22.6
NI[d]	3.1	1.5	1.9	1.9	1.3	1.9	3.0	3.7	1.2	1.3
ω6/ω3	0.78	0.79	0.83	0.61	0.93	0.46	0.52	0.56	0.38	0.42

[a]DMA = Dimethyl acetal.
[b]tr = trace, less than 0.1%.
[c]– = not detected.
[d]NI = not identified.

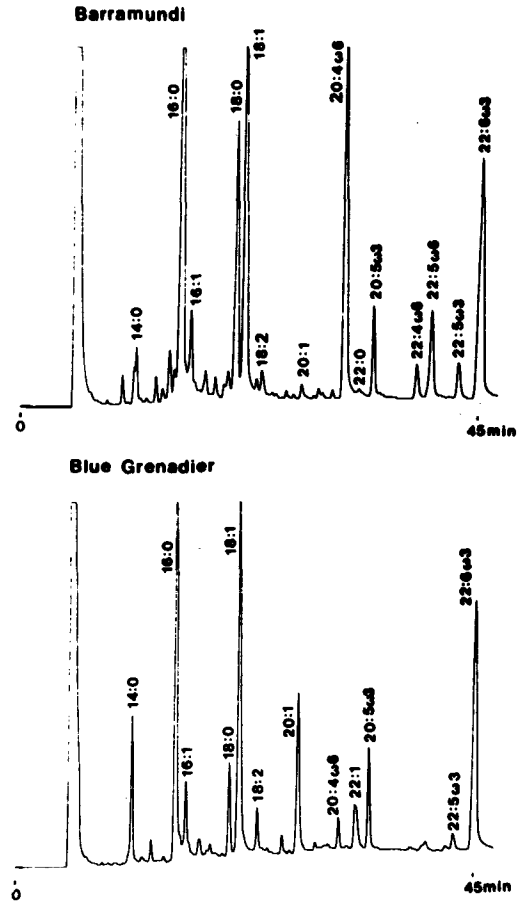

FIG. 1. Separation of fatty acid methyl esters from barramundi and blue grenadier muscle lipids on an OV-275 SCOT capillary column. The ω6 to ω3 ratio for the barramundi was 1.21 and for the blue grenadier 0.14.

that the marine food chain in this region contains substantial levels of ω6 fatty acids, particularly arachidonic acid. At present there is insufficient data for a complete understanding of the food chain or the reasons why arachidonic acid accumulates in this food chain. Although the potential physiological significance of these observations to human nutrition is not yet clear, it should be appreciated that arachidonic acid is a major component of cell membranes and a precursor of the prostaglandins, including those involved in hemostatic function (15).

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METHODS

Stereospecific Analysis of Triglycerides of *Glycine max, Glycine soya, Avena sativa* and *Avena sterilis* Strains[1]

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ABSTRACT

A rapid method for the stereospecific analysis of triglycerides based on enzymatic hydrolysis on thin layer plates was applied to a number of *Glycine max, Glycine soya, Avena sativa* and *Avena sterilis* strains. The percentage of each fatty acid on the *sn*-1-, *sn*-2- and *sn*-3-positions was linearly related to the total percentage of the fatty acid in the triglyceride. Large deviations from the common triglyceride pattern were not found.
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INTRODUCTION

The physical properties (1), stability (2) and nutritional value (3) of fats and oils may depend on the triglyceride structure, and it may be possible to modify triglyceride structure of plant oils by breeding (4). Fatemi and Hammond examined the glyceride structure of 17 soybean varieties by a stereospecific analysis (5) and silver ion chromatography (6). They concluded that the stereospecific analysis predicted the results of the silver ion chromatography fairly well. Plots of the percentage of a fatty acid on the *sn*-1-, *sn*-2- or *sn*-3-position vs the percentage of the fatty acid in the whole triglyceride gave straight lines for the varieties examined. One variety, PI 68-788, gave values that deviated significantly more than the others from this straight-line relation and also gave deviant values by silver ion chromatography. Fatemi and Hammond suggested that this variety might be a genetic deviant and that it should be possible to search for genetic deviants by performing stereospecific analyses. One obstacle to this goal is that previous methods for stereospecific analysis have been time-consuming and laborious. We wish to report an adaptation of stereospecific analysis that is significantly faster and easier to perform than those previously reported. It is based on the on-plate hydrolysis procedures of Dutta et al. (7,8).

This rapid technique has been applied to 48 plant introductions of soybeans *(Glycine max)* and 47 plant introductions of the wild relative of the soybean, *Glycine soya.* Because little variation was found in the glyceride structure of soybeans and oats tend to be less uniform in fatty acid composition than soybeans (9,10), the technique also was applied to 60 varieties of the cultivated oat *(Avena sativa)* and 43 varieties of its wild relative *Avena sterilis.*

METHODS

The following material was obtained from the Agronomy Department at Iowa State University: strains of *G. soya*, progeny from the cultivar Steel that had been treated with ethyl methanesulfonate (EMS), samples of PI (plant introduction) 68-788, Amsoy, crosses of PI 68-788 and Amsoy and strains of *A. sativa* and *A. sterilis.* Other plant introductions of *G. max* were obtained from the U.S. Regional Soybean Laboratory, Urbana, IL. Seeds of Steel treated with EMS (11) were advanced two generations in Puerto Rico and once in Ames, and individual plants were harvested. PI 68-788 was crossed with Amsoy in Ames and advanced one generation in Puerto Rico and one generation in Ames, and individual plants were harvested.

To extract the triglyceride, 5 g of soybeans or oats were crushed with a hydraulic press in plastic cups fitted with stainless-steel cylinders (11). The crushed material was soaked in 12 ml of hexane for 6 hr, the hexane was decanted, and the residual meal was washed twice with 8 ml of hexane. The soybean extract was used without further purification, but the oat triglycerides were purified by thin layer chromatography on Silica Gel G plates developed with hexane/ether/acetic acid (50:50:0.5, v/v/v). The triglyceride was visualized by spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol and viewing under ultraviolet light. The triglyceride band was eluted with ether. This was necessary for oats because of the considerable amounts of diglyceride in the extracts.

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The lipid hydrolysis procedure was an adaptation of that of Dutta et al. (7). Ca. 25 mg pancreatic lipase (Sigma Chemical Co., St. Louis, MO) in 0.25 ml water was applied to a Silica Gel G (Analabs, New Haven, CT) plate 0.5 mm thick with a streaker (Applied Science, State College, PA). Ca. 25 mg of triglyceride in hexane (1:1, v/v) was overlaid as evenly as possible on the enzyme band. The plate was placed immediately on a hot plate at 40 C. After 3 min, the plate was exposed to the vapors from concentrated HCl in a closed chamber for 1 min to stop the lipase reaction. Excess HCl was removed by blowing a stream of air across the plate for 1 min. The lipid material was moved from the reaction zone by developing the plate three times with diethyl ether to a height 2 cm above the line of application. The ether was removed by blowing a stream of air across the plate, and the plate was developed to the top with hexane/ether/acetic acid (50:50:0.5, v/v/v). The lipid bands were visualized with dichlorofluorescein as before. Bands representing 2-monoglyceride and *sn*-1,2(2,3)-diglyceride (R_f 0.12 and 0.27, respectively) were scraped from the plate and eluted with ether.

The *sn*-1,2(2,3)-diglycerides, which usually amounted to 6-7 mg, were phosphorylated according to Brockerhoff et al. (12) as follows. The diglycerides in 0.2 ml of ether were added dropwise to a solution of 0.2 ml ether, 0.2 ml of pyridine and 0.1 ml phenyl dichlorophosphate (Sigma Chemical Co.). After 1 hr at room temperature, 1 ml of pyridine, 0.6 ml ether and several drops of water were added with cooling. The reaction mixture was mixed with 6 ml of methanol, 5 ml of chloroform and 5 ml of distilled water in a separatory funnel, and the lower layer was recovered and evaporated.

The synthetic phosphatide was hydrolyzed on a silica gel plate by a modification of the method of Dutta and Das (8). A phospholipase A_2 solution (2.5 mg of *Ophiophagus hannah* venom, Sigma Chemical Co., in 0.25 ml water) was applied to a 0.5-mm Silica Gel G plate. The phosphatide in ether/methanol (95:5, v/v) was applied evenly over the enzyme band, and the plate was placed immediately in a chamber saturated with ether vapors for 40 min at room temperature. After hydrolysis of the phosphatides, the plate was developed to the top with chloroform/methanol/14 M ammonium hydroxide (85:15:2, v/v/v). The phosphatide bands were revealed by spraying with 0.1% Rhodamine 6G in ethanol. The lysophosphatidylphenol (R_f 0.15) was scraped from the plate and eluted with chloroform/methanol (2:1, v/v).

The triglyceride, monoglyceride and lysophosphatidylphenol were converted to methyl esters by transesterification in 1 N methanolic sodium methoxide for 1 hr at room temperature. The fatty acid composition was determined by gas chromatography on a Beckman GC5 instrument fitted with a flame detector and a 1.8 m x 3.3 mm column packed with 15% EGSSX on 100/200 mesh Chromosorb P (Applied Science). The column temperature was 180 C, and nitrogen was the carrier gas at 50 ml/min. Peaks were integrated electronically with a Commodore Pet 2001 computer (Commodore Business Machines, Santa Clara, CA).

RESULTS AND DISCUSSION

Our procedure for stereospecific analysis makes it possible for one person to complete ca. 10 analyses a day. If it were not necessary to remove the diglycerides from the thin layer plate for phosphorylation, the method would be considerably faster; however, on-plate phosphorylation was unsuccessful. We also tried to adapt the diglyceride kinase method of Lands et al. (13) to on-plate methods, but we were not able to prepare enzyme concentrates that were sufficiently active, and the enzyme was not active at all in the presence of silica gel.

It is generally agreed that diglycerides generated by lipase are less representative than those generated by Grignard reagent (14); however, analyses of 3 soybean triglycerides by our method and Brockerhoff's method (15) gave almost identical results for the *sn*-1-position. Palmitic acid on the *sn*-2-position of soybean triglyceride is believed to be an artifact of rearrangement during lipase hydrolysis. The average amount for the 48 *G. max* analyses by the on-plate method was 0.73 ± 0.06. This may be compared with 15 analyses by Fatemi and Hammond (5) using conventional lipase hydrolysis which gave 0.80 ± 0.17.

Analyses were completed on 48 soybean plant introductions *(G. max)* and 47 strains of the closely related species, *G. soya.* The statistical analyses are summarized in Table 1. The percentage of each fatty acid on the 3 glycerol positions is plotted vs the percentage of the fatty acid in the whole triglyceride for *G. max* in Figures 1-5. Some of the lines in the figures show less than the 48 or 47 points because some points were too close together to plot separately.

The results are similar to those reported by Fatemi and Hammond (5) for *G. max* and *G. soya* and for other vegetable oils (14-19). There is very little saturated fatty acid on the *sn*-2-position, and the *sn*-1-position is consistently richer in palmitic and linolenic acids

TABLE 1

Linear Regression of the Percentage of Fatty Acids at the 3 Positions of Glycerol vs the Amount in the Whole Triglyceride

		Glycine max							
		Plant introductions				Steel EMS			
Fatty acid	Position	Slope	Intercept	R^2	No.[a] of deviants	Slope	Intercept	R^2	No.[a] of deviants
16:0[b]	1	1.67 ± 0.02	0.83 ± 1.21	0.82	3				
	3	1.27 ± 0.10	−0.92 ± 1.21	0.72	3				
18:0[b]	1	1.52 ± 0.10	0.42 ± 0.37	0.85	4				
	3	1.41 ± 0.09	−0.21 ± 0.36	0.83	4				
18:1	1	0.86 ± 0.02	0.46 ± 0.63	0.97	4				
	2	0.84 ± 0.03	2.03 ± 0.87	0.94	3	0.96 ± 0.10	0.18 ± 2.69	0.72	3
	3	1.30 ± 0.04	−2.49 ± 0.96	0.97	2				
18:2	1	0.82 ± 0.04	1.76 ± 2.31	0.88	1				
	2	0.79 ± 0.05	26.92 ± 2.42	0.87	1	1.08 ± 0.14	0.78 ± 7.28	0.61	1
	3	1.39 ± 0.05	−28.69 ± 2.42	0.95	1				
18:3	1	1.01 ± 0.04	0.69 ± 0.25	0.94	4				
	2	0.98 ± 0.03	−0.30 ± 0.21	0.96	3	0.93 ± 0.25	−0.06 ± 1.89	0.27	1
	3	1.00 ± 0.04	−0.39 ± 0.30	0.92	3				

		Glycine max				*Glycine soya*			
		PI 68-788 Amsoy							
Fatty acid	Position	Slope	Intercept	R^2	No.[a] of deviants	Slope	Intercept	R^2	No.[a] of deviants
16:0[b]	1					1.52 ± 0.14	3.94 ± 1.68	0.71	1
	3					1.42 ± 0.15	−4.13 ± 1.71	0.68	1
18:0[b]	1					1.29 ± 0.11	1.25 ± 0.40	0.74	2
	3					1.64 ± 0.11	−1.04 ± 0.39	0.83	3
18:1	1					0.83 ± 0.04	1.81 ± 0.72	0.91	2
	2	1.02 ± 0.04	−2.16 ± 1.10		4	1.05 ± 0.06	−0.45 ± 1.11	0.87	1
	3					1.12 ± 0.07	−1.36 ± 1.28	0.85	2
18:2	1					0.98 ± 0.07	−11.81 ± 4.00	0.80	3
	2	1.03 ± 0.05	14.49 ± 2.58		3	0.70 ± 0.07	31.09 ± 3.80	0.69	3
	3					1.37 ± 0.10	−19.23 ± 5.30	0.80	3
18:3	1					1.18 ± 0.06	−0.36 ± 0.76	0.89	3
	2	1.59 ± 0.09	2.38 ± 0.73		2	0.86 ± 0.08	0.51 ± 0.95	0.74	3
	3					0.96 ± 0.09	−0.15 ± 1.12	0.71	1

[a]Individuals falling > 2 standard deviations from the regression line.

[b]The amount of 16:0 and 18:0 at the *sn*-2-position was less than the experimental error.

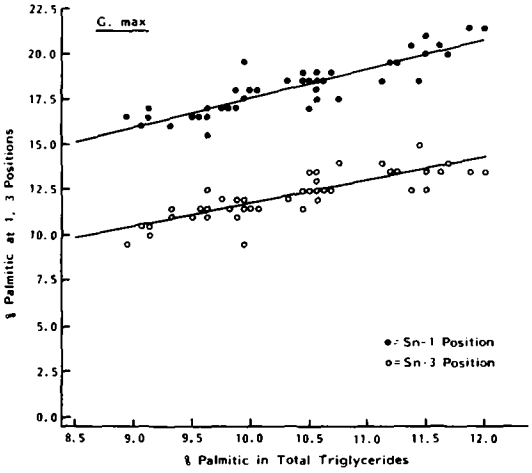

FIG. 1. The percentage of palmitic acid on the *sn*-1- and *sn*-3-positions of glycerol vs the percentage of palmitic acid in the whole triglyceride for *G. max*.

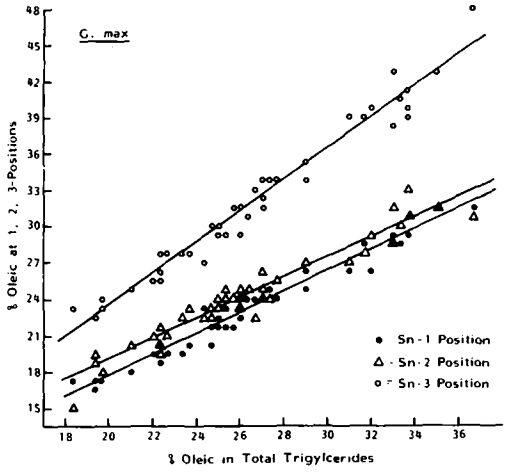

FIG. 3. The percentage of oleic acid on the *sn*-1-, *sn*-2- and *sn*-3-positions of glycerol vs the percentage of oleic acid in the whole triglyceride for *G. max*.

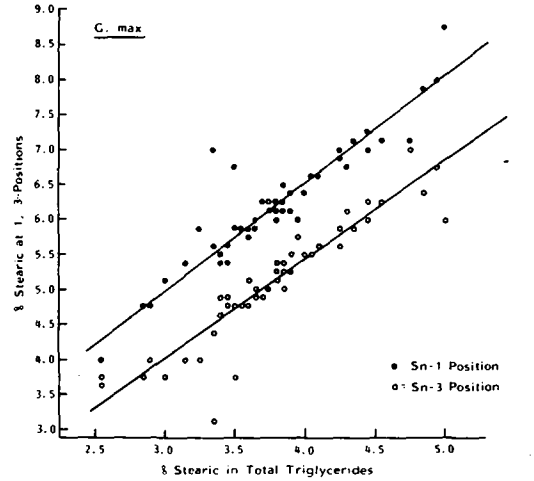

FIG. 2. The percentage of stearic acid on the *sn*-1- and *sn*-3-positions of glycerol vs the percentage of stearic acid in the whole triglyceride for *G. max*.

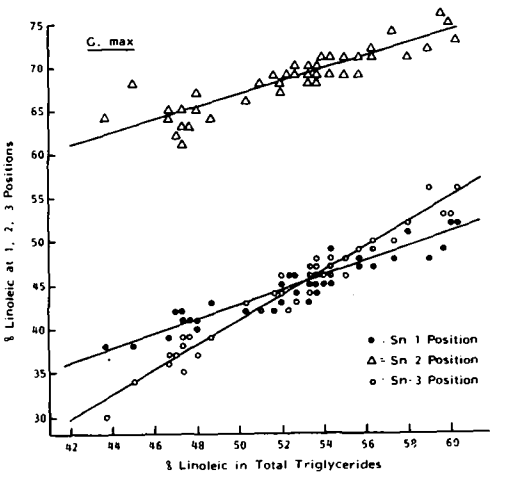

FIG. 4. The percentage of linoleic acid on the *sn*-1-, *sn*-2- and *sn*-3-positions of glycerol vs the percentage of linoleic acid in the whole triglyceride for *G. max*.

than is the *sn*-3-position. The *sn*-2-position is enriched with linoleic acid.

We also applied our method for the *sn*-2-position only to 40 samples of the variety Steel that had been treated with the mutagen EMS and to PI 68-788, Amsoy and crosses of PI 68-788 with Amsoy. Linear regressions for this material are also given in Table 1. The slopes for the *sn*-2-position obviously vary with the population chosen. Probably the data for the 48 plant introductions is a good estimate of the regressions for a random sample of soybeans. The results for *G. max* and *G. soya* are similar.

The results of the stereospecific analyses for 60 strains of *A. sativa* and 47 strains of *A. sterilis* are presented in Table 2, and plots of the percentage of each fatty acid on the three glycerol positions vs the percentage in the whole fat is given for *A. sativa* in Figures 6-10. In *A. sativa*, an average of 3.09 ± 1.11% palmitic

acid was found on the *sn*-2-position. This may reflect rearrangement of the diglycerides during lipolysis. But there is ca. 1.5-2 times more palmitic in oats than soy, and the amount of palmitic found on the *sn*-2-position is more than twice that found in soybeans. As in other vegetable oils, the *sn*-2-position is consistently richer in unsaturated fatty acids, but in contrast to *Glycine* sp., oleic acid is favored over linoleic acid on the *sn*-2-position. The *sn*-3-position usually contains slightly more saturated fatty acids than the *sn*-1-position. The percentage of linolenic acid tends to be greater on the *sn*-3-position than on *sn*-1. The results for *A. sativa* and *A. sterilis* are similar.

The data are fitted reasonably well by simple linear regressions in all instances. R^2 values tended to be less for *G. soya* than for *G. max* and less for *Avena* sp. than *Glycine* sp. The algebraic sum of the intercepts for each fatty acid is very close to zero, and the average of the slopes is nearly 1. This is required for this sort of plot if the linear regression fits the data well because the 3 ordinate values for a fatty acid

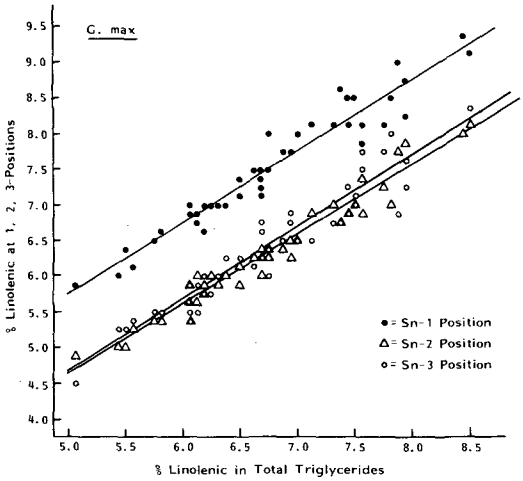

FIG. 5. The percentage of linolenic acid on the *sn*-1-, *sn*-2- and *sn*-3-positions of glycerol vs the percentage of linolenic acid in the whole triglyceride for *G. max*.

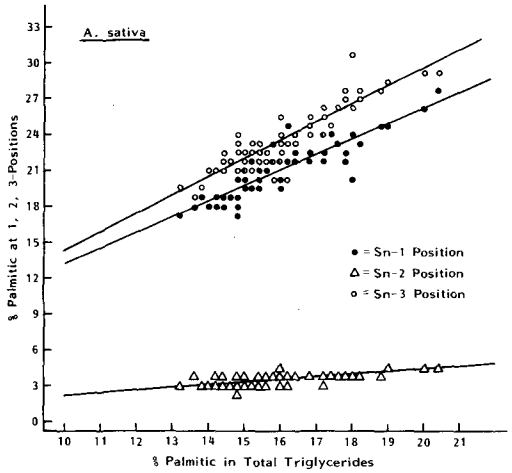

FIG. 6. The percentage of palmitic acid on the *sn*-1-, *sn*-2- and *sn*-3-positions of glycerol vs the percentage of palmitic acid in the whole triglyceride for *A. sativa*.

TABLE 2

Linear Regression of the Percentage of Fatty Acid at the 3 Positions of Glycerol vs the Amount in the Whole Triglyceride

Fatty acid	Position	*Avena sativa* Slope	Intercept	R^2	No.[a] of diviants	*Avena sterilis* Slope	Intercept	R^2	No.[a] of deviants
16:0	1	1.28 ± 0.09	0.47 ± 1.40	0.79	4	1.02 ± 0.12	3.56 ± 2.08	0.66	3
	2	0.21 ± 0.03	0.19 ± 0.42	0.52	3	0.31 ± 0.03	−1.20 ± 0.58	0.70	3
	3	1.51 ± 0.10	−0.66 ± 1.59	0.80	4	1.66 ± 0.13	−2.14 ± 2.35	0.80	2
18:0[b]	1	1.47 ± 0.11	−0.26 ± 0.24	0.74	2	1.22 ± 0.11	0.43 ± 0.31	0.75	2
	3	1.10 ± 0.14	0.92 ± 0.30	0.50	2	1.41 ± 0.12	0.00 ± 0.33	0.78	2
18:1	1	1.17 ± 0.16	−9.34 ± 6.40	0.49	1	1.01 ± 0.08	−3.78 ± 3.54	0.79	3
	2	0.93 ± 0.15	12.37 ± 5.98	0.41	3	0.78 ± 0.09	19.38 ± 3.94	0.64	3
	3	0.91 ± 0.17	−2.97 ± 7.10	0.32	4	1.21 ± 0.12	−15.60 ± 4.96	0.73	3
18.2	1	0.91 ± 0.13	0.45 ± 5.12	0.46	3	0.85 ± 0.07	3.35 ± 2.29	0.79	1
	2	1.09 ± 0.12	1.70 ± 4.87	0.57	3	0.89 ± 0.07	10.09 ± 2.45	0.78	1
	3	1.00 ± 0.18	−2.31 ± 7.09	0.35	3	1.26 ± 0.10	−13.44 ± 3.28	0.80	2
18:3	1	1.03 ± 0.08	−0.16 ± 0.16	0.75	4	0.97 ± 0.07	−0.01 ± 0.18	0.84	3
	2	0.66 ± 0.09	0.42 ± 0.19	0.49	5	0.77 ± 0.05	0.17 ± 0.14	0.84	2
	3	1.31 ± 0.08	−0.25 ± 0.17	0.82	2	1.26 ± 0.09	0.16 ± 0.25	0.82	2

[a]Individuals falling > 2 standard deviations from the regression line.

[b]The amount of 18:0 at the *sn*-2-position was less than the experimental error.

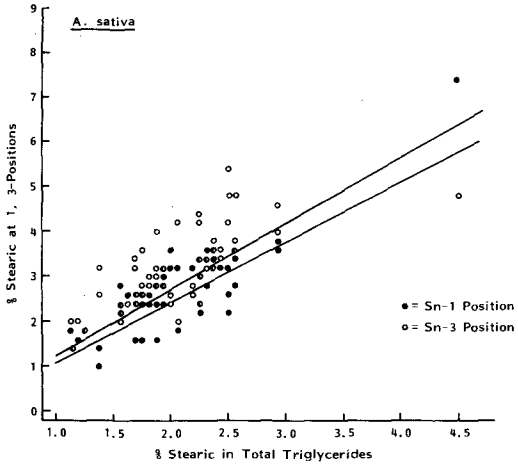

FIG. 7. The percentage of stearic acid on the *sn*-1- and *sn*-3-positions of glycerol vs the percentage of stearic acid in the whole triglyceride for *A. sativa.*

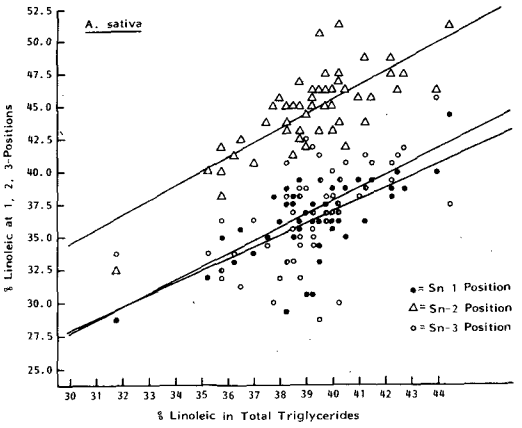

FIG. 9. The percentage of linoleic acid on the *sn*-1-, *sn*-2- and *sn*-3-positions of glycerol vs the percentage of linoleic acid in the whole triglyceride for *A. sativa.*

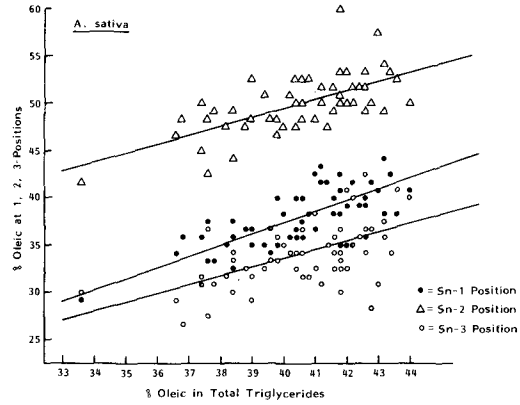

FIG. 8. The percentage of oleic acid on the *sn*-1-, *sn*-2- and *sn*-3-positions of glycerol vs the percentage of oleic acid in the whole triglyceride for *A. sativa.*

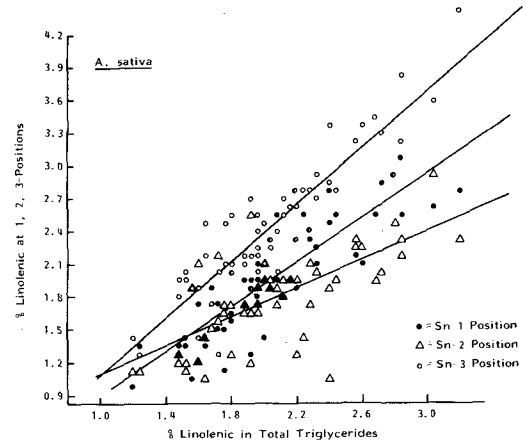

FIG. 10. The percentage of linolenic acid on the *sn*-1-, *sn*-2- and *sn*-3-positions of glycerol vs the percentage of linolenic acid in the whole triglyceride for *A. sterilis.*

must average to its value on the abscissa. The slopes and intercepts of the linear regressions for *G. max* triglycerides are quite different from those reported by Fatemi and Hammond (5). The most striking difference is that in our data there are quite large intercepts for linoleic acid in the *sn*-2- and *sn*-3-positions, and the slopes are less than 1. Fatemi and Hammond found a slope of 1.34 and intercept −0.84 for *sn*-2 and slope 0.94 and intercept −3.45 for *sn*-3. These differences seem to be caused by the particular plant materials chosen for the plots. Fatemi and Hammond selected material from a breeding program to give a wide range of fatty acid compositions. Their regressions also are based on fewer strains.

Although the data are fitted well by a linear regression, plots such as those given in Figures 1-10 obviously must go through 0,0 and 100,100 because when there is 0 or 100% of a fatty acid present, its amounts on the 3 glycerol positions must be 0 or 100%, respectively. Only a line with a slope of 1 can go through these points and have the same slope from 0 to 100. Thus, the lines passing through our data cannot be linear all the way from 0 to 100% of a fatty acid because frequently the slopes $\neq 1$. A slope of 1 means that the amount of a fatty acid placed on a glycerol position is exactly proportional to the amount in the triglyceride. A slope greater than 1 means that increasing proportions of a fatty acid are being placed on a particular position as the percentage in the triglyceride increases. When an intercept is positive,

it means that placement of a fatty acid on that position is favored, and if the intercept is negative, it means that there is a bias against placement on that position. For example, for *G. max* and the *sn*-2-position, linoleic acid had an intercept of 26.9 and a slope 0.79. This indicates that, in the range of our data, as the linoleic acid percentage in the oil increased, the proportion being placed on the *sn*-2-position was decreasing because the slope of the line was less than 1. But the large positive intercept means that the placement of linoleic acid on the *sn*-2 position is strongly favored. Possibly, if our data covered a larger range, we could observe a significant curvature in the lines.

Figures 1-10 show that none of the individual analyses deviated markedly from the regression lines. Similar results were obtained for the data not plotted here. Tests indicated that the individual analyses were normally distributed about the regression lines. Individuals that deviated from the regression by more than 2 standard deviations were identified. The number of such deviants should be ca. 2 in a normally distributed sample of the size used, and this is about the number identified. Such individual selections would need to be regrown and tested again to see if their deviation from the regression was genetically controlled. PI 68-788, identified as a possible deviant by Fatemi and Hammond (5), deviated from the regression lines for the *sn*-2-position of *G. max* by ca. 2 standard deviations, an amount comparable to that found by Fatemi and Hammond. Thus, its deviation does seem to be reproducible, although not very large. The analyses for PI 68-788 were made before our method for the *sn*-1-position was perfected, so data for the *sn*-1- and *sn*-3-positions are not available. One must conclude that large deviations from the common triglyceride pattern are rare in *G. max* and *G. soya* and that the incidence of such deviations is not greatly increased by treatment with the mutagen EMS.

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Ceramides from the Sponge *Dysidea etheria*[1]

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ABSTRACT

Ceramides have been isolated from the polar organic extracts of the sponge *Dysidea etheria*. The ceramides proved to be amides of sphingosine with either normal, saturated fatty acids or saturated α-hydroxy fatty acids. Sphingosine was identified by isolation from the hydrolysis reaction mixture, acetylation and comparison of its physical and spectral properties with those reported for authentic sphingosine triacetate. The acids were identified by gas chromatography-mass spectrometry analysis of their respective methyl esters.

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INTRODUCTION

Our investigations of the chemistry of Bermudian sponges (1,2) have recently focused on *Dysidea etheria*, a distinctively blue-colored inhabitant of the shallow, low light zones of Bermuda's calm, inshore waters. In the course of isolating the new sesquiterpene lactone 1(3) from the weakly antibiotic polar fractions of the dichloromethane-soluble extracts, we encountered modest quantities of a white solid which ultimately proved to be a mixture of ceramides.

In this report, we describe this first isolation of sphingosine derivatives from a sponge and their characterization as amides of sphingosine and saturated fatty acids and α-hydroxy fatty acids.

EXPERIMENTAL

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 250 MHz multinuclear Fourier transform spectrometer, using $CDCl_3$, d_6-acetone, d_5-pyridine, pyridine or d_8-THF as solvent and internal standard; chemical shifts are reported in ppm (δ units) relative to tetramethylsilane (δ = 0). Mass spectra were obtained with a VG MM-16F mass spectrometer operating at 70 eV in the electron impact mode or a VG SR-7070 HE operating in the fast atom bombardment mode. IR spectra were obtained with a Beckman IR-20 spectrophotometer and optical rotations with a Carl Zeiss circle polarimeter.

Collection and Extraction of *Dysidea etheria*

Specimens (dry wt 350 g) were collected from a variety of shallow water (2-6 m deep) locations in Harrington Sound and Castle Harbour, Bermuda, in August 1982. The sponges were stored in acetone at −5 C prior to extraction. The acetone extracts were decanted and filtered; the sponges were then ground in a Waring blender and extracted with acetone (twice, 24 hr each), then with dichloromethane (3 times, 24 hr each). The combined acetone extracts were reduced, in vacuo, to an aqueous suspension. The dichloromethane extracts were then equilibrated with the aqueous suspension; subsequent evaporation of the dichloromethane phase gave 17.4 g of crude extract.

Isolation of Ceramides

The dichloromethane-soluble extract (17.4 g) was chromatographed on Florisil (350 g), eluting with combinations of hexane, ethyl acetate and methanol of gradually increasing polarity. EtOAc/MeOH (97:3), EtOAc/MeOH (95:5) and EtOAc/MeOH (90:10) eluted 3 fractions (302, 276 and 51 mg, respectively), each of which contained the ceramides as indicated by thin layer chromatography (TLC) (SiO_2, THF/hexane, 1:1) R_f 0.26 and 0.35. Detection was best achieved with phosphomolybdic acid. The crude fractions were individually submitted to gel permeation on Sephadex LH-20 (125 × 2 cm), using CH_2Cl_2/MeOH (1:1), to give 64-, 160- and 23-mg fractions, respectively, each highly enriched in sphingosine derivatives (as indicated by TLC). These 3 fractions were combined, washed with cold methanol and then purified by low pressure chromatography (100 g, SiO_2, 37-53μ,N_2, 25 psi), using hexane/tetrahydrofuran (1:1) to obtain 2 fractions. The minor fraction (32 mg, R_f 0.35) contained a mixture of N-acylsphingosines whose spectral data were similar to those previously reported (4). The major fraction (44 mg, R_f 0.26) contained a mixture of α-hydroxy-N-acylsphingosines, ν_{max}^{KBr} 3330, 2912, 2842, 1624, 1517, 1447 cm^{-1}; ^{1}H-NMR (d_8-

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THF): δ7.39 1H, br d, J = 8.9), 5.70 (1H, dt, J = 15.5, 6.3), 5.49 (1H, dd, J = 15.5, 6.3), 4.79 (1 OH, d, J = 4.8), 4.43, (1 OH, br d, J = 5.2), 4.13 1H ddd, J = 6.3, 5.2, 5.0), 3.97 (1H, m), 3.77-3.90 (3H, overlapping m), 3.58 (1H, m), 2.04 (2H, m), 1.95 (2H, m), 1.45-1.65 (XH*, m), 1.30 (XH*, br s), 0.88 (6H, br t); ^{13}C-NMR (pyridine): δ175.00 (s), 132.15 (d), 131.58 (d), 72.50 (d), 71.94 (d), 61.32 (t), 55.46 (d), 35.18 (t), 32.16 (t), 31.53 (2C,t), 29.41-29.03 (XC*, t), 25.26 (t), 22.32 (2C, t), 13.66 (2C, q).

Acetylation of α-Hydroxy-N-acylsphingosines

A mixture of α-hydroxy-N-acylsphingosines (11 mg) was added to 0.5 ml pyridine, 2 mg 4-N,N-dimethylaminopyridine and 0.5 ml acetic anhydride. The flask was flushed with nitrogen, heated to 35 C for 1.5 hr and left at room temperature overnight. The organic solvents were evaporated and the residue separated on Sephadex LH-20 (125 x 2 cm) by elution with CH_2Cl_2/MeOH (1:1) to yield 11 mg of the triacetylated N-acylsphingosine, $\nu_{max}^{CHCl_3}$ 3409, 2887, 2839, 1723, 1668, 1347 cm^{-1}; ^{1}H-NMR (d_6-acetone): δ7.13 (1H, brd, J = 8.5), 5.78 (1H, dt, J = 15.3, 6.5), 5.46 (1H, dd, J = 15.3, 6.5), 5.31 (1H, dd, J = 6.7, 6.5), 4.95 (1H, br t, J = 6.7), 4.35 (1H, m, J = 7.9, 6.7, 4.7, 8.5), 4.21 (1H, dd, J = 11.6, 7.9), 4.07 (1H, dd, J = 11.6, 4.7), 2.13† (3H, s), 2.02† (3H, s), 2.00† (3H, s), 1.97 (2H, m, J = 6.5), 1.72 (2H, m, J = 6.7), 1.28 (XH*, br s), 0.83 (6H, br t, J = 8.0) ^{13}C-NMR ($CDCl_3$): δ 171.45 (s), 169.96 (s), 169.91 (s), 169.77 (s), 130.77 (d), 124.01 (d), 74.16 (d), 73.74 (d), 62.13 (t), 50.98 (d), 32.26 (t), 31.88 (2C, t), 29.66-29.30 (XC*, t), 28.82 (t), 24.79 (t), 22.63 (2C, t), 21.12 (q), 21.10 (q), 20.77 (q), 13.99 (2C, q).

Acetylation of N-Acylsphingosine

A mixture of N-acylsphinogosine (4 mg) was acetylated and then separated as described above to yield 3 mg of the diacetylated N-acylsphingosine derivative, ^{1}H-NMR ($CDCl_3$): δ5.77 (1H, dt, J = 14.9, 6.2), 5.58 (1H, br d, J = 8.6), 5.37 (1H, dd, J = 14.9, 6.5), 5.26 (1H, dd, J = 7.0, 6.5), 4.43 (1H, m, J = 8.6, 7.0, 6.2, 3.9), 4.29 (1H, dd, J = 12.3, 6.2), 4.02 (1H, dd, J = 12.3, 3.9), 2.15 (2H, t, J = 7.1), 2.00 (2H, dt, J = 6.24, 6.83), 2.05 (3H, s), 2.07 (3H, s), 1.58 (6H, br m), 1.27 (XH, br s), 0.88 (6H, br t, J = 8.0).

*X indicates indeterminate number due to mixture of acylamides.

†Measured in $CDCl_3$; the acetone impurity in d_6 acetone precluded observation in that solvent.

Hydrolysis of α-Hydroxy-N-acylsphingosines

A portion of the mixture of α-hydroxy-N-acylsphingosines (28 mg), in 7 ml of THF, was hydrolyzed with 7 ml 1.2 M H_2SO_4 in 85% MeOH at gentle reflux for 4 hr. The organic solvents were then evaporated, yielding an acidic aqueous suspension which was extracted twice with hexane to remove the α-hydroxy fatty acid methyl esters (16 mg). The aqueous phase was then basified with 2 M KOH and extracted twice with hexane/CH_2Cl_2 (1:1) to remove the sphingosine (8 mg). The fatty acid esters were separated by gel permeation on Sephadex LH-20 with CH_2Cl_2/MeOH (1:1) to yield one major fraction (14 mg) which contained esters of n-C_{22} (MS: m/z 370, 311), n-C_{23} (MS: m/z 384, 325) and n-C_{24} (MS: m/z 398, 339) acids in ca. 9:1.5:1 ratio, $[\alpha]_D^+$ 19.5° ($CHCl_3$, c 0.61).

Hydrolysis of N-Acylsphingosine

A portion of the mixture of N-acylsphingosines (27 mg) was hydrolyzed exactly as above. The fatty acid methyl esters (10 mg) were separated on Sephadex LH-20 by CH_2Cl_2/MeOH (1:1) to yield 3 major fractions. The first two (2 and 4 mg, respectively) contained esters of n-C_{22} (MS: m/z 354, 311), n-C_{23} (MS: m/z 368, 325), and n-C_{24} (MS: m/z 382, 339) acids in an overall ratio of ca. 8:1.5:1. The third fraction (1 mg) contained pentadecanoic (MS: m/z 256, 213), hexadecanoic (MS: m/z 270, 227), heptadecanoic (MS: m/z 284, 241), and octadecanoic (MS: m/z 298, 255) acid methyl esters in ca. 1:11:5:3 ratio.

Acetylation of Sphingosine

Sphingosine (8 mg) was added to 0.5 ml pyridine, 0.5 ml acetic anhydride and 1 mg 4-N,N dimethylpyridine. The mixture was sealed under nitrogen and then heated to 40 C for 2 hr. After cooling overnight, the solvent and excess reagent were evaporated and the residue separated by gel permeation on Sephadex LH-20 with CH_2Cl_2/MeOH (1:1) to yield one major fraction, 6 mg of sphingosine triacetate, $[\alpha]_D$-8.3° ($CHCl_3$, c 0.58) [lit. (5) $[\alpha]_D$-11.7° ($CHCl_3$, c 1.0); MS, m/z (relative intensity): 426(3), 394(3), 370(4), 369(4), 366(2), 352(2), 338(7), 277(30), 75(100), 57(100); ^{1}H-NMR ($CDCl_3$): δ 5.76 (1H, dt, J = 15.3, 6.9), 5.63 (1H, br d, J = 9.0), 5.37 (1H, dd, J = 15.3, 6.5), 5.25 (1H, dd, J = 6.5, 6.9), 4.32 (1H m), 4.27 (1H, dd, J = 11.7, 5.9), 4.02 (1H, dd, J = 11.7, 3.7), 2.05 (3H, s), 2.04 (3H, s), 1.96 (3H, s), 1.32 (2H, m), 1.24 (22H, br s), 0.86 (3H, br t, J = 7.2).

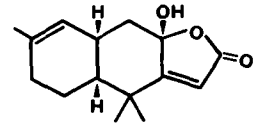
RESULTS

Florisil chromatography of the dichloromethane-soluble extracts of *Dysidea etheria* concentrated the weak antimicrobial activity of the extracts in three polar fractions. In the course of isolating the sesquiterpene lactone 1 by gel permeation chromatography, we encountered a tan solid which was appreciably soluble only in tetrahydrofuran, pyridine and methanol/dichloromethane (1:1). This material could not be fully dissolved in hexane, diethyl ether, chloroform, dichloromethane, acetone, ethyl acetate or methanol. Purification by low pressure column chromatography yielded 2 distinct fractions, each an amorphous white solid.

The ^{1}H-NMR spectra, recorded in deuterated tetrahydrofuran, suggested moderately functionalized molecules, each with a long aliphatic chain. The minor fraction, R_f 0.35 in silica gel TLC, contained 3 exchangeable protons, and the major fraction, R_f 0.26, had 4 such protons. However, acetylation of the minor fraction gave only a diacetate, whereas the major fraction yielded a triacetate. An absorption in the IR spectrum (KBr pellet) at 1623 cm^{-1} for the minor fraction and 1624 cm^{-1} for the major fraction suggested a secondary amide functionality. Thus, to account for the number of deuterium exchangeable protons in each molecule, it appeared that a secondary amide, in addition to a diol for the minor fraction and a triol for the major fraction, was present.

Since the overlap of numerous low and midfield signals precluded unequivocable information from decoupling experiments, structure elucidation was ultimately accomplished on the acetylated compounds. ^{1}H-NMR decoupling experiments on these derivatives established part structure 2a for the minor fraction and 3a for the major fraction. The ^{13}C-NMR of 2 and 3 indicated one carbonyl, 2 olefinic carbons and 3 heteroatom-bearing carbons (2 doublets and a triplet), for 2, and one carbonyl, 2 olefinic carbons and 4 heteroatom-bearing carbons (3 doublets and a triplet) for 3. A coupling constant of 15.3 Hz between the olefinic protons suggested a *trans* geometry and ^{1}H chemical shift considerations led to the 1,3-diacetoxy, 2-amido groupings, thus establishing both fractions as sphingosine-type compounds. The upfield region of inverse-gated and off-resonance decoupled ^{13}C-NMR spectra of 3 in pyridine indicated ca. 32 methylenes (all but 7 between δ29.41 and 29.03) and 2 methyls (see Scheme 1).

Hydrolysis of both 2 and 3 gave sphingosine and a mixture of fatty acid methyl esters. The ^{1}H-NMR of the sphingosine from both fractions were identical in all respects to that reported previously (4). The sphingosine was converted to its triacetate and identified by comparison with literature values ($[\alpha]_D$, ^{1}H-NMR) (4,5). Fast atom bombardment mass spectral studies indicated an M+H ion at m/z 426 and diagnostic fragments at m/z 366 (loss of O_2CCH_3) and 352 (loss of $CH_2O_2CCH_3$) verified the presence of sphingosine triacetate. The fatty acid methyl esters of 3 were shown to be α-hydroxylated by the ^{1}H-NMR (δ 4.17, 1H, dd, J = 7.8, 3.6) and MS (M-59, loss of CO_2CH_3) spectra and to be composed predominantly of n-C_{22} acids with minor amounts of n-C_{23} and n-C_{24} acids. The fatty acid methyl esters of 2 were shown by mass spectrometry to be composed mainly of n-C_{22}, n-C_{23} and n-C_{24} acids, with smaller amounts of n-C_{15}, n-C_{16}, n-C_{17} and n-C_{18} acids also present (see Table 1). The aliphatic chains of the sphingosine triacetate and the fatty acid methyl esters from 2 and 3 were proven to be normal (unbranched) by virtue of the fragmentation patterns in their respective mass spectra (m/z 29, 43, 57, 71, 85, etc.).



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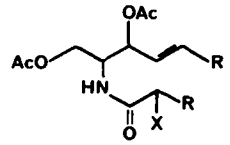
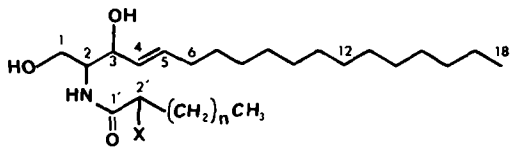


2a R = alkyl, X = H

3a R = alkyl, X = OAc



2 X = H , n = 12,13,14,15,19,20,21

3 X = OH, n = 19,20,21

SCHEME 1

TABLE 1

Observed Quantities (mg) of Fatty Acids from Ceramide Hydrolysis

Acid	Source 2[a]	3[b]
C_{15}	0.05	–[c]
C_{16}	0.55	–[c]
C_{17}	0.25	–[c]
C_{18}	0.15	–[c]
C_{22}	4.6	11.0
C_{23}	0.9	1.8
C_{24}	0.6	1.2

[a]From 27 mg ceramides.

[b]From 28 mg ceramides.

[c]Not detected.

The optical rotation, $[\alpha]_D$ + 19.5°, of the hydroxy acid methyl esters suggested an *S* configuration at C-2 on the basis of comparison with literature data (see Table 2). Although the observed rotations vary slightly with concentration (17-19.5°), the magnitude is consistently and inexplicably higher than those reported for similar esters with the *R* configuration.

The ceramides of fully saturated acids, 2, appear to be responsible for the antibacterial activity observed in the extracts and crude fraction. In a qualitative impregnated disk assay, 2 inhibited, albeit weakly, the growth of *Corynebacterium michiganense*, but 3 did not. the lactone 1, isolated from the same fraction, was also inactive against gram-positive bacteria, but did exhibit marginal inhibition of the yeast *Rhodoturula glutinus* and the fungus *Curvularia lunata*.

DISCUSSION

This isolation of sphingosine derivatives is significant for several reasons.

Ceramides have recently been found in a variety of marine organisms, including the first isolation of such compounds from plants–red (4) and green (10,11) algae–as well as the electric organ of the ray *Torpedo marmorata* (12), the rectal gland of the spiny dogfish (13), the starfish *Patiria pectinifera* (14), octopus (15) and the shellfish *Pinctada martensii* (16), but they have thus far not been reported from sponges.

The fatty acids, both hydroxylated and saturated, represented in the ceramides of *Dysidea etheria* were predominantly C_{22} or larger, in keeping with recently described profiles of fatty acids (17,18) and their derivatives (2) in the Porifera.

This report would seem to represent the first isolation of α-hydroxy acids with an *L* (or *S*) configuration from a natural source. Although some studies (19,20) suggested that the α-oxidative degradation of fatty acids in plants proceeded through an *L*-2-hydroxy acid intermediate, more recent evidence (21) indicates that *D*-2-hydroxy acids are the actual intermediates and the the *L* enantiomers are not formed. The apparent presence of the *S (L)* enantiomers in *Dysidea etheria* raises the question of an alternative pathway for their production in sponges.

Since sponges harbor and support considerable microbial communities, it is not impossible that the ceramides could be of bacterial or fungal origins, but the finding of very similar quantities of these compounds in two major collections (1979 and 1982), each a sampling of numerous sites, leads us to believe that they are, in fact, produced by the sponge.

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TABLE 2

Optical Rotations, Esters of 2-Hydroxy Acids[a]

Compound[b]	$[\alpha]_D$ (°)	Solvent	Reference
S-methyl valerate	+16.6	$CHCl_3$	6
S-methyl octanoate	+11	$CHCl_3$	7
R-methyl tetradecanoate	−3.6	$CHCl_3$	8
R-methyl hexadecanoate	−3.6	$CHCl_3$	8
R-methyl octadecanoate	−2.1	$CHCl_3$	9

[a]This topic has been reviewed (22).

[b]All esters have hydroxyl substituents at C-2.

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A Simple Synthesis of 5,8,11-Eicosatrienoic Acid

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ABSTRACT

A straighforward synthesis of Z,Z,Z-5,8,11-eicosatrienoic acid is described that gives a pure product without chromatography. The C_{20}-polyacetylenic acid is purified by recrystallization and then reduced by a Lindlar catalyst in an aprotic solvent with a trace quantity of quinoline.
Lipids 18:894-895, 1983.

We are interested in 5,8,11-eicosatrienoic acid. This acid has been prepared by the partial reduction of methyl arachidonicate with hydrazine and purified by preparative gas liquid chromatography (GLC) (1). It has also been synthesized by Hammarström (2) who used the general procedure of Osbond (3). The final product was purified by reverse-phase high performance liquid chromatography (HPLC).

We now wish to report a straightforward synthesis of eicosatrienoic acid that is suitable for scale-up since it does not require liquid ammonia steps and gives a pure product without chromatography (Fig. 1). 5,8-Nonadiynoic acid was prepared by coupling 5-hexynoic acid to propargyl bromide. 1-Bromo-2-undecyne was then coupled to the 5,8-nonadiynoic acid to produce 5,8,11-eicosatriynoic acid, which was purified by crystallization and then reduced. By using hexane/ethyl ether as a solvent and a Lindlar catalyst with quinoline as a modifier, hydrogenation ceased after 30 min to give chromatographically pure 5,8,11-eicosatrienoic acid.

Analyses of the reaction product by thin layer chromatography (TLC) and high-resolution mass spectrometry (MS) and analyses of the methyl ester by gas chromatography-mass spectrometry (GC-MS) showed the product to be analytically pure.

MATERIALS AND METHODS

Melting points were determined on a Thomas-Hoover capillary melting point apparatus. ^1H Nuclear magnetic resonance (NMR) spectra were taken with a Perkin-Elmer 521 and UV spectra with a Varian Super Scan 3. Low resolution MS were conducted on a Finnigan 3200 GC-MS with a 6100 data system using a 3% OV1 column programmed at 4 C/min starting at 120 C. High resolution MS were conducted on a Finnigan MAT 731 double focus with xenon fast bombardment. The TLC was conducted on 0.2 mm silica gel sheets, No. 5775, EM Reagents (from MC/B, Cincinnati, OH).

Synthesis of 5,8-Nonadiynoic Acid

The general procedure of Fryer (4) was followed. To 5.0 g (0.045 mol) of 5-hexynoic acid in 100 ml of dry tetrahydrofuran (THF, Aldrich, Milwankee, WI) under nitrogen on an ice bath was added, over 5 min, 32 ml of 2.8 M ethyl magnesium bromide (Aldrich). The 5-hexynoic acid was prepared by the method of Ferrier (5). After warming (1 hr) to room temperature, 250 mg of copper(I) cyanide of (Aldrich) was added and after 20 min, 6.6 g (0.044 mol) of propargyl bromide (80% in toluene, Aldrich) dissolved in 40 ml of THF was added. After 5 hr, an additional 250 mg of copper(I)cyanide was added. The reaction was stirred overnight and poured into a mixture of 100 ml of 3 N sulfuric acid and 200 g of ice. The product was extracted with methylene chloride. The organic layer was washed successively with 5% ethylenediaminetetraacetic acid, water, and then dried (Na_2SO_4). After removing the solvent on a rotary evaporator, distillation gave 2.8 g (bp 105-110 C, 0.1 mm) (41% yield). ^1H NMR (CCl_4) δ 11.2 ppm (s, 1 H), 3.1 ppm (q, 2 H), 1.5-2.7 ppm (m, 6 H).

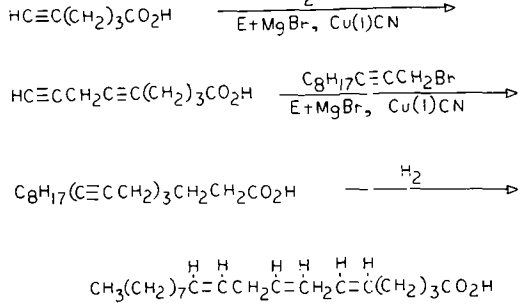

FIG. 1. Synthetic scheme for 5,8,11-eicosatrienoic acid.

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Synthesis of 5,8,11-Eicosatriynoic Acid

Ethyl magnesium bromide (9 ml, 0.0025 mol, Aldrich) was added over 5 min to 1.7 g (0.011 mol) of 5,8-nonadiynoic acid in 70 ml of THF on an ice bath under nitrogen. After 20 min, 2.5 g (0.011 mol) of 1-bromo-2-undecyne was added in 5 ml of THF. 1-Bromo-2-undecyne was synthesized by the method of Fryer (4). After 17 hr, the reaction mixture was processed as described for 5,8-nonadiynoic acid.

The product was dissolved in 5 ml of dry ethyl ether under nitrogen and after 18 hr at -10 C, 0.82 g of light brown solid was obtained which was crystallized from cyclohexane with charcoal to give 0.56 g (15% yield) of 5,8,11-eicosatriynoic acid, mp 69.5-71 C. ^{1}H NMR (CCl_4) δ11.4 ppm (s, 1 H), 3.0 (s, 4 H), 0.6-2.8 (m, 23 H).

Synthesis of all-*cis*-5,8,11-Eicosatrienoic Acid

5,8,11-Eicosatriynoic acid (0.50 g), 50 ml of hexane, 35 ml of dry ethyl ether, 0.2 g quinoline, and 0.3 g of Lindlar catalyst (Aldrich) were stirred for 1 hr at room temperature (28 C) with hydrogen. After 30 min, no more hydrogen was absorbed. TLC (developed with ethyl acetate/heptane, 1:1, v/v) showed the reaction to be complete with no side reactions: R_f product 0.60, R_f starting compound 0.28, R_f quinoline 0.42.

After filtration, the mixture was extracted with 1 N HCl to remove to quinoline, dried (Na_2SO_4), and evaporated to a thick oil which solidified in an ice bath. No further purification was carried out since spectral data and TLC showed the product to be pure.

IR (neat) 3500-3100 (broad OH), 1710 (C=0) cm^{-1}. No triple bond at 2100-2140 cm^{-1}, no *trans* double bond at 965 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ10.2 ppm (m, 6 H, 3 x CH=CH), 2.7 (t, 4 H, 2 x C=CH$_2$-C=C), 0.5-2.5 (m, 23 H, aliphatic H); MS (low resolution of methyl ester of product): m/z (rel intensity): 321 (M^+ + 1, 50%), 349 (M^+ + 29, 5%), 289 (M^+ + 1 - MeOH), 195, 181, 149, 137, 123, 109, 95, 81 (base). Observed mass of pure acid at high resolution: m/z = 307.2668 (m + 1), calcd. for $C_{20}H_{34}O_2$ = 307.2637 (m + 1). The GC-MS of the methyl ester showed the sample to be analytically pure.

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A Complete Separation of Lipids by Three-Directional Thin Layer Chromatography[1]

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ABSTRACT

A novel three-directional thin layer chromatography (TLC) method is reported by which all the polar and neutral lipids are isolated on a single TLC plate. Following resolution of the phospholipids by two-directional TLC, lipids are visualized by ultraviolet light after spraying with 2',7'-dichlorofluorescein. A line is drawn across the plate, parallel to the second direction of development, separating the resolved phospholipids and the neutral lipids concentrated along the solvent front. The TLC plate is then chromatographed in the reverse direction of the second development to resolve the neutral lipids. By exposing the lipids to HCl fumes after the first development, the plasmalogen content of the lipids may also be determined. This new technique is rapid and lends itself to qualitative and quantitative analyses of total lipids.
Lipids 18:896-899, 1983.

Thin layer chromatography (TLC) has been universally accepted as an effective method to separate complex lipid mixtures into constituent lipid classes (1-4). A number of one- and two-directional separations have been reported in attempts to separate as many lipid components as possible. The neutral lipids are successfully separated by development in one direction only (5-8), while two-directional TLC provides good resolution of polar lipids (9-15). A complete analysis of both neutral and polar lipids at present requires either prior separation of neutral and polar lipids by column chromatography, or elution of the neutral lipids from the solvent front following two-directional separation of polar lipids and rechromatography of neutral lipids, or separate analyses of neutral and polar lipids. All these methods are time-consuming and subject to error because of excessive handling. In this paper, a three-directional TLC method is reported by which all lipid classes are resolved on one TLC plate quickly and in which the relative ratio between lipid classes is preserved without loss of any lipid component.

METHOD

All solvents were of analytical grade and used without further purification. Precoated Silica Gel G plates (Redi-plates), 20 x 20 cm and 250 μm in thickness, were purchased from Fisher Scientific Co. (Ottawa, Ontario). All TLC plates were prewashed by developing the plates in chloroform/methanol (2:1, v/v), and a horizontal line was drawn across the top of each plate (ca. 2 cm from top) to isolate the impurities concentrated in this region during washing. Lipid extracts of rat tissues were prepared as previously published (16). Authentic standards of neutral lipids were obtained from NuChek Prep, Elysian, MN, and of polar lipids from Supelco Inc., Bellefonte, PA.

The prewashed TLC plates were activated at 110 C for 1 hr. The plate, while hot, was placed immediately in a preparation box (Canlab, Ottawa, Ontario), flushed with nitrogen, and the lipid sample was spotted on the lower left-hand corner of the plate, 2 cm in from both edges. Ca. 3 mg of total lipid extract dissolved in 0.1 ml chloroform/methanol (2:1, v/v) was applied. The plate was quickly transferred to a TLC developing chamber lined on all sides with filter paper saturated with the solvent mixture. The solvent mixture for the first direction was chloroform/methanol/28% aqueous ammonia (65:25:5, v/v/v) according to Rouser et al. (11). The solvent front was allowed to reach the top of the plate. The TLC plate was then placed in the preparation box and dried by flushing with nitrogen for 10 min.

The plate was developed in the second direction using the solvent mixture chloroform/acetone/methanol/acetic acid/water (50:20:10:15:5, v/v/v/v/v) (14). Development was stopped when the solvent front reached ca. 2 cm from the top of the plate. The TLC plate was then dried for 10 min under a stream of nitrogen, and the lipid classes were visualized under UV light (longwave) after spraying the plate with a 0.1% solution of 2',7'-dichlorofluorescein in methanol. A line was drawn in the same direction as the second development separating the resolved phospholipids and the neutral lipids concentrated along the solvent front. A 1.5-cm

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wide band of adsorbent was then removed above the solvent front of the phospholipid region after the second development.

The TLC plate was finally developed in the third direction which was the reverse direction of the second development, using the solvent mixture hexane/diethyl ether/acetic acid (85:15:1, v/v/v). With removal of the adsorbent above the phospholipid region, only the neutral lipids were resolved in the third direction. After development, the TLC plate was again dried under a stream of nitrogen and all spots were visualized by charring the plate following spraying with sulfuric acid/ethanol (1:1, v/v).

Confirmation of the identity of all lipid classes was established by removing the spots from the three-directional TLC plate and co-chromatographing these lipids with authentic standards using one-directional TLC developments. For neutral lipids, the solvent mixture hexane/diethyl ether/acetic acid (85:15:1, v/v/v) was used, and chloroform/methanol/water (65:25:4, v/v/v) was used for the phospholipids. In addition, several spray reagents were used to identify sterols (sulfuric acid reagent), sugars (α-naphthol-sulfuric acid reagent) and amino groups (ninhydrin reagent) (2). The relative position of the lipid classes on the three-directional TLC was consistent enough to identify them in subsequent separations.

To determine in addition the alk-1-enyl ether content of the phospholipids, the partially resolved lipids were exposed to HCl fumes (17) after the first development. Most of the TLC plate was covered with a glass plate except for a 4-cm wide band containing the partially resolved lipids to avoid deactivation of the entire plate by HCl fumes. The TLC plate was then passed through HCl fumes made by heating concentrated hydrochloric acid in a crystallization dish. Following exposure to HCl fumes, the TLC plate was placed in the preparation box and flushed with nitrogen for 10 min. Development in the second and third directions is as described above.

RESULTS AND DISCUSSION

Figure 1 shows typical chromatographic separations obtained from total lipid extracts of selected rat tissue using the three-directional TLC chromatographic procedure described here. The phospholipid and neutral lipid classes were well resolved on a single TLC plate when as much as 6 mg of total lipid extract was applied. The chromatographic separations provide a quick comparison of the lipid profile of different fat mixtures and a semiquantitative estimate of the lipid classes. By hydrolyzing the alk-1-enyl ethers with HCl prior to the second development, the plasmalogen lipid content in the phospholipids was determined as well as the products of hydrolysis, i.e., 2-lysophospholipids and aldehydes. To separate aldehydes and free fatty acids, the mixture was methylated to obtain dimethyl acetals and methyl esters, respectively, which were then separated by TLC using 1,2-dichloroethane as developing solvent (18). Figure 2 shows the chromatographic separations of the same lipid mixtures shown in Figure 1 using the additional acid hydrolysis step in the three-directional TLC method. More complex lipid mixtures, containing phospho-, glyco- and neutral lipids can also be resolved successfully using this new chromatographic technique as seen in Figure 3. The identity of several steryl glucosides (X_1, X_2 and X_3) was not determined, but the spots gave positive sugar and sterol stains.

To maintain consistent and reproducible phospholipid separations, lipid samples were applied onto the TLC plate while the plate was hot to prevent deactivation of the TLC adsorbent which is a problem particularly on humid days. A combination of previously published solvent mixtures was found to give the best resolution of the phospholipids. Development in the first direction was according to Rouser et al. (11) using chloroform/methanol/28% aqueous ammonia (65:25:5, v/v/v). Ammonia was chosen in this solvent system rather than methylamine (14) because the former could be removed with ease, and did not require exposure to HCl fumes to neutralize, as is the case for the latter. Exposure to HCl fumes also hydrolyzes the alk-1-enyl ethers of plasmalogens, not an undesirable feature, but it eliminates the choice of retaining the plasmalogen lipids or hydrolyzing them. Development in the second direction was according to Yavin and Zutra (14) using chloroform/acetone/methanol/acetic acid/water (50:20:10:15:5, v/v/v/v/v). The reduced proportion of acetone in this solvent mixture, compared to that used by Rouser et al. (11), improved the resolution of phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SP). The developing solvent in the third direction consisted of hexane/diethyl ether/acetic acid (85:15:1, v/v/v). Streaking occurred if the sample is high in neutral lipids and/or they were concentrated too much along the solvent front after the second development. To spread the neutral lipids, just barely let the first developing solvent reach the top of the plate.

The advantages of the three-directional TLC system described here are numerous. It provides a rapid, reproducible and economical method

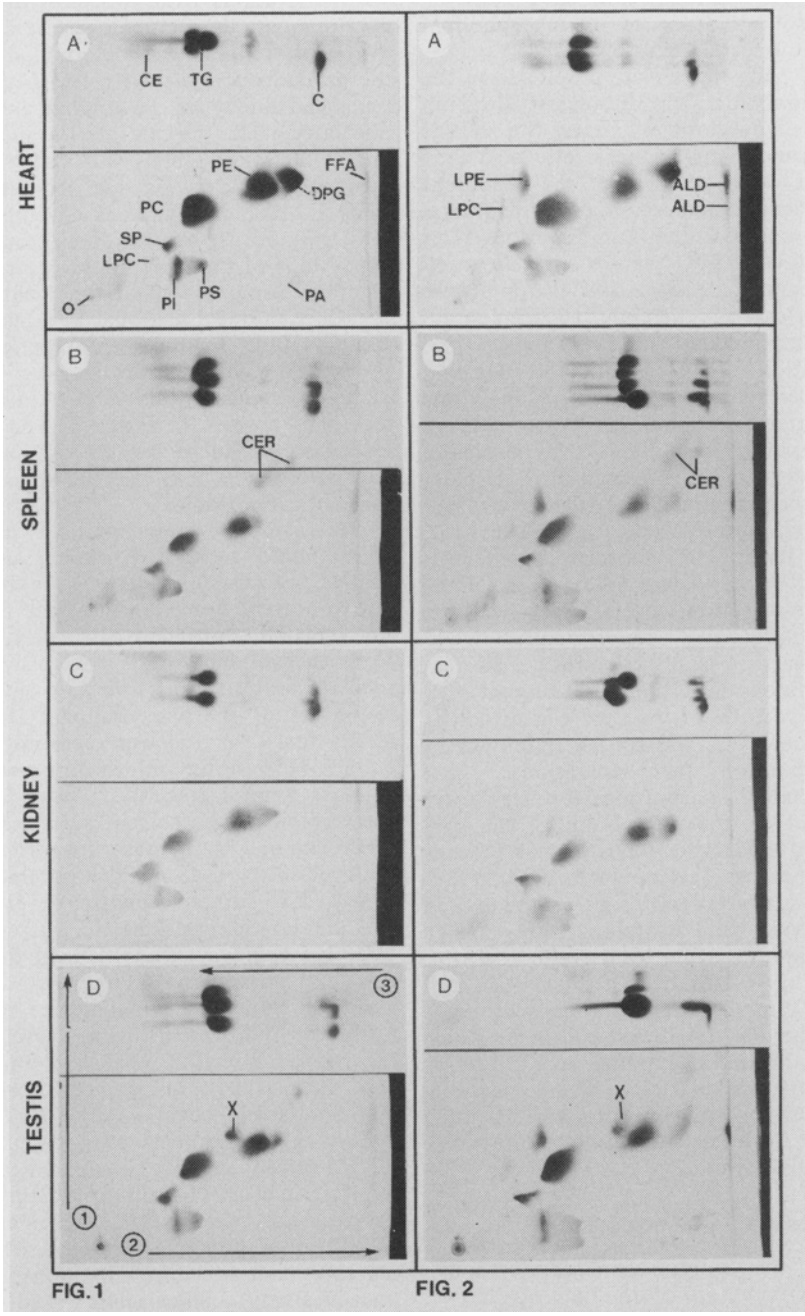

FIG. 1. Three-directional TLC separation of heart, spleen, kidney and testis lipids of rats fed diets containing 20% fat for 16 weeks. Direction of development, see Figure 1D. Solvents: 1st direction, chloroform/methanol/28% aqueous ammonia (65:25:5); 2nd direction, chloroform/acetone/methanol/acetic acid/water (50:20:10:15:5); 3rd direction, hexane/diethyl ether/acetic acid (85:15:1). CE, cholesteryl ester; TG, triglyceride; C, cholesterol; FFA, free fatty acid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SP, sphingomyelin; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; CER, cerebroside; X, unknown; O, origin.

FIG. 2. Same as Figure 1, except that the chromatogram was exposed to fumes of HCl after the first development. LPE, lysophosphatidylethanolamine; ALD, aldehyde.

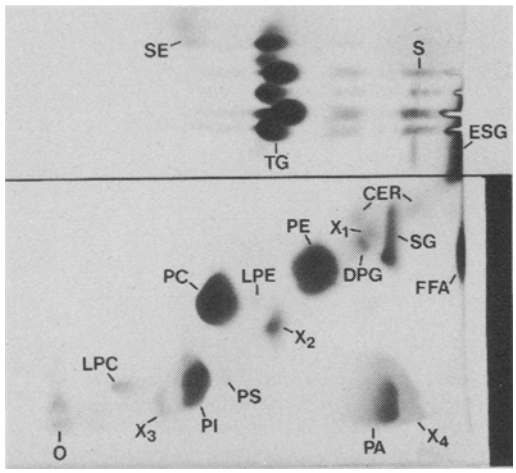

FIG. 3. Three-directional TLC separation of crude soybean lecithin. Solvents and direction of development as in Figure 1. SE, steryl ester; S, sterol; ESG, esterified steryl glucoside; SG, steryl glucoside; X_1, X_2 and X_3, unknowns which stain sugar and sterol positive; X_4, unknown phospholipid.

of resolving both neutral and polar lipids on a single TLC plate. No prior separation by column chromatography (4) or separate analyses of neutral and polar lipids is required. A single TLC plate provides a complete lipid analysis in which the relative proportion of all lipid classes is preserved.

The three-directional TLC system will have distinct advantages in radioactive isotope work because all the lipid classes can be investigated simultaneously. Naturally, this semiquantitative technique can be quantitative by scraping off the spots and subjecting them to subsequent chemical or gas chromatographic analysis. The acyl and alk-1-enyl composition of the lipid classes can also be determined. It was found that up to 6 mg of total lipids could be adequately resolved with this three-directional TLC method, which gave sufficient material for most subsequent lipid analyses.

Since this manuscript was submitted for publication, another "three-way" TLC method for analyzing total lipid mixtures has appeared (19). These authors used plastic-backed precoated TLC plates which can be cut in a manner to permit separate chromatographic separations. It should be noted, however, that this leads to poor resolution of neutral lipids (ref. 19, Fig. 2), and possible elution by the second developing solvent of cholesterol and monoglycerides after cutting off the side arm. Figure 1C from reference 19 could prove to be an improvement of this method (no results provided) but the problem remains of where to cut the chromatogram. Usually, diphosphatidylglycerol, cerebroside, free fatty acid, monoglyceride diglyceride and free sterol migrate very close and often overlap after the first development in a two-directional analysis of phospholipids. The method we have described is much simpler, requires no special plates, avoids loss of any single lipid component, permits the use of any detecting reagent, gives good resolution of all lipid classes, and is suitable for separating larger amounts of lipids.

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COMMUNICATIONS

Metazoan Lipids: An Unusual Association of Saturated Sterols with Relatively Saturated Fatty Acids in the Cilia of *Ciona intestinalis*

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ABSTRACT

Cilia from the tunicate *Ciona intestinalis* have been analyzed for their biochemical compositions. Cholestanol is found to be a major steroidal component, whereas the phospholipids are composed mainly of C_{14}-C_{18} saturated fatty acids. The saturated nature of the lipids may be related to specific requirements of the ciliary membrane.
Lipids 18:900-901, 1983.

Until comparatively recently, saturated sterols (stanols) have been considered to be products of the normal diagenesis of natural product sterols in sediments (1). With improved analytical techniques, however, it has become obvious that, although their function remains unclear, stanols are themselves important natural product components of many marine organisms. (2-9). More detailed analysis of their distribution in several species of tunicates has shown that stanols are particularly abundant in the gills and endostyle (10,11). A common feature of these tissues is that they are rich in cilia. Here we present a detailed biochemical analysis of cilia from the tunicate *Ciona intestinalis* and show cholestanol to be a major steroidal component. This is thought to be the first report of such compounds being associated with a specific membrane, and indeed the first detailed lipid analysis of pure metazoan cilia.

It is usually difficult to obtain samples of metazoan cilia that are free from their cells of origin, so that structural lipids of the ciliary membrane can be analyzed. We have examined cilia which are appropriately long enough and abundant enough that it is possible to shave the cilia from the cells bearing them and so obtain isolated pure ciliary preparations. The sessile tunicate *Ciona* secretes a mucous filter from its endostyle, and the median cilia of the endostyle from an elongate fence enclosed by the secretory cells of the endostyle (12). The cilia are ca. ½ mm long, and so after removal of the endostyle side walls it is possible to obtain isolated cilia with the use of fine scissors. Adult *Ciona* were taken from the breeding population at the Plymouth laboratory. Several animals were used to give one sample of cilia and three separate samples were collected over a period of 6 months. The total lipids were immediately extracted from the cilia with chloroform/methanol (13) and the residue analyzed for total protein (14) and carbohydrate (15). The major lipid classes were quantified by chromarod-flame ionization detection techniques (16,17) and the component phospholipids and sterols separated (18). Detailed fatty acid and sterol analyses were performed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) techniques (10).

The three samples were analyzed separately and similar results were obtained. The cilia were composed mainly of protein (>60% dry weight) and lipid (10-20% dry weight) with much lower levels of carbohydrate (<5% dry weight). Phospholipids (65-75% total lipid) and sterols (20-30% total lipid) were the only major lipid components.

The phospholipids were composed mainly of C_{14}-C_{18} saturated fatty acids (>65% total fatty acids) with lower amounts of C_{16}-C_{18} unsaturated acids (see Table 1). Only cholesterol (64-75% total sterols) and cholestanol (25-36% total sterols) were found in the sterol fraction, agreeing with previous analyses of whole tissues taken from this same population of *Ciona* (10), although the relative levels of cholestanol in the cilia are significantly higher than the levels reported for the whole tissues.

In addition to membrane, cilia also consist of dynein, tubulin and a little cytoplasm. Although we cannot discount the possibility that some lipids may be associated with the

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TABLE 1

Fatty Acid Composition of the Component Phospholipids from *Ciona* Cilia

Fatty acid	% Composition
14:0	12.7
16:0	41.1
16:1	12.9
18:0	13.2
18:1	13.5
18:2	4.9
18:3	1.5

axonemal proteins, we presume that most of the lipids are associated with the ciliary membrane. We believe the sterol data indicate that there is a specific concentration of saturated sterols in the cilia of *Ciona*. Their saturated nature, and their association in the membrane with largely short-chain, saturated phospholipids, may contribute to specific structural or permeability requirements of the ciliary membrane.

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Occurrence of Long-Chain Alkan-diols and Alkan-15-one-1-ols in a Quaternary Sapropel from the Eastern Mediterranean

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ABSTRACT

C_{28}-C_{32} alkandiols and keto-ols are reported in an S_1 sapropel from the Eastern Mediterranean. Mediterranean. Their occurrence in this sediment and in certain other sedimentary environments may provide a clue to their source which is thought to be a specific marine organism or group of marine organisms.
Lipids 18:902-905, 1983.

Discrete horizons of organic-rich sediment, known as sapropels, are commonly found in both piston and gravity cores taken from the eastern basin of the Mediterranean (1,2). They are believed to be the legacy of past anoxic events, and most theories of their origin have invoked the formation of stagnant conditions in the deep water basins, leading to enhanced preservation of sedimentary organic matter (1,3-6). We have undertaken an extensive study of a core containing an S_1 sapropel layer (the most recently deposited, 7,000-9,000 years B.P. (1,2,10). Major components of the sapropel lipids (up to 5,756 ng/g dry sediment) were series of C_{28}-C_{32} alkandiols and keto-ols. These unusual lipids have been previously reported in sapropellic (quaternary) Black Sea sediments (7) but were absent in the Recent organic-rich contemporary sediments underlying the high productivity areas off Southwest Africa and Peru (8,9). They may prove to be valuable biological markers for a specific organism or groups of organisms and hence may ultimately assist in defining the type of water column that was associated with sapropel formation in the Eastern Mediterranean.

The sediment core was taken from the northern arm of the Hellenic Outer Ridge in the Ionian Sea (36 09.63′ N 20 28.50′ E) (11). The core was 15 cm^2 × 130 cm long and contained a single dark-grey/black sapropel layer from 28 to 101 cm, which ^{14}C dating indicated to be an S_1 layer (1,2,10) (Table 1). Five sections were taken for organic geochemical analysis. The lipids were extracted and analyzed as described elsewhere (12). Significant components of these lipids were homologous series of C_{28}-C_{32} diols and C_{28}-C_{32} keto-ols (Table 2 and Fig. 1).

Identification of the components was achieved from their gas chromatographic retention times and mass spectra; such data have been published for the C_{30}, C_{31} and C_{32} compounds (7), which were originally identified by comparison with synthetic standards (7). No molecular ions were obtained for any of the diols, although M-15, M-90 and M-105 fragments were observed in some cases, and structural assignment was based on the major

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TABLE 1

Selected Data for Sapropel-Containing Core from the Eastern Mediterranean (11)

Core section (depth in cm)	% Sediment dry weight: Total carbon	Organic carbon	Lipid extract	Age[a] (years B.P.)	Sediment type
0-7	6.7	0.2	0.005	2,590	Light-brown calcareous ooze
29-36	7.9	2.2	0.194	6,395	Black
53-60	9.3	2.5	0.182	7,460	sapropellic
78-85	–	2.5	0.116	8,210	mud
104-111	6.3	<0.1	0.003	12,320	Light-grey marl

– = not determined.
[a] ^{14}C-dating of organic carbon fraction, except for 104-111 cm section where the carbonate carbon was dated.

TABLE 2

Long-Chain Diols and Keto-ols in an Eastern Mediterranean Quaternary Sapropel – Abundances in ng/g of Sediment Dry Weight

Compound[a]		Core section (depth in cm)					MS Diagnostic ion (m/z)	
		0-7	29-36	53-60	78-85	104-111	a[b]	a'[b]
28:0	1,13-diol	–	43	160	35	–	359	313
28:0	1,14-diol	–	15	57	12	–	373	299
28:0	1,15-diol	–	7	35	8	–	387	285
29:0	1,13-diol	–	3	16	tr	–	359	327
29:0	1,14-diol	–	2	7	tr	–	373	313
29:0	1,15-diol	–	6	50	5	–	387	299
30:1	1,14-diol	–	21	118	–	–	371	327
30:0	1,15-diol	1	2142	5,756	1,050	3	387	313
31:0	1,15-diol	–	84	184	32	–	387	327
32:0	1,15-diol	–	80	166	28	–	387	341
							M+	M-15
28:0	? keto-ol	–	tr	tr	tr	–	496	481
29:0	15-keto-1-ol	–	27	69	14	–	510	495
30:0	15-keto-1-ol	tr	1162	2,185	399	1	524	509
31:0	15-keto-1-ol	–	35	74	10	–	524	509
32:1	15-keto-1-ol	–	99	103	15	–	550	535
32:0	15-keto-1-ol	–	230	285	40	–	552	537

[a]Structures expressed as x:y, where x = carbon chain length and y = number of double bonds.

[b]Fragment ions as shown in Figure 2.

tr = trace component (<1 ng/g dry weight); – = not detected.

fragment ions in the mass spectrum (7) (Fig. 2). The chain length, degree of unsaturation and position of the midchain hydroxyl group could be determined from the fragment ions a and a' (the base peak) in Figure 2. The presence of different isomers of the C_{28} and C_{29} diols, given in Table 2, was inferred from the occurrence of different a and a' ion pairs in the mass spectrum. These isomers were not separated under the conditions used, and their quantitation was based on the relative abundances of these ions, assuming that displacement of the midchain hydroxyl group by one or two carbons would not significantly change the fragmentation pattern.

The mass spectra of the C_{30}-C_{32} compounds indicated that they consisted of only one isomer. The presence of a double bond in the compound assigned as a 30:1 monounsaturated-1,14-diol was indicated by a decrease of 2 mass units for several characteristic ions, compared to the other spectra; the formation of m/z 371 (rather than m/z 373) locates this double bond between C-1 and C-14. A number of other minor compounds were present whose mass spectra showed "diol-like" characteristics (see Fig. 1), but the data were inadequate for the assignment of likely structures.

The keto-ols all showed a base peak in the mass spectrum at m/z 130, interpreted as a rearrangement ion by de Leeuw et al. (7), and the structural assignments are based on molecular ions, M-15 and M-90 fragment ions. The fragmentation pattern is more complex than for the diols (7), and we have assumed that the C_{28}-C_{32} keto-ols consist principally of the 15-keto-1-ol compounds, by analogy with the diols.

C_{30}-C_{32} saturated 1,15-diols and 15-keto-1-ols were first found in Black Sea sediments although, unlike here, no shorter chain lengths or unsaturated components were reported (7). Similar compounds have subsequently been identified in the Middle America Trench (13), the Japan Trench (14) and several other deep-sea drilling project sites (S.C. Brassell, personal communication), including a range of C_{28}-C_{32} diols and keto-ols in three other sapropels from the Eastern Mediterranean (15). These sediments all have several features in common; they are organic-rich deposits, believed to have been formed under conditions of high marine biological productivity, whose organic contents have not been extensively degraded. However, no long-chain diols or keto-ols were detected in Recent organic-rich diatomaceous oozes from the highly productive Namibian and Peruvian upwelling areas (8,9), which suggests that their presence may be confined to particular types of sedimentary environments.

These compounds may derive from organisms which thrive only under the peculiar water column and/or sedimentary conditions associated with formation of the type of de-

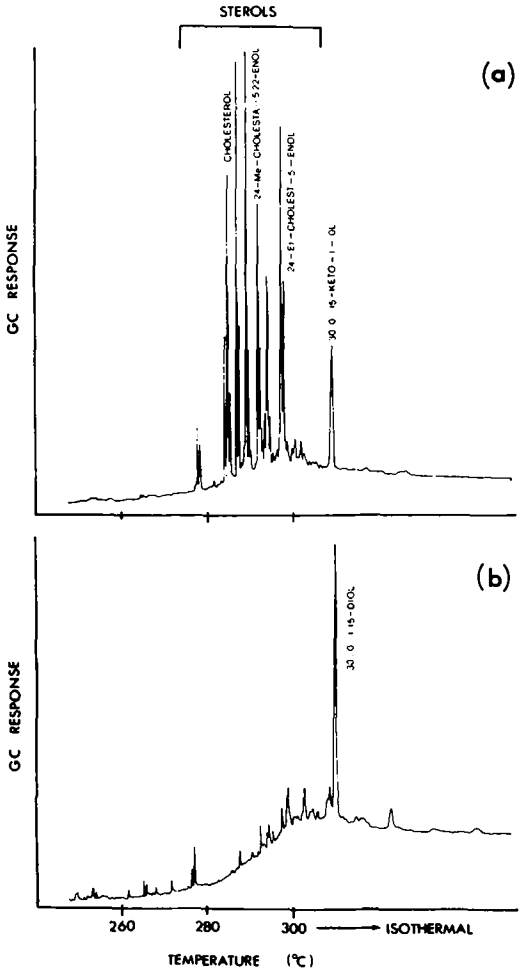

FIG. 1. Partial gas chromatograms of: (a) sterol plus keto-ol fraction, (b) alkandiol fraction from sapropel section 29-36 cm. Gas chromatographic conditions: 25 m OV-1 capillary column, temperature programmed from 80 to 300 C at 4 C/min, with helium carrier gas at 1.2 kg/cm^2.

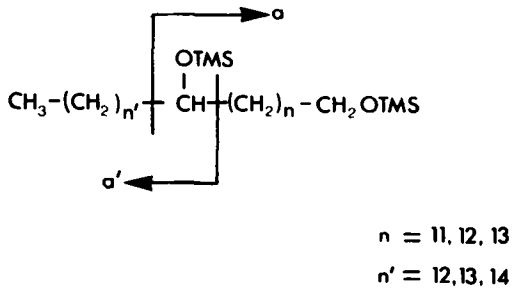

FIG. 2. Major fragmentations observed in mass spectra of alkandiols, as di(trimethylsilyl) ethers.

posit described above. Identifying their source may therefore provide an insight into the formation of such deposits. Other lipid data (12) suggest an organic input dominated by marine organisms, especially phytoplankton. Although some compounds thought to derive from higher plant waxes were present in the sapropel (12), to our knowledge no reported analyses of such waxes have included these diols and keto-ols. Furthermore, these compounds have not been found in sediments dominated by a higher plant input such as lakes (16) and hence a marine origin seems most likely.

The sapropel described here contained pteropods, planktonic foraminifera, coccoliths, plant debris, siliceous spines and shell fragments as recognizable fossil debris (11). A variety of organisms from which such debris derive has been analyzed, in particular phytoplankton, but none has yet been reported to contain these diols or keto-ols. Much work, however, remains to be done concerning the origins of sedimentary organic matter. The sapropel lipids suggest a significant contribution from coccolithophorids and dinoflagellates; these groups of phytoplankton were also important in the formation of the Black Sea sapropels (7,17). Therefore, these groups should be a primary target in searching for a biological source for the diols and keto-ols. On the other hand, diatoms (the most extensively studied phytoplankton group), are not significant contributors to the Mediterranean sapropel (11,12). It is, therefore, unlikely that they are the source. Two indirect origins for the diols and keto-ols have been suggested previously; a possible derivation from certain bacterial lipids (7), or from polyhydroxy compounds found in some Cyanophyceae (15).

These long-chain diols and keto-ols form an interesting group of sedimentary lipids, with potential as useful biomarkers. Their possible presence should be considered in future examinations of organisms and sediments. Certainly knowledge of their distribution in nature may help explain the mechanism of sapropel formation in the Eastern Mediterranean and elsewhere.

ACKNOWLEDGMENTS

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Fatty Acids in Plasma and Red Cell Membranes in Normal Humans

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ABSTRACT

A detailed study was made of the fatty acid composition of plasma triglycerides, free fatty acids, phospholipids, red cell total phospholipids, phosphatidylcholine and phosphatidylethanolamine in 32 normal males and 18 normal females. No sex differences could be detected. There were substantial differences in the compositions of the various fractions and long-chain polyunsaturated fatty acids were particularly important in the red cells.
Lipids 18:906-908, 1983.

INTRODUCTION

Essential fatty acids of the n-6 series are important constituents of all tissues and the n-3 series are important in many organs. In spite of this, there is surprisingly little information about the fatty acid composition of plasma and cell membranes in normal individuals. Many studies have reported the amounts of 18:2n-6 and 20:4n-6 and some have reported 20:5n-3 but the others have been almost ignored. The only detailed survey is that by Holman et al. (1). They studied fatty acid levels in the serum of patients attending a hospital who were not thought to be suffering from any essential fatty acid related disease. Individuals of all ages and both sexes were investigated and information about age trends documented. We now report fatty acid levels in both plasma and red cell membranes in a population of truly normal young adults.

METHODS

Thirty-two male and 18 female students (mean age of each group, 20) were recruited from Acadia University, Wolfville, Nova Scotia. They were all free of known acute or chronic illnesses and denied taking either therapeutic or recreational drugs. Individuals with a personal or immediate family history of atopy were excluded because atopic disorders may be associated with abnormal essential fatty acid metabolism (2). Venous blood was collected into a tube pretreated with ethylene diamine tetraacetic acid (EDTA) as an anticoagulant and centrifuged. The plasma was separated off and the red cells washed with isotonic saline.

Plasma samples were extracted with chloroform/methanol (2:1). The extract was filtered through sodium sulfate, evaporated to dryness and taken up in 0.5 ml chloroform/methanol. The lipid fractions were separated by thin layer chromatography on silica gel plates (E. Merck, Darmstadt, West Gemary). The phospholipid, triglyceride and free fatty acid fractions were methylated using boron trifluoride/methanol. The resulting methyl esters of the fatty acids 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 gas was helium (30 ml/min). Oven temperature was programmed to rise from 165 C to 190 C at 2 C/min. Detector temperature was 220 C and injector temperature 200 C. Retention times and peak areas were automatically computed by a Hewlett-Packard Level 4 integrator. Peaks were identified by comparison with standard methyl fatty acid esters from NuChek Prep Inc, Elysian, MN.

Red blood cells were washed and lipids were extracted according to the procedure of Dodge and Phillips (3). High performance liquid chromatographic (HPLC) grade reagents from Fisher Scientific Co. (Ottawa, Ontario) were used throughout. Total phospholipids and the individual phospholipid classes were separated by thin layer chromatography. The solvent systems used were petroleum ether/diethyl ether/acetic acid/methanol (85:15:2.5:1) for total phospholipids and chloroform/methanol/water (60:40:4) for separation of the phospholipid classes. Individual phospholipid classes were made visible under UV light by spraying with a 0.01% solution of rhodamine in methanol. Phosphatidylcholine and phosphatidylethanolamine were identified by comparison with authentic standards supplied by Sigma Chemical Co., St. Louis, MO. The phospholipid bands were scraped off immediately and analyzed for fatty acids as described for plasma.

RESULTS

The results are shown in Table 1-3. Only

TABLE 1

Levels of Fatty Acids in Total Plasma Phospholipids

Fatty acid	Present study	Holman female	Holman male
18:2n-6	21.45 ± 2.81	20.14 ± 3.72	20.24 ± 3.57
20:3n-6	3.06 ± 0.60	3.38 ± 1.42	3.46 ± 1.02
20:4n-6	11.36 ± 1.67	11.89 ± 2.45	11.08 ± 2.34
22:4n-6	0.73 ± 0.26	1.62 ± 0.78	1.47 ± 1.06
22:5n-6	1.12 ± 0.67	0.58 ± 0.72	0.46 ± 0.36
20:5n-3	1.01 ± 0.36	1.19 ± 0.62	1.06 ± 0.73
22:5n-3	0.93 ± 0.27	0.71 ± 0.51	0.73 ± 0.59
22:6n-3	3.54 ± 0.89	2.33 ± 1.27	1.80 ± 1.14
18:1n-9	13.50 ± 2.20	13.06 ± 2.34	13.51 ± 2.60
16:0	23.90 ± 7.02	26.81 ± 4.41	27.33 ± 3.55
18:0	11.61 ± 1.32	12.59 ± 1.75	13.37 ± 2.18

In the present study, no differences were found between males and females and so the two were grouped together. The values of Holman et al. are taken from ref. 1 and show levels corrected for a 20-year-old age group. Vaolues are mean percentages of the total fatty acids present ± SD.

TABLE 2

Levels of Fatty Acids in Plasma Free Fatty Acids and Plasma Triglycerides

Fatty acid	Free fatty acids	Triglycerides
18:2n-6	14.80 ± 0.44	17.33 ± 0.72
20:4n-6	–	1.21 ± 0.06
18:3n-3	–	0.90 ± 0.09
18:1n-9	37.20 ± 0.89	40.00 ± 0.78
16:0	24.55 ± 0.48	23.16 ± 0.57
18:0	13.18 ± 0.48	3.70 ± 0.32

Levels expressed as percentages of the total amount of fatty acids present. Values are means ± SEM. Only fatty acids present at more than 0.5% are shown.

fatty acids which were consistently detected at the level of 0.5% of the total or more are shown. We found no differences between the sexes and so males and females are grouped together. Table 1 compares the values for percentages of individual fatty acids in total plasma phospholipids obtained by ourselves and by Holman et al. (1).

DISCUSSION

Table 1 shows a considerable degree of agreement between our values and those obtained by Holman et al. (1). There were only three modest differences. Our values for sat-

TABLE 3

Amounts of Fatty Acids Present in Red Blood Cell Total Phospholipids Phosphatidylcholine and Phosphatidylethanolamine

Fatty acid	Total phospholipid	Phosphatidylcholine	Phosphatidylethanolamine
18:2n-6	9.78 ± 0.24	16.01 ± 0.27	6.22 ± 0.27
20:3n-6	1.37 ± 0.05	1.86 ± 0.06	0.84 ± 0.06
20:4n-6	15.13 ± 0.29	12.49 ± 0.25	21.46 ± 0.35
22:4n-6	5.54 ± 0.20	1.87 ± 0.13	5.41 ± 0.15
22:5n-6	3.99 ± 0.15	0.77 ± 0.09	–
20:5n-3	0.65 ± 0.03	–	0.76 ± 0.07
22:5n-3	2.53 ± 0.13	1.42 ± 0.08	3.52 ± 0.14
22:6n-3	4.20 ± 0.15	3.85 ± 0.13	5.47 ± 0.22
18:1n-9	14.83 ± 0.24	17.16 ± 0.24	19.39 ± 0.34
16:0	20.68 ± 0.26	23.60 ± 0.32	15.42 ± 0.35
18:0	14.71 ± 0.22	18.07 ± 0.31	9.87 ± 0.36

Amounts expressed as percentages of the total amount of fatty acids present. Values are means ± SEM.

urated fats were slightly lower. 20:3n-9 which was present at ca. 1% in Holman's samples was consistently less than 0.5% in ours. Saturated fatty acid levels were somewhat lower in our samples, whereas 22:6n-3 was substantially higher. There are at least two possible explanations for these relatively minor discrepancies. First, the patients studied by Holman et al. were attending hospital and may have suffered from diseases which have hitherto unnoticed effects on fatty acid metabolism. Second, our population of students in a maritime university may have consumed a diet rather different from Holman's patients living in the middle of the continent. The presence of 22:6n-3 in seafood could have accounted for the differences in levels of that fatty acid.

More important than these small discrepancies is the overall agreement which means that these sets of figures can be used as reliable guides by those investigating fatty acid metabolism in populations with various disease states.

We would like to draw attention to the obvious major importance of the C_{22} polyunsaturated fatty acids which make up 16-17% of the fatty acids in red cell membrane total phospholipids. The amounts present and their degrees of unsaturation mean that these acids must make major contributions to determining the physical properties of cell membranes, affecting fluidity, permeability and the functioning of receptors and membrane-bound enzymes. It is unfortunate that so many investigators turn off their gas chromatographs before the C_{22} fatty acids emerge, leading to loss of much potentially valuable information.

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Hydroxylation of Secondary Bile Acids in the Perfused Prairie Dog Liver

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ABSTRACT

Taurolithocholic acid and deoxycholic acid were perfused into isolated prairie dog livers. Taurolithocholic acid was 7α-hydroxylated to form taurochenodeoxycholic acid, whereas deoxycholic acid was conjugated and 7α-hydroxylated to form taurocholic acid. The low concentrations of secondary bile acids observed in prairie dog bile are due, at least in part, to active bile acid 7α-hydroxylase(s) in the liver of these animals.
Lipids 18:909-912, 1983.

The bile acid composition of bile is a composite of newly synthesized bile acids formed in the liver and secreted into bile and bile acids absorbed from the intestinal tract and preserved via the enterohepatic circulation (1). In the prairie dog, the major biliary bile acids are cholic acid (85%) and chenodeoxycholic acid (13%) (2). The proportions of the secondary bile acids in bile are very small (lithocholic acid [0-1%] and deoxycholic acid [1-2%]) (2). We have investigated the fate of the secondary bile acids (taurolithocholic acid and deoxycholic acid) in the isolated perfused prairie dog liver to determine possible reasons for their virtual absence from the bile.

EXPERIMENTAL PROCEDURES

Experimental Animals

Adult prairie dogs (*Cynomys ludovicianus*) of both sexes (trapped in the wild) were supplied by Fur and Feather Game Farm, Green Bay, WI. The animals were fed Purina rodent chow for 14-21 days and given food and water ad libitum. All animals were operated on between 9 and 10 a.m.; the perfusion was done immediately thereafter.

Liver Perfusion

The methods used were those previously described for the rabbit, with some modifications (3,4). The bile acid (1 mg, 1 μCi/mg) was dissolved in 200 μl of ethanol. The ethanol was dispersed into the perfusate which contained washed rabbit red cells, 3% serum albumin(fraction V), heparin (10 μg/ml) and Krebs-Henseleit bicarbonate buffer containing amino acids (387 μmol/100 ml) and administered over a 2-min period at time 0. Bile was collected from the liver for 30 min prior to administration of the bile acid (30-0 min, perfusion, fraction A). The bile acid was administered at time 0 and bile samples collected as follows: fraction B, 0-60 min; fraction C, 60-120 min; and fraction D, 120-180 min. The bile volumes were measured and the bile was deproteinized immediately after the perfusion.

Viability of the perfused liver was examined periodically during the perfusion using the following parameters: (a) bile flow, (b) urea production, (c) O_2 consumption, and (d) lactate and pyruvate levels.

Thin Layer Chromatography

All solvents were reagent grade and used without purification. Chromatography was carried out on 500-μ Silica Gel G plates from Analtech, Newark, DE. Solvents A or B (see below) were used to separate the bile acids.

Labeled Compounds

[24-^{14}C] Taurolithocholic acid (sodium salt) (California Bionuclear Corp., Sun Valley, CA) was found to be greater than 98% pure by thin layer chromatography (TLC) on Silica Gel G plates with butanol/acetic acid/water, 75:20:5, v/v/v (solvent A). [24-^{14}C] Deoxycholic acid (Amersham Corp., Arlington Heights, IL) was found to be greater than 99% pure by TLC using isooctane/ethyl acetate/acetic acid, 5:5:1, v/v/v (solvent B). All radioactive bile acids were reexamined by high pressure liquid chromatography (HPLC) after TLC analyses. Each pure radioactive bile acid, [24-^{14}C] taurolithocholic acid and [24-^{14}C] deoxycholic acid (10,000 dpm) was mixed with 25 μg of unlabeled bile acid in the HPLC solvent. Analyses were carried

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out on a Waters C_{18} Bondapak column (3.9 mm x 30 cm) using conditions described below (flow rate 1.0 ml/min). Each bile acid peak (deoxycholic acid 9.3 min; taurolithocholic acid 22.7 min) was collected, solvents were evaporated and the counts recovered determined by scintillation counting. In each case, more than 99% of the isotope was recovered with the bile acid carrier.

Reference Compounds

Conjugated bile acids were purchased from Steraloids, Inc., Wilton, NH. They were found to be better than 98% pure by TLC using solvents A or B.

High Pressure Liquid Chromatography

The HPLC procedures used were those of Shaw et al. (5) with modifications. One hundred μl of bile were added to a 20-fold excess of methanol and left at -20 C for 24 hr. The bile was centrifuged and the supernatant solution was removed. The precipitate was washed with 1 ml of methanol, centrifuged and the supernatant solution was removed and pooled. The combined solutions were evaporated and the resulting residue was dissolved in 1.00 ml of isopropanol/phosphate buffer (8.8 mmol, pH 7.0), 1:2, v/v. The material was filtered to remove solids (Waters organic filter 26875, Waters Associates, Milford, MA) and analyzed by HPLC (5). The analytical conditions were: C_{18} Bondapak column in a Waters Z module; solvent – isopropanol/phosphate buffer (8.8 mmol, pH=7.0), 1:2, v/v; flow – 2.5 ml/min; detector – Varichrom 50 (Varian Associates, Palo Alto, CA), 195 nm. Peaks corresponding to taurocholic acid (4.9 min), taurochenodeoxycholic acid (9.0 min), taurodeoxycholic acid (11.0 min) and taurolithocholic acid (19.2 min) were recorded and quantitated on a Hewlett-Packard 3390A integrator. Each peak was collected and the radioactivity was measured by liquid scintillation counting (see below).

Liquid Scintillation Counting

Radioactivity measurements were carried out using a Beckman LS 8000 liquid scintillation system. All samples were dissolved in Aquasol 2 (New England Nuclear, Boston, MA). Corrections for quenching and background were made using appropriate blanks and standards.

RESULTS

In the perfused prairie dog liver, secondary bile acids were 7α-hydroxylated to primary bile acids. These studies explain the virtual absence of secondary bile acids from prairie dog bile.

Metabolism of [24-^{14}C] Taurolithocholic Acid

Liver perfusions were performed in duplicate. One mg of [24-^{14}C] taurolithocholic acid (sp act 1 μCi/mg) was perfused into an isolated prairie dog liver after a 30-min preperfusion period. Most of the radioactivity was recovered in the bile during the first hour (69.9% and 71% for perfusions 1 and 2, respectively) with the

TABLE 1

Distribution of Radioisotope in Bile Acids by HPLC Analysis (%)[a]

Perfusion and substrate	Period	Taurocholic acid	Taurodeoxycholic acid	Taurochenodeoxycholic acid	Taurolithocholic acid
[24-^{14}C] Taurolithocholic acid	30-0	–	–	–	–
	0-60	–	–	64 (44)	36 (25)
	60-120	–	–	50 (13)	50 (13)
	120-180	–	–	51 (3)	49 (2)
[24-^{14}C] Taurolithocholic acid	30-0	–	–	–	–
	0-60	–	–	57 (41)	43 (34)
	60-120	–	–	52 (13)	48 (12)
	120-180	–	–	–	–
[24-^{14}C] Deoxycholic acid	30-0	–	–	–	–
	0-60	65 (6)	35 (3)	–	–
	60-120	93 (64)	7 (5)	–	–
	120-180	89 (14)	11 (8)	–	–
[24-^{14}C] Deoxycholic acid	30-0	–	–	–	–
	0-60	50 (4)	50 (4)	–	–
	60-120	85 (56)	15 (10)	–	–
	120-180	95 (25)	5 (1)	–	–

[a]Numbers represent the percentage of isotope present in each bile acid during each period. Numbers in parentheses represent the fraction of each bile acid calculated for the entire perfusion study.

remainder excreted during the second (25.7% and 23.8%) and third hours (4.4% and 4.7%), respectively. No isotope was detected in the liver at the end of the perfusion. Analysis of the biliary bile acids by HPLC (Table 1) revealed that a portion of the taurolithocholic acid was 7α-hydroxylated to taurochenodeoxycholic acid. In the first hour, 64% of the isotope in perfusion 1 and 57% of the isotope in perfusion 2 was present as taurochenodeoxycholic acid. Significant amounts of taurochenodeoxycholate were still present during the second hour of the perfusion (Table 2). Analysis of the biliary bile acid composition (Table 2) revealed that taurocholic acid was the major component in most fractions (taurocholic acid comprised more than 80% of total biliary bile acids) during the entire perfusion.

Matabolism of [24-^{14}C] Deoxycholic Acid

Two perfused livers were given 1 mg of [24-^{14}C] deoxycholic acid (sp act 1 μCi/mg). This bile acid was secreted into the bile at a slower rate than taurolithocholic acid; most of the isotope (68.6% and 65.4% in perfusions 3 and 4, respectively) was found in the bile during period C (60-120 min). The remainder of the isotope was secreted during periods B (9.8% and 8.7%) and D (21.6% and 25.9%), respectively. No isotope remained in the liver. Deoxycholic acid was extensively 7α-hydroxylated to cholic acid (Table 2) and appeared in the bile as taurocholic acid. Taurodeoxycholic acid comprised a total of 15-16% of the total isotope recovered in the bile; the remainder was present as taurocholic acid (averaging ca. 85% of the total biliary bile acid, Table 2); the remainder of the biliary bile acid was taurochenodeoxycholic acid.

DISCUSSION

In some mammals, such as the prairie dog, hamster and guinea pig, secondary bile acids are almost completely absent from the bile (2,6,7). These animals either fail to absorb the bile acids from the intestinal tract or absorb them with subsequent hydroxylation in the liver. In this report, we examined the fate of secondary bile acids in the perfused prairie dog liver, an isolated system in which hydroxylation can be studied without the participation of intestinal microorganisms and without complications due to differential intestinal absorption.

The major biliary bile acids in the prairie dog fed a Purina chow diet are reported to be taurocholic acid (85%) and taurochenodeoxycholic acid (13%) with taurolithocholic acid

TABLE 2

Distrubution of Bile Acids in Bile by HPLC Analysis (%)[a]

Perfusion and bile acid infused	Period A 30-0 min				Period B 0-60 min				Period C 60-120 min				Period D 120-180 min			
	LA	DA	CDA	CA	LA	DA	CDA	CA	LA	DA	CDA	CA	LA	DA	CDA	CA
Taurolithocholic	8	–	39	53	45	–	29	26	–	–	23	77	–	–	–	100
Taurolithocholic	–	–	14	86	1	–	12	87	–	–	8	92	–	–	6	94
Deoxycholic	–	–	8	92	–	1	8	91	–	1	7	92	–	1	8	91
Deoxycholic	–	–	19	81	–	–	18	82	–	–	19	81	–	–	7	93

[a]Numbers represent the percentage of bile acids in each fraction based on total mass present (as determined by HPLC).
Abbreviations: LA, lithocholic acid; DA, deoxycholic acid; CDA, chenodeoxycholic acid; CA, cholic acid.

and taurodeoxycholic acid comprising the remaining 2% of the bile acids (2). When prairie dogs were fed chenodeoxycholic acid, 90% of the biliary bile acids was taurochenodeoxycholic acid with the remainder being taurocholic acid 9-10%) and taurolithocholic acid (0-1%). Our experiments were designed to determine why taurolithocholic acid was essentially absent from the bile. We found that, in the perfused prairie dog liver, taurolithocholic acid was hydroxylated at the 7α-position; chenodeoxycholic acid was formed and excreted into the bile as its taurine conjugate. Thus, detoxification of lithocholic acid in the prairie dog occurs via 7α-hydroxylation. Hydroxylation of lithocholic acid at the 7α-position has previously been reported in the hamster. In this species, lithocholic acid was converted to chenodeoxycholic acid (9), whereas, in the rat, lithocholic acid underwent 6β-hydroxylation (10). Thus, we postulate that, in the prairie dog, lithocholic acid was detoxified via "rehydroxylation" to chenodeoxycholic acid. It is unknown whether the 7α-hydroxylation of conjugated lithocholic acid is carried out by the same 7α-hydroxylase enzyme which converts cholesterol to bile acids.

The 7α-hydroxylation of deoxycholic acid has been previously reported in the rat (10,11). We have now observed a similar transformation in the prairie dog. Following the infusion of [24-^{14}C] deoxycholic acid, the major product was [24-^{14}C] taurocholic acid. Most of the labeled taurocholate appeared in the bile during the second hour of the perfusion. Presumably, deoxycholic acid had to be conjugated with taurine before hydroxylation took place. This conclusion is based on the results of in vitro studies demonstrating that, in the rat, the microsomal 7α-hydroxylase is relatively inactive with unconjugated deoxycholic acid (11). Nevertheless, hydroxylation of deoxycholic acid was more efficient than that of taurolithocholic acid (ca. 85-86% of the [24-^{14}C] deoxycholic acid was recovered as taurocholic acid). These results explain why there are minimal quantities of deoxycholic acid detected in prairie dog bile. At present, it is not known whether 7α-"rehydroxylation" of the two secondary bile acids is catalyzed by the same hydroxylase. The prairie dog is the only mammalian species studied to date which efficiently 7α-hydroxylates both lithocholic acid and deoxycholic acid to the corresponding primary bile acids.

ACKNOWLEDGMENT

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LETTER TO THE EDITOR

Sir:

In recent years there have been a number of papers in which the name "vaccenic" has been applied to *cis*-11-octadecenoic acid, and "*trans*-vaccenic" used to refer to *trans*-11-octadecenoic acid. Examples of this usage may be found in a number of issues of *Lipids*, and several of the major suppliers of lipid standards list vaccenic as the *cis* isomer. Vaccenic acid was the trivial name originally given by Bertram (1) to the *trans* form, and is so used in *Chemical Abstracts.* The *cis* isomer is commonly termed *cis*-vaccenic, although Pryde (2) lists the trivial name of asclepic acid.

This simple transposition of trivial names may seem itself somewhat trivial, but when it obscures the identity of the compound meant, it adds to the difficulty of an already complex subject. *Lipids* reviewers and editors should be made aware of this problem, and authors encouraged to use some less ambiguous nomenclature at least once to remove any uncertainty regarding identity. If trivial names are used, vaccenic should be used only for *trans*-11-octadecenoic acid, and *cis*-vaccenic (or possibly asclepic) for *cis*-11-octadecenoic acid.

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[Received September 13, 1983]

ERRATUM

In the article "Reduced Plasma Lecithin Cholesterol Acyl Transferase Activity in Rats Fed Iron-Deficient Diets" by G.A. Rao, R.T. Crane and E.C. Larkin (*Lipids* 18:673-676, 1983), a portion of text was misplaced. The first column on page 674 and the top three lines of the second column on page 674 belong in the second paragraph under Results and Discussion on page 675. On the following two pages are reproduced pages 674 and 675 as they should have appeared.

washed syringes and needles. Whole blood was centrifuged at 1500 rpm at room temperature in a Dynac centrifuge for 7 min, and plasma was removed. Red cells were washed 3 times with resuspension in standard incubation medium (10) and centrifugation at 1500 rpm for 7 min.

Lipids were extracted from 1-ml aliquots of plasma and red cells as described by Folch et al. (11) and by Rose and Oklander (12), respectively. Free cholesterol (CH) and CE contents were analyzed by the O-phthalaldehyde method (13). Plasma triglyceride and phospholipid contents were determined by analyzing their fatty acid composition by gas liquid chromatography and using methyl pentadecanoate as an internal standard (14).

Assay of LCAT was carried out according to the procedure by Stokke and Norum (15). Plasma (100 μl) was incubated at 37 C with albumin-[4-^{14}C] cholesterol emulsion (100 μl) and Ellman reagent (1.4 mM in 0.2 M phosphate buffer, pH 7.1, 20 μl) for 2 hr to allow the equilibration of added labeled CH with the plasma lipoprotein cholesterol. Mercaptoethanol (0.1 M, 20 μl) was then added and incubation was continued for an additional 2-hr period. The reaction was stopped by the addition of 5 ml chloroform/methanol (2:1, v/v), lipids were extracted, separated by thin layer chromatography and the (^{14}C) activities in the CH and CE fractions were quantitated. [4-^{14}C] Cholesterol (52.5 mCi/mmol, 0.01 mCi) was purchased from New England Nuclear, Boston, MA.

RESULTS AND DISCUSSION

In the present study, although rats were fed different iron-deficient diets, only those in the CO-Fe group became moderately iron-deficient as indicated by their reduced blood Hb and Hct levels (ca. 8.4 g % and 34%, respectively, as compared to ca. 14 g % and 44% in other ± Fe groups) (8). In humans, the CH content of red blood cells (RBC) has been reported to be increased markedly due to anemia (16,17). We found that the CH content of RBC was significantly ($p<0.01$) increased only in the CO-Fe group (1.41 ± 0.13 mg/ml as compared to 1.10 ± 0.18 mg/ml in the CO+Fe group). The cholesterol content of RBC of rats in the other groups was not altered significantly (FF+Fe, 1.0 ± 0.18; FF-Fe, 1.13 ± 0.09; HCNO + Fe, 1.13 ± 0.16; HCNO-Fe, 1.0 ± 0.22).

Rats maintained on the FF-Fe or HCNO-Fe diets were deprived of essential fatty acids and iron. On the other hand, those fed the CO-Fe diet were exposed only to a low intake of dietary iron. In rats on the FF-Fe and HCNO-Fe diets, the plasma lipids contained appreciable levels of eicosatrienoic acid, a sign of essential fatty acid deficiency, although they were less depleted of 18:2 and 20:4 as compared to the iron-supplemented controls (7).

As reported previously (7), when rats were fed the FF-Fe diet, their growth was not reduced markedly as compared to those fed the FF+Fe diet. The final body weights of rats on the FF-Fe and FF+Fe diets were 300 ± 28 g and 326 ± 36 g, respectively. A marked effect on growth by dietary deprivation of iron was also not observed in rats maintained on the HCNO diet. On the contrary, in the case of rats fed the CO diet, omission of iron caused a significant reduction in growth (7). The weights of rats fed the CO-Fe and CO+Fe diets were 379 ± 24 g and 484 ± 11 g, respectively.

Plasma LCAT Activity

In previous studies which reported that the LCAT activity was reduced in severely but not moderately iron-deficient rats (9), for the enzyme assays, labeled CH was not preincubated with plasma to allow for its equilibration with endogenous cholesterol. Such preincubations are vital to obtain optimal LCAT activity (15). Furthermore, the evaluation of LCAT was based only on the radioactivity of CE and not on the amount of CE produced (9). In the present study, the amount of CE produced was measured after the substrate CH and plasma CH were allowed to equilibrate. Our results show that when rats consumed low iron diets, their plasma had reduced LCAT activity regardless of whether they were iron-deficient or not (Table 1). It is possible that in earlier experiments, plasma LCAT would have been found to be decreased in severe as well as moderate iron-deficient rats if the enzyme activity was measured based on the amount of CE produced. Significant differences in the specific activity of labeled CH can occur due to the variations in the cholesterol content of plasma in rats fed the various diets (Table 2). These observations also suggest that the reduction of LCAT found in severely anemic rats would have been further amplified if the dilution effect of CH was taken into consideration (9).

Plasma CH and CE Levels

It is not known whether the CE/CH ratio in plasma is altered as would be expected by the reduction of LCAT activity since these parameters have not been examined previously in the same iron-deficient rat. It would be logical to

TABLE 1

Lecithin Cholesterol Acyl Transferase Activity in Plasma of Rats Fed Iron-Deficient or Iron-Supplemented Diets

Diet[a]	LCAT Activity[b]
FF-Fe	560 ± 79[c]
FF+Fe	1047 ± 151
HCNO-Fe	668 ± 43[c]
HCNO+Fe	1041 ± 72
CO-Fe	689 ± 97[d]
CO+Fe	1096 ± 116

[a]Diet abbreviations are: FF, fat-free; HCNO, 14% hydrogenated coconut oil diet; CO, 14% corn oil diet. Iron-deficient and iron-supplemented diets are given as -Fe and +Fe, respectively.

[b]LCAT activity is given as nmol cholesterol esterified/min/l plasma. Values are mean ± SE from duplicate determinations with plasma sample from each of the 6 rats in the diet groups.

[c]Significantly different with $p<0.01$ using 2-tailed t-test as compared to the value in the corresponding +Fe group.

[d]Significantly different with $p<0.02$ as compared to the value in the CO+Fe group.

expect that, when LCAT activity is reduced in animals fed iron-deficient diets (Table 1), the relative level of CE to CH in plasma would also be reduced as compared to the levels in iron-supplemented controls. However, our results showed that CE/CH ratio was increased significantly rather than decreased when compared to the controls (Table 2). It would appear that the LCAT activity is low under conditions when the relative level of CE to CH in the plasma is already high. On the other hand, enzyme activity is high in iron-supplemented animals when CE/CH ratio is reduced. An evaluation of the data from earlier studies also suggests that it is difficult to correlate plasma LCAT and CE/CH levels in iron-deficient animals. The relative level of CE to CH in plasma lipids had been found to be either decreased (2, 18, 20) or unchanged (3, 19) in iron-deficient rats. Furthermore, the values for CE/CH decreased mostly due to increased levels of CH and not due to decreased levels of CE (2, 18, 20). In some experiments, even though the blood Hb levels were decreased to 4.1 g % (vs 15.9 g % in controls), plasma cholesterol contents have remained unchanged (6). Hence, further experiments are needed to understand the relationship between iron deficiency, plasma CE/CH levels and LCAT activity.

Hyperlipidemia

Another reported effect of iron deficiency is the production of hyperlipidemia. However, iron deficiency during both pregnancy and lactation causes hyperlipidemia in 18-day-old offspring but not in maternal rats (1). When male weanling rats were fed iron-deficient diets for 5 weeks, their blood triglyceride levels were 5-fold greater than in those in animals fed an iron-supplemented diet (6). Marked lipemia was also observed in weanling male and female rats and chicks when they were fed iron-deficient diets (4). However, hyperlipidemia appears to be dependent on the amount and type of dietary fat fed and also on the strain of rat used (5). In some experiments, hyperlipidemia was not produced and instead the serum triglyceride levels were depressed markedly (60-75%) by iron deficiency (3,5). The present study supports this finding since the analysis of the plasma lipid contents showed that the triglyceride levels were significantly reduced in rats fed iron-deficient diets as com-

TABLE 2

Cholesterol and Cholesteryl Ester Contents of Plasma from Rats Fed Iron-Deficient or Iron-Supplemented Diets[a]

Diet[b]	Cholesterol	Cholesteryl ester	Cholesteryl ester / Cholesterol
FF-Fe	17.6 ± 1.3	30.4 ± 1.8	1.76 ± 0.1[c]
FF+Fe	23.4 ± 1.6	21.4 ± 4.4	0.97 ± 0.23
HCNO-Fe	18.6 ± 0.4	23.9 ± 2.5	1.40 ± 0.11[c]
HCNO+Fe	26.3 ± 3.6	15.7 ± 4.9	0.72 ± 0.24
CO-Fe	19.0 ± 2.7	31.5 ± 4.0	1.64 ± 0.26[c]
CO+Fe	27.8 ± 6.4	29.8 ± 5.2	0.82 ± 0.10

[a]Plasma cholesterol and cholesteryl ester contents are given as mg %. these are mean ± SE obtained from duplicate analysis with plasma sample from each of the 6 rats in the diet groups.

[b]See Table 1 for diet designation.

[c]Significantly different from the corresponding value in the +Fe group with $p<0.001$ using the 2-tailed t-test.

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